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C-reactive protein/serum amyloid P promotes pro-inflammatory function and induces M1-type polarization of monocytes/macrophages in mudskipper, *Boleophthalmus pectinirostris*

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ABSTRACT

C-reactive protein (CRP) and serum amyloid P (SAP) play essential roles in the phagocytic cell-mediated innate immune response of mammals. In-depth studies into CRP and SAP have been completed in mammals; however, such studies, particularly those relating to the functions of CRP and SAP, are rare in fish species. In this study, a homolog of CRP/SAP (BpCRP/SAP) was identified in mudskipper (*Boleophthalmus pectinirostris*), which had the typical characteristics of a fish short pentraxin protein. Phylogenetic tree analysis revealed that BpCRP/SAP was most closely related to mudskipper CRP/SAP-l3. BpCRP/SAP transcripts were detected in all tested tissues, with the highest level observed in the liver; transcripts in the immune tissues and protein expression in the serum were induced in response to *Edwardsiella tarda* infection. The active recombinant BpCRP/SAP (rBpCRP/SAP) was able to augment the mRNA expression of pro-inflammatory cytokines and attenuate the mRNA expression of anti-inflammatory cytokines in monocytes/macrophages (MO/MΦ). In addition, phagocytosis and bacterial killing of *E. tarda* by mudskipper MO/MΦ were boosted by rBpCRP/SAP stimulation. rBpCRP/SAP also promoted M1-type MO/MΦ polarization, but inhibited M2-type polarization. In conclusion, the present research describes the pro-inflammatory function of BpCRP/SAP in mudskipper against *E. tarda* infection.

1. Introduction

Innate immunity is the first line of defense against invading pathogens, and involves the recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) with cell-associated or soluble pattern-recognition receptors (PRRs) [1]. Pentraxins (PTXs) are evolutionarily conserved soluble PRRS, which share a common primary motif of approximately 200 amino acids (aa) in length called a pentraxin signature (His-x-Cys-x-Ser/Thr-x-Ser). PTXs can be divided into two subfamilies: long pentraxins and short pentraxins, with serum amyloid P component (SAP) and C-reactive protein (CRP) belonging to the latter and sharing 51% amino acid sequence homology [2].

Short PTXs participate in tissue development, disease development, immune regulation, and cell differentiation. SAP is involved in the formation of the dragged-wing phenotype in *Drosophila* [3], and can also promote the formation of amyloid deposits and the development of lung fibrosis [4–6]. In addition, SAP and CRP can exert immune-related effects via the recognition of various ligands. By binding to PAMPs (e.g. glycosaminoglycans, lipopolysaccharide [LPS], peptidoglycan [PGN], and viral hemagglutinin) or DAMPs (e.g. DNA, chromatin, and histones), SAP and CRP can protect the host from invading pathogens or autoimmune responses [7–12]. Furthermore, SAP and CRP can bind to complement (C1q) to modulate the classical complement pathway and compete with IgG to bind the Fcγ receptor (FcγRI, FcγRIIa), thus regulating phagocytes [13,14]. SAP is also involved in regulating the polarization of M1-type or M2-type macrophages, which is dependent on the exact macrophage type and biological environment encountered [15]. For example, in mouse models of tuberculosis, SAP promoted differentiation of lung resident M1 macrophages; in models of systemic lupus erythematosus, SAP promoted M2 macrophage differentiation of kidney macrophages [13,16]. Proteomic analysis has also revealed that human CRP can affect the state of monocytes [17].

In contrast to the in-depth studies of SAP and CRP performed in

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mammals, the role of these proteins in teleosts remains largely unexplored. In contrast to mammals, there is no clear distinction between CRP and SAP either in terms of sequence homology or in phylogenetic tree analysis in fish [18]. Currently, SAP and CRP (CRP/SAP) homologs have been identified in many fish species, such as zebrafish (*Danio rerio*), common carp (*Cyprinus carpio*), Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), and Atlantic cod (*Gadus morhua*) [19–22]. Most of those previous studies focused primarily on the expression patterns of SAP or CRP in healthy and pathogen-infected tissues. Most recently, the ligand-binding activity of a CRP/SAP homolog, and its' regulatory roles in the phagocytosis of monocytes/macrophages (MO/MΦ), were elucidated in ayu (*Plecoglossus altivelis*) [23]. However, as SAP/CRP is important in immune regulation, systematic studies investigating its effects on immune cell function are needed.

The mudskipper is an amphibious fish that can breathe through its skin, lining of its mouth (the mucosa), and throat (the pharynx) like an amphibian, and can adapt to a wide range of temperatures and salinities. Although the mudskipper inhabits intertidal mudflats containing abundant and diverse microbial populations, they rarely suffer from severe bacterial diseases. *Edwardsiella tarda*, a well-known intracellular bacterium, is pathogenic to mudskipper [24–26]. Hence, it is necessary to investigate the anti-pathogen mechanisms of mudskipper to gather information that may be used for the immune-related prevention or treatment of aquaculture diseases. In the present study, a CRP/SAP homolog (BpCRP/SAP) was identified from mudskipper. The expression patterns of *BpCRP/SAP* were determined in healthy and *E. tarda*-infected fish. In addition, the effects of recombinant BpCRP/SAP (rBpCRP/SAP) on phagocytosis, bacterial killing, and cytokine expression of mudskipper MO/MΦ were determined. Moreover, the regulatory role of rBpCRP/SAP in MO/MΦ polarization was analyzed.

2. Materials and methods

2.1. Experimental fish

Healthy mudskipper individuals weighing 30–40 g were purchased from a commercial farm in Ningbo city, China. Fish were reared in a recirculating system with defluorinated brackish water (salinity 10‰) at 23–25 °C for 2 weeks to allow acclimatization to laboratory conditions. All experiments were performed in accordance with the Experimental Animal Management Law of China and were approved by the Animal Ethics Committee of Ningbo University.

2.2. Molecular characterization of BpCRP/SAP cDNA

The cDNA sequence of *BpCRP/SAP* was retrieved from the transcriptome data of mudskipper kidney-derived MO/MΦ, and was authenticated by further cloning, sequencing, and comparison to other similar sequences using a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The information on BpCRP/SAP and on related sequences used for analyses is presented in Table 1. The signal peptide was predicted using the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). Multiple sequence alignment was generated using ClustalW (http://www.genome.jp/tools/clustalw/). Phylogenetic trees of protein sequences were analyzed using MEGA version 5.

2.3. In vivo bacterial challenge and tissue collection

Healthy mudskipper individuals were infected with *E. tarda* as previously described [26]. Briefly, *E. tarda* was cultured at 28 °C in Tryptic soy broth medium (TSB) (Sigma-Aldrich, Saint Louis, USA) and harvested at the logarithmic growth phase. Bacteria were washed, resuspended, and diluted to 1.0×10^5 colony-forming units (CFU)/mL in sterile phosphate-buffered saline (PBS). Each fish was intraperitoneally injected with 1.0×10^4 CFU live *E. tarda*. Fish injected with PBS alone were used as a control. To analyze the expression pattern of *BpCRP/SAP*

Table 1
SAP-, CRP-, and pentraxin-like protein sequences used in this study.

Accession number	Species		Protein
	Latin name	English name	
MH796661	*Boleophthalmus pectinirostris*	mudskipper	CRP/SAP
XM_020937340	*B. pectinirostris*	mudskipper	CRP/SAP-l1
XM_020923671	*B. pectinirostris*	mudskipper	CRP/SAP-l2
XM_020937238	*B. pectinirostris*	mudskipper	CRP/SAP-l3
KP329195	*Plecoglossus altivelis*	ayu	CRP/SAP
FJ940746	*Gadus morhua*	Atlantic cod	CRP-I
P86689[a]	*G. morhua*	Atlantic cod	CRP-II
NM_001124721	*Oncorhynchus mykiss*	rainbow trout	CRP/SAP-1a
XM_021565334	*O. mykiss*	rainbow trout	CRP/SAP-1b
XM_021557381	*O. mykiss*	rainbow trout	CRP/SAP-1c
XM_021580214	*O. mykiss*	rainbow trout	CRP/SAP-2
NM_001123671	*Salmo salar*	Atlantic salmon	CRP/SAP-1a
BT079947	*Esox lucius*	northern pike	SAP
BT074711	*Osmerus mordax*	rainbow smelt	SAP
JX914666	*Cynoglossus semilaevis*	half-smooth tongue sole	CRP
KT025856	*C. semilaevis*	half-smooth tongue sole	SAP
GU441682	*Epinephelus coioides*	orange-spotted grouper	CRP
XM_006630971	*Lepisosteus oculatus*	spotted gar	SAP-1
XM_003966161	*Takifugu rubripes*	tiger puffer	SAP
BT082602	*Anoplopoma fimbria*	sablefish	SAP
AB665331	*Oplegnathus fasciatus*	rock bream	SAP-2
AB665328	*O. fasciatus*	rock bream	SAP-1
XM_005472765	*Oreochromis niloticus*	Nile tilapia	SAP
XM_004541412	*Neolamprologus brichardi*	fairy cichlid	SAP
XM_004541412	*Maylandia zebra*	zebra mbuna	SAP
CAAE01014679	*Tetraodon nigroviridis*	spotted green pufferfish	PTX
XM_693995	*Danio rerio*	zebrafish	CRP-1
NM_001025297	*D. rerio*	zebrafish	CRP-2
AB028455	*Cyprinus carpio*	common carp	PTX
JQ010977	*C. carpio*	common carp	CRP-1
XM_019271265	*Larimichthys crocea*	large yellow croaker	CRP
XM_019271266	*L. crocea*	large yellow croaker	SAP
XM_004077855	*Oryzias latipes*	Japanese ricefish	CRP
FJ547474	*Ctenopharyngodon idella*	grass carp	PTX
NM_001639	*Homo sapiens*	human	SAP
NM_000567	*H. sapiens*	human	CRP
NM_011318	*Mus musculus*	mouse	SAP
X13588	*M. musculus*	mouse	CRP
NM_213887	*Sus scrofa*	pig	SAP
NM_213844	*S. scrofa*	pig	CRP
XM_002715288	*Oryctolagus cuniculus*	rabbit	SAP
L47237	*O. cuniculus*	rabbit	CRP
NM_001039564	*Gallus gallus*	chicken	CRP
XM_002198407	*Taeniopygia guttata*	zebra finch	SAP
XM_006115236	*Pelodiscus sinensis*	Chinese softshell turtle	CRP
XM_008122047	*Anolis carolinensis*	lizard	CRP
XM_003228401	*A. carolinensis*	lizard	SAP
NM_001008174	*Xenopus tropicalis*	tropical clawed frog	SAP
XM_002934081	*X. tropicalis*	tropical clawed frog	CRP-1
NM_001172215	*Xenopus laevis*	African clawed frog	CRP-1
M14026	*Limulus polyphemus*	horseshoe crab	CRP-1.1
AY066022	*L. polyphemus*	horseshoe crab	SAP

[a] Full-length sequence used from Ref. [22].

Table 2
Oligonucleotide primers used for RT-qPCR analysis.

Primers	Gene	Accession number	Nucleotide sequence (5′–3′)	Amplicon size (bp)
BpCRP/SAP-F	*CRP/SAP*	MH796661	GCTGTGGGTCAATGGAAAAC	151
BpCRP/SAP-R			AATCCTGCTCCTGTCCAAG	
Bp-18S–F	*18S RNA*	KX492896	ATTGGAGGGCAAGTCTGGTG	180
Bp-18S-R			CAGCTAAGAGCATCGAGGGG	
BpIL-1β-F	*IL-1β*	KX492895	ACGAGTGGTGAATGTGGTCA	163
BpIL-1β-R			GAACTGAGGTTGTGCTGCAA	
BpTNF-α-F	*TNF-α*	KX492896	GGACAACAACGAGATCGTGA	155
BpTNF-α-R			GTTCCACCGTGTGACTGATG	
BpIL-10-F	*IL-10*	XM020936977	GTGGAGGGGTTCCCTCTAAG	179
BpIL-10-R			GTGCGGAGGTAAAAGCTCAG	
BpTGF-β-F	*TGF-β*	XM020928521	TCAAAGGACACTTGCACAGC	183
BpTGF-β-R			CAGGGCCAAGATCTGTGAAT	

after infection, fish were euthanized 0, 4, 8, 12, 24, and 48 h post infection (hpi) and tissues from the liver, spleen, and kidney were collected and stored at −80 °C until use. Healthy fish tissues from the gill, spleen, intestine, heart, skin, brain, kidney, blood, and liver were also collected for subsequent analysis of tissue expression patterns. To examine the levels of serum BpCRP/SAP induction after infection, *E. tarda*-infected fish were euthanized, and blood was collected 0, 4, 8, 12, 24, and 48 hpi. Serum was isolated and stored for subsequent western blot analysis.

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

Total RNA was isolated using RNAiso (TaKaRa, Beijing, China), treated with DNase I (TaKaRa), and reverse-transcribed into first-strand cDNA using AMV reverse transcriptase (TaKaRa) following the manufacturer's protocol. RT-qPCR was performed on an ABI StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA) using SYBR premix Ex *Taq*II (TaKaRa), as previously described [27]. The primers used are listed in Table 2. The RT-qPCR amplification conditions were as follows: 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, followed by melting curve analysis at 94 °C for 30 s, 72 °C for 30 s, and 94 °C for 30 s. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and the data were normalized against *Bp18S* rRNA. Each PCR trial was performed in triplicate and repeated at least three times.

2.5. Prokaryotic expression and antibody preparation

The primers used were BpCRP/SAPep(+): 5′-GGGGTACCGCTTCC TCAAGATCTGTCTGGAAAGATGC-3′ and BpCRP/SAPep(−): 5′-CGGG ATCCACACTGATCTGCTACATCCTTG-3′, and were designed to amplify the complete open reading frame (ORF) sequence of *BpCRP/SAP*. The amplicons were digested by *Kpn* I and *BamH* I, and then inserted into the enzyme-digested pMAL-c2e vector to construct the prokaryotic expression plasmid pMAL-BpCRP/SAP. The constructed plasmid was then transformed into *Escherichia coli* BL21 (DE3) pLysS. The expression of recombinant BpCRP/SAP protein with an N-terminal MBP-tag and a C-terminal His-tag was induced by isopropyl-β-D-thiogalactopyranoside (IPTG). The protein was purified using amylose resin (New England Biolabs, Beijing, China) and then further digested by enterokinase (Sangon, Shanghai, China) to remove the MBP-tag. The recombinant BpCRP/SAP protein with a C-terminal His-tag (rBpCRP/SAP) was purified using a nickel-nitrilotriacetic acid (Ni-NTA) column (TaKaRa), according to the manufacturer's instructions. Possible contamination of the rBpCRP/SAP preparation with endotoxins was evaluated using the *Limulus amebocyte* lysate test. Endotoxins in the recombinant proteins were at levels of less than 0.1 EU/mg after toxin removal with an endotoxin-removal column (Pierce, Rockford, USA). Then, rBpCRP/SAP was used as an antigen to immunize rabbits for subsequent production of the antiserum. The specificity of antiserum was tested by western blotting and then visualized using an enhanced chemiluminescence (ECL) kit (Advansta, Menlo Park, USA).

2.6. Isolation of mudskipper kidney-derived MO/MΦ and E. tarda stimulation

Kidney-derived MO/MΦ from mudskipper were isolated as previously described [26]. The mudskipper kidney was isolated, washed, and disassociated in RPMI 1640 medium (Gibco, Shanghai, China) supplemented with 2% fetal bovine serum (FBS) (Gibco), penicillin (100 U/mL, Gibco), streptomycin (100 μg/mL, Gibco), and EDTA (500 μg/mL, Sangon). The kidney leukocyte-enriched fractions were obtained using a Ficoll (1.077 g/mL, GeneHealthcare, Chicago, USA) density gradient, according to the manufacturer's instructions. The cells were then seeded in 12-well plates at a density of 1×10^7/mL in RPMI 1640 medium supplement with 0.1% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL), and EDTA (500 μg/mL) before being cultured overnight at 24 °C with 5% CO_2. After non-adherent cells were removed by washing, the attached cells were incubated with complete RPMI 1640 medium (10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin) and then cultured under the same conditions.

To evaluate the role of BpCRP/SAP in the expression of inflammatory cytokines upon *E. tarda* stimulation, the isolated MO/MΦ were pre-treated with rBpCRP/SAP or PBS for 8 h and then infected with live *E. tarda* at a multiplicity of infection (MOI) of 10. Cells were collected at 0.25, 0.5, 1, 2, 3, 4, 8, 12, 24, and 48 h after infection. Then, the mRNA expression of cytokine genes including interleukin-1β *(IL-1β),* tumor necrosis factor-α *(TNF-α),* interleukin-10 *(IL-10),* and transforming growth factor-β *(TGF-β)* was determined by RT-qPCR. The primers used are listed in Table 2.

2.7. In vitro phagocytosis assay

Phagocytosis of mudskipper MO/MΦ was performed as previously described [26]. Logarithmic-phase *E. tarda* were labeled with fluorescein isothiocyanate (FITC) (Sigma-Aldrich) according to the manufacturer's protocol and were designated as *E. tarda*-FITC (*Et*-FITC). MO/MΦ seeded in 12-well plates (1×10^6 cell/well) were pre-treated with 20 μg/mL rBpCRP/SAP for 8 h. Cells treated with PBS alone were used as a control. *Et*-FITC were added at an MOI of 10 and then incubated for 30 min before being treated with trypan blue (0.4%, Sangon) to remove any fluorescence outside the cell membranes. The MO/MΦ were washed, harvested, and resuspended in 500 μL PBS. The engulfed bacteria were examined using a MACSQuant® Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany) and then analyzed using MACSQuant Analysis Software (Miltenyi Biotec). Relative mean fluorescence

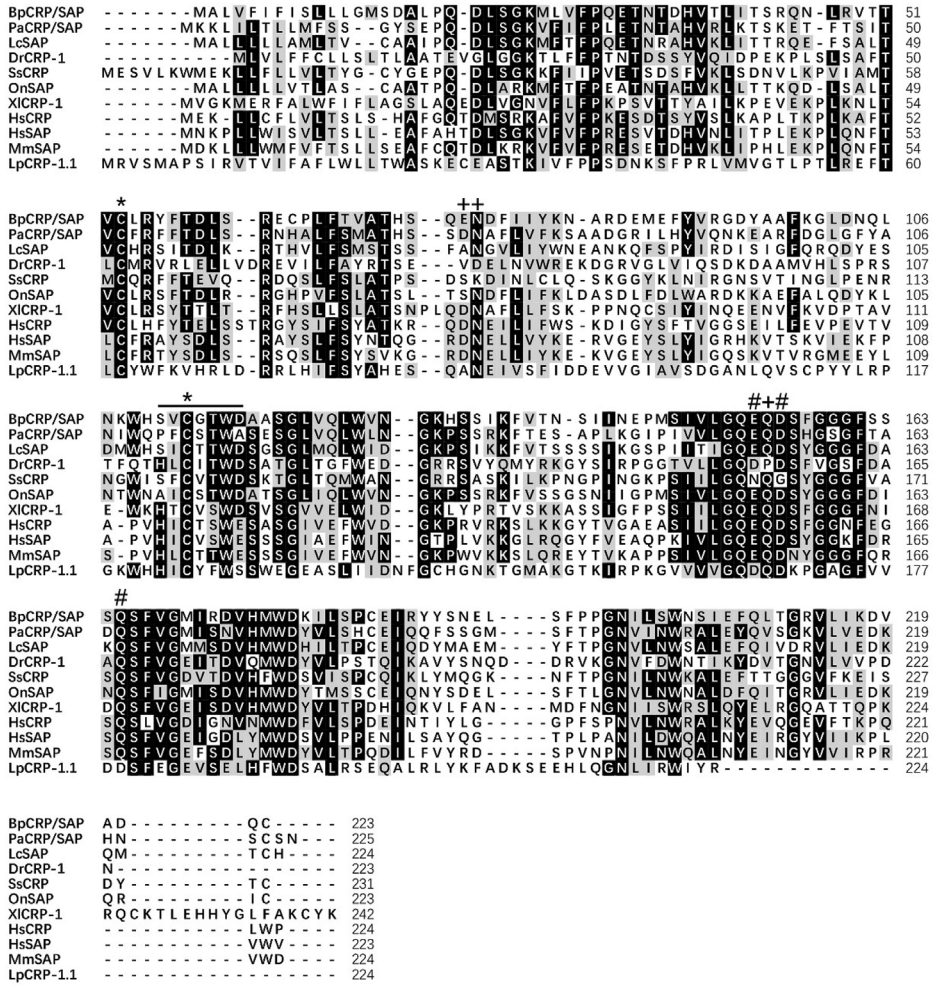

Fig. 1. Multiple alignment of the CRP and SAP amino acid sequences from fish and other animals. The threshold for shading is > 60%; similar residues are denoted by gray shading, identical residues are denoted by black shading, and alignment gaps are denoted as "-". PaCRP/SAP: ayu CRP/SAP; LcSAP: large yellow croaker SAP; DrCRP-1: zebrafish CRP-1; SsCRP: Atlantic salmon CRP; OnSAP: Nile tilapia SAP; XlCRP-1: African clawed frog CRP-1; HsCRP: human CRP; HsSAP: human SAP; MmSAP: mouse SAP; LpCRP-1.1: horseshoe crab CRP-1.1. The accession numbers for these sequences are listed in Table 1. The two conserved cysteine residues in all selected sequences are denoted by "*". The conserved PTX signature is indicated by a horizontal line. The two binding sites for calcium ions are denoted by "+" (site 1) and "#" (site 2).

intensity (MFI) of the rBpCRP/SAP-treated group was expressed as fold-change relative to that of the PBS-treated group (assigned a value of 100).

2.8. In vitro bacterial killing assay

A bacterial killing assay was performed as previously described [26]. Mudskipper MO/MΦ were pre-treated with 20 μg/mL rBpCRP/SAP protein or PBS for 8 h, and then infected with live *E. tarda* at an MOI of 10. MO/MΦ phagocytosis of bacteria proceeded at 24 °C in RPMI 1640 (10% FBS) with 5% CO_2 for 30 min. The remaining bacteria in the culture were killed using gentamicin (50 μg/mL) and MO/MΦ were washed with sterile PBS. The medium was replaced with RPMI 1640 (10% FBS, 50 μg/mL gentamicin) and each set of MO/MΦ was divided into two groups. One group (the uptake group) was immediately lysed with 1% Triton X-100 and plated onto tryptone soya agar (TSA) plates to evaluate bacterial uptake values, and the remaining group (the kill group) was incubated for a further 1.5 h before being lysed and plated on TSA plates to evaluate bacterial kill values. Bacterial survival was determined by dividing the colony forming units (CFUs) of the kill group by those of the uptake group. Three independent experiments were performed.

2.9. MO/MΦ polarization assay

The role of rBpCRP/SAP in mudskipper MO/MΦ polarization was further analyzed. The LPS-induced M1 type and the cAMP-induced M2 type MO/MΦ were prepared as previous described [28]. Mudskipper MO/MΦ were pre-stimulated with rBpCRP/SAP (20 μg/mL) or PBS for 8 h and subsequently treated with rBpCRP/SAP (20 μg/mL), LPS (50 μg/mL, *E. coli*, Sigma-Aldrich), or a cAMP analog (dibutyryl cAMP, 500 μg/mL, Sigma-Aldrich) for 6 h. The mRNA expression of M1-type cytokines (pro-inflammatory cytokines TNF-α and IL-1β) and M2-type cytokines (anti-inflammatory cytokines TGF-β and IL-10) was determined.

The activities of induced nitric oxide synthase (iNOS; a marker of M1 polarization) and arginase activity (a marker of M2 polarization) were also evaluated. The activity of iNOS was measured using the Nitric Oxide Synthase Assay Kit (fluorescence probe method, Beyotime, Shanghai, China). rBpCRP/SAP- (20 μg/mL) and PBS-stimulated cells were further treated with LPS and rBpCRP/SAP, respectively, for 18 h at 24 °C, and the PBS-only group was used as a negative control. The relative iNOS activity of each group was expressed as a fold-change of the value in the negative control.

Arginase activity was measured using the Arginase Activity Assay Kit (Sigma-Aldrich). The rBpCRP/SAP- (20 μg/mL) and PBS-stimulated cells were further treated with either cAMP or rBpCRP/SAP, respectively, for 18 h at 24 °C, and the PBS-only group was used as a negative control. The absorbance was read at 430 nm, and arginase activity (units/L) was calculated by comparing with a urea standard curve.

2.10. Statistical analysis

All data are described as the means ± standard error of the mean (SEM). Study results were analyzed by one-way analysis of variance (ANOVA) or the Students *t*-test using SPSS version 13.0 (SPSS Inc,

Chicago, IL, USA). The following p values were considered statistically significant: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3. Results

3.1. Molecular characterization of BpCRP/SAP

The cDNA of *BpCRP/SAP* was 1055 nucleotides (nt) in length, which included a 672 nt ORF encoding a peptide of 223 aa (calculated molecular weight 25.3 kDa). The protein comprised a 17 aa signal peptide and a 206 aa mature protein (MW 23.4 kDa) with a theoretical p*I* of 5.31. Multiple sequence alignment showed that BpCRP/SAP had an atypical PTX signature with the canonical SxCxS/TWxA sequence modified into HxCxS/TWxS. This type of Ser to His change is common in teleosts, and there are two cysteine residues at amino acids 53 and 113, which are commonly conserved in all teleost PTXs. Two conserved binding sites for calcium ions were also found in BpCRP/SAP: one at Glu75, Asn76, and Gln154, whereas the second, loosely located at Glu153, Asp155, and Gln165, with a buffer-derived acetate lattice bridging the two together (Fig. 1).

Sequence comparison revealed that BpCRP/SAP protein shared the highest amino acid identity (59.8%) with mudskipper CRP/SAP-l3. Phylogenetic tree analysis of protein amino acid sequences showed that the CRP and SAP of mammals, reptiles, and birds grouped together to form a distinct clade (Fig. 2). The CRP and SAP homologs of amphibians and most fish formed another clade, which included BpCRP/SAP (Fig. 2). The CRP and SAP homologs of some fish, including zebrafish CRP-1 and CRP-2, grass carp PTX, common carp CRP-I and PTX, Atlantic cod CRP-II, and spotted green pufferfish PTX formed another clade, which was distantly related to the two larger clades (Fig. 2). BpCRP/SAP was found to be most closely related to mudskipper CRP/SAP-l3 (Fig. 2).

3.2. Tissue distribution and expression analysis of BpCRP/SAP

RT-qPCR revealed that *BpCRP/SAP* transcripts were constitutively expressed in all tested tissues of healthy mudskipper, including the gill, spleen, intestine, heart, skin, brain, kidney, and liver, with the highest transcript levels found in the liver (Fig. 3A). Upon *E. tarda* infection, *BpCRP/SAP* transcripts in the liver significantly increased at 4 and 8 hpi, and subsequently decreased to a level lower than that of the control at 12 hpi before increasing again at 48 hpi (Fig. 4B). *BpCRP/SAP* transcripts in the kidney decreased significantly at 4 and 8 hpi, then increased at 12 and 24 hpi and returned to the control level at 48 hpi (Fig. 4C). *BpCRP/SAP* transcripts in the spleen decreased significantly at 8 hpi, then increased at 12 and 24 hpi and returned to the control level at 48 hpi (Fig. 4C).

3.3. Production of rBpCRP/SAP and antiserum

After the pMAL-BpCRP/SAP plasmid was constructed and subsequently transformed into *E. coli* BL21(DE3), overexpression of the fusion protein rBpCRP/SAP-MBP was induced by IPTG. The protein was then purified using amylose resin and examined by SDS-PAGE. The size of rBpCRP/SAP-MBP observed in SDS-PAGE was approximately 70 kDa, which was similar to the value expected based on previous calculations (43.8 kDa MBP-tag + 23.4 kDa BpCRP/SAP mature peptide + 2.86 kDa 8 × His-tag) (Fig. 4A). The purified rBpCRP/SAP-MBP was then digested by enterokinase to remove the MBP-tag, and the rBpCRP/SAP was further purified using an Ni-NTA column (Fig. 4A). The anti-BpCRP/SAP antiserum was subsequently produced by immunizing rabbits with the purified protein. Using this antiserum, western blot analysis revealed that the size of native BpCRP/SAP in mudskipper serum was approximately 23 kDa (Fig. 4B), which was similar to the calculated value. This suggested that BpCRP/SAP existed in the serum as a protein without N-linked glycosylation, which was consistent with the sequence analysis detailed above. Alternation of BpCRP/SAP in the serum of mudskipper upon *E. tarda* infection was also determined by western blot using the antiserum. The results showed that the level of serum BpCRP/SAP protein increased significantly at 4 hpi (~2.9-fold), 8 hpi (~5.6-fold), and 12 hpi (~5.0-

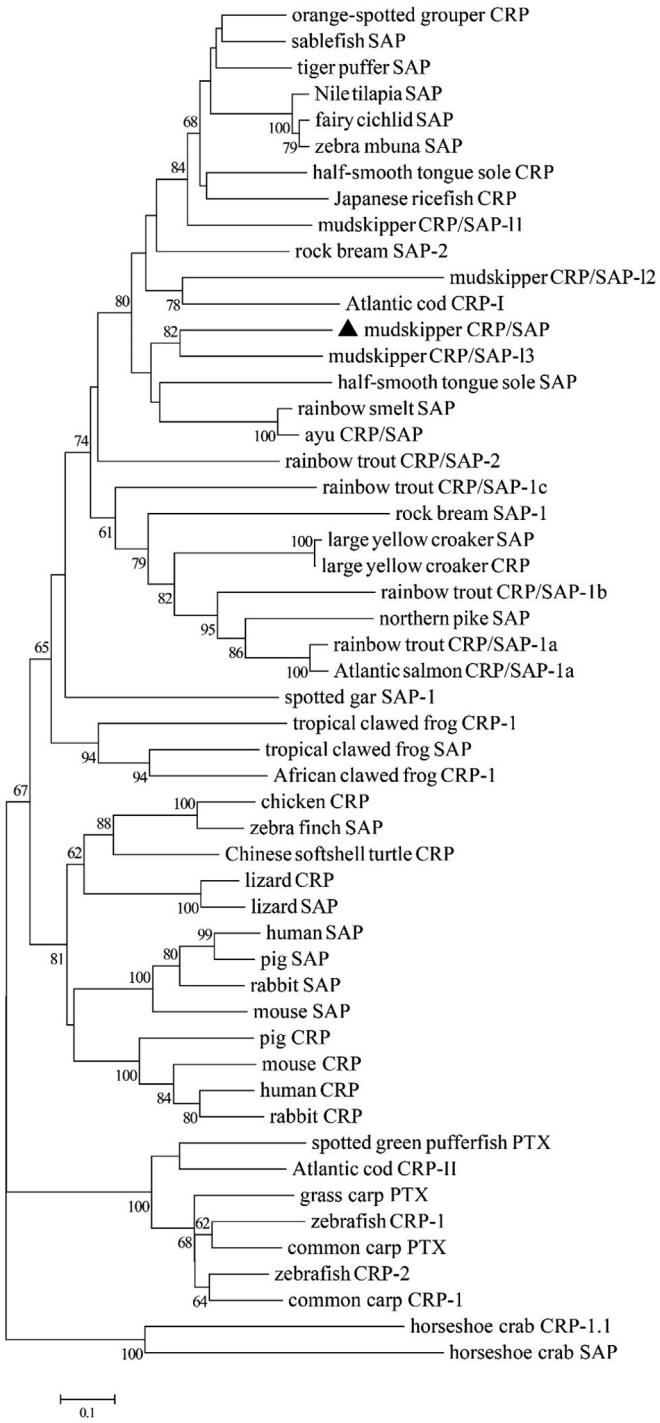

Fig. 2. Phylogenetic tree analysis of the complete amino acid sequence of BpCRP/SAP with other known SAP, CRP, and pentraxin-like homologs using the neighbor-joining method. The sequences of horseshoe crab CRP-1.1 and SAP were used as the outgroup to root the tree. The values at the forks indicate the percentage of trees in which this grouping occurred after bootstrapping the data (1000 replicates; shown only when > 60%). The scale bar shows the number of substitutions per base. Accession numbers of sequences used are listed in Table 1. The site of BpCRP/SAP is marked with ▲.

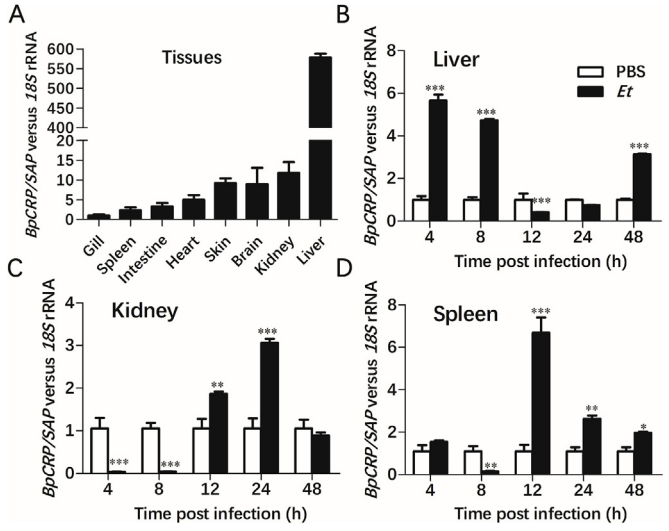

Fig. 3. Relative mRNA expression of BpCRP/SAP in tissues of healthy (A) and *E. tarda*-challenged mudskipper (B–D). The control group was intraperitoneally injected with PBS. Fish were euthanized at 0, 4, 8, 12, 24, and 48 h post-infection (hpi). *Bp18S* rRNA were used as the internal control. Data are expressed as the mean ± SEM of the results from three fish. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

fold) compared with the control (0 hpi), and had returned to those of the control at 24 hpi (Fig. 4C).

3.4. rBpCRP/SAP affects cytokine mRNA expression in E. tarda-stimulated mudskipper MO/MΦ

The mRNA levels of typical proinflammatory cytokine genes (*TNF-α* and *IL-1β*) and anti-inflammatory cytokine genes (*IL-10* and *TGF-β*) were determined to elucidate the effect of rBpCRP/SAP on MO/MΦ (Fig. 5). *E. tarda* stimulation promptly and significantly upregulated the expression of *TNF-α* and *IL-1β*, and rBpCRP/SAP co-treatment further augmented the expression of those genes, especially during the first 3 h of infection (Fig. 5A and B). Expression of the anti-inflammatory cytokine *IL-10* was markedly induced from 4 h after infection; rBpCRP/SAP co-treatment attenuated its expression dramatically (Fig. 5C). Slight fluctuations were observed in the expression of *TGF-β*, which was downregulated following administration of with rBpCRP/SAP (Fig. 5 D).

3.5. rBpCRP/SAP promotes phagocytosis of mudskipper MO/MΦ

As MO/MΦ is essential for bacterial clearance, the potential role of rBpCRP/SAP in the regulation of phagocytosis by mudskipper MO/MΦ was further investigated. rBpCRP/SAP treatment increased the MO/MΦ phagocytosis of FITC-labeled *E. tarda* 1.6-fold compared with the PBS-treated group (Fig. 6A). In addition, measurement of intracellular *E. tarda* CFUs in mudskipper MO/MΦ showed that the bacterial survival rate of the rBpCRP/SAP-treated group (14.10 ± 1.63%) was lower than that of the PBS group (46.43 ± 2.96%) (Fig. 6B).

3.6. Effect of rBpCRP/SAP on mudskipper MO/MΦ polarization

MO/MΦ polarization is known to play an important role in modulating the proinflammatory response in fish [29]. Therefore, the involvement of rBpCRP/SAP in the regulation of LPS-induced M1-type polarization and cAMP-induced M2-type polarization of MO/MΦ was further investigated. rBpCRP/SAP stimulation induced the expression of pro-inflammatory cytokine genes (*IL-1β* and *TNF-α*) and upregulated iNOS activity in mudskipper MO/MΦ, as observed in the LPS-treated

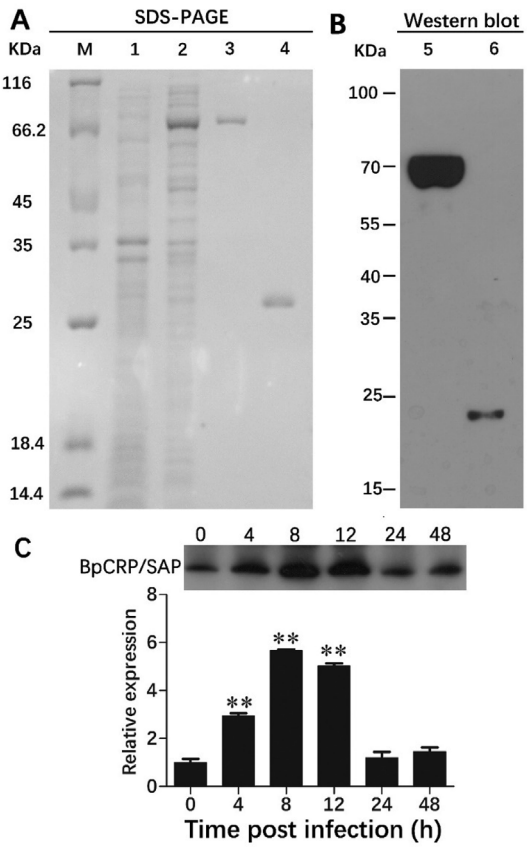

Fig. 4. Prokaryotic expression, antibody preparation, and western blot analysis of BpCRP/SAP. (A) 12% SDS-PAGE analysis of the bacterial lysates and purified rBpCRP/SAP. Lane M: protein marker; 1: the lysates of pMAL-BpCRP/SAP/BL21 before IPTG induction; 2: the lysates of pMAL-BpCRP/SAP/BL21 after IPTG induction; 3: purified rBpCRP/SAP-MBP; 4: purified rBpCRP/SAP. (B) Western blot analysis of rBpCRP/SAP-MAP and native BpCRP/SAP in mudskipper serum; 5: lysates of pMAL-BpCRP/SAP/BL21 after IPTG induction; 6: serum from healthy mudskipper. (C) Western blot analysis of changes in serum BpCRP/SAP protein expression in mudskippers upon *E. tarda* infection. Data are expressed as the mean ± SEM of the results from three fish. $*p < 0.05$ and $**p < 0.01$.

group (Fig. 7A–C). Furthermore, co-administration of rBpCRP/SAP and LPS significantly enhanced the production of NO, compared with the groups administered rBpCRP/SAP or LPS alone (Fig. 7 A). Conversely, rBpCRP/SAP stimulation had no significant effect on the expression of anti-inflammatory cytokine genes (*IL-10*) or on the induction of arginase activity compared with the PBS-treated group (Fig. 7D–F). Furthermore, co-administration of rBpCRP/SAP and cAMP significantly reduced the upregulation of anti-inflammatory cytokines and arginase activity induced by cAMP alone (Fig. 7D–F).

4. Discussion

Short-chain PTXs such as CRP and SAP play key roles in innate immune responses and are involved in pathogen reorganization and clearance [13–15]. In mammals, CRP and SAP are distinct proteins [14,30]. CRP and SAP are produced in the liver in response to IL-6 and are main acute phase reactants in humans and mice, respectively [14]. Structurally, SAP is a plasma glycoprotein which comprises 5 or 10 subunits and human SAP carries a single N-glycosylation site at Asn51, a characteristic which is not shared by CRP [31]. Functionally, SAP and CRP are both soluble PRRs in the serum and are involved in the infection, inflammation and tissue damage processes. They can recognize danger associated molecular patterns (DAMPs) and initiate

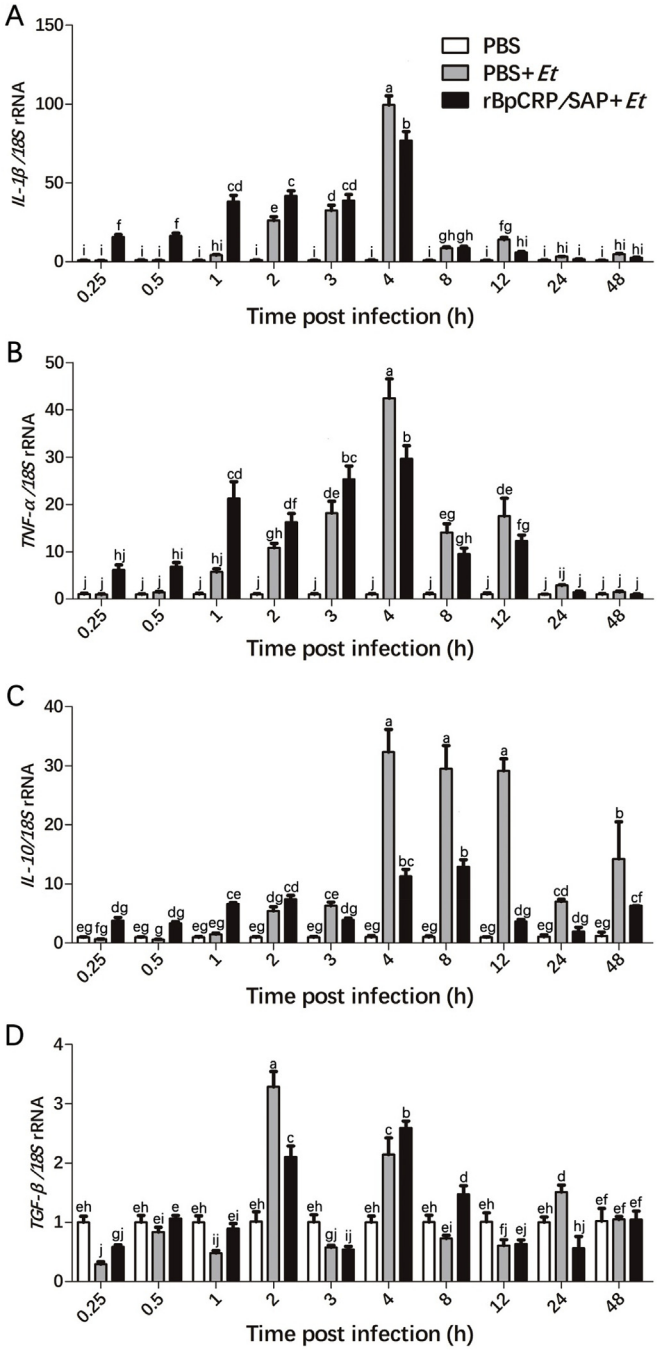

Fig. 5. Effect of rBpCRP/SAP on the mRNA expression of cytokines in *E. tarda*-stimulated mudskipper MO/MΦ. Mudskipper MO/MΦ were pre-treated with PBS or rBpCRP/SAP for 8 h before *E. tarda* infection. Cells were collected at 0.25, 0.5, 1, 2, 3, 4, 8, 12, 24, and 48 h post-infection, and the relative expressions of *IL-1β* (A), *TNF-α* (B), *IL-10* (C), and *TGF-β* (D) were analyzed using *Bp18S* rRNA as a control. Data represent the means ± SEM of three fish. Different letters denote significant differences ($p < 0.05$).

inflammatory signaling pathways [32]. Besides, they are also involved in pathogen and apoptotic cells clearance [33]. Despite the above mentioned functional similarities, CRP and SAP sometimes have opposite effects. For example, SAP has been reported to affect several aspects of innate immune system to reduce fibrosis, while CRP potentiate fibrosis [34]. Also, CRP but not SAP or PTX3 could inhibit proliferation and induce apoptosis of leukemia cell [35]. Despite the in depth study of SAP and CRP in mammals, however, no clear distinction between CRP and SAP is observed in fish either by sequence homology or by phylogenetic tree analysis; thus, it has been suggested to refer to them jointly as CRP/SAP [18,23]. In the present study, a CRP/SAP-like gene from mudskipper with an atypical PTX signature was investigated. BpCRP/SAP shared the highest amino acid identity with mudskipper CRP/SAP-l3, and phylogenetic tree analysis showed that BpCRP/SAP belonged to a clade consisting of CRP and SAP homologs from amphibians and most fish. The highly conserved nature of these short chain PTXs suggests that these molecules were important during vertebrate evolution.

The short-chain PTXs are typical acute-phase proteins (APPs), and many fish CRP/SAPs have proven to be positive acute-phase reactants [36,37]. In Atlantic salmon, the serum SAP level was found to significantly increase in response to *Aeromonas salmonicida* infection [38]. In half-smooth tongue sole, SAP mRNA expression was significantly increased in the liver, kidneys, and spleen following infection with *Vibrio harveyi* or megalocytivirus [39]. In rock bream, the mRNA expression of SAP1 and SAP2 was markedly upregulated in the liver upon *E. tarda*, *Streptococcus iniae* or red seabream iridovirus infection [40,41]. In the present study, the highest level of *BpCRP/SAP* mRNA expression among the healthy mudskipper tissues was detected in the liver. Upon *E. tarda* infection, *BpCRP/SAP* mRNA expression levels were altered in immune tissues at most time points studied. Additionally, the levels of serum BpCRP/SAP protein increased in a time-dependent manner following *E. tarda* infection. Taken together, these results suggested that BpCRP/SAP is involved in the immune response of mudskipper against pathogens.

Previous research into SAP/CRP in fish has mainly focused on their interaction with pathogens [23,40–44], and studies focusing on the influence of SAP/CRP on MO/MΦ function have been rare [23]. In the present study, rBpCRP/SAP co-treatment was found to augment the *E. tarda*-induced expression of pro-inflammatory cytokines and markedly attenuate the expression of anti-inflammatory cytokines, indicating that BpCRP/SAP may have a pro-inflammatory role with regard to regulating MO/MΦ function during infection. In mammals, SAP and CRP can promote phagocytosis by interacting with Fcγ receptors to activate spleen tyrosine kinase (syk). Then, they can regulate actin dynamics to increase the phagocytosis of MO/MΦ [45–47]. In the present study, BpCRP/SAP was found to promote MO/MΦ phagocytosis and subsequent bacterial killing. In fish, the Fcγ receptor has not been identified in the genome database; however, an Fcγ-like receptor that was suggested to exist in Japanese seabass, was able to promote phagocytosis following treatment with mouse anti-FcγR antibodies [48,49]. Recently, ayu CRP/SAP was reported to inhibit complement-mediated opsonophagocytosis by MO/MΦ, and CRP/SAP alone had no significant influence on phagocytosis in the same species [23]. Therefore, further studies investigating the molecular mechanisms of MO/MΦ activation by BpCRP/SAP are needed.

As reported before, mononuclear phagocytes can also be polarized into M1 and M2 types [50]. The M1 types are referred to as classical activated macrophages, and are induced by IFN-γ either alone or in conjunction with microbial products such as LPS, cytokines such as TNF-α, or unknown pro-inflammatory cytokines that produce toxic intermediates (nitric oxide, reactive oxygen intermediates) [51]. iNOS produce M1-type macrophages. M2 types are referred to as activated macrophages; these aid wound healing and tissue repair, and inhibit damaging immune system processes through the secretion of anti-inflammatory cytokines such as IL-10. The M2 type can be induced by IL-4, IL-13, toll like receptors (TLRs), IL-1R agonists, IL-10, and cAMP (in fish), and is characterized by arginase activity [52–54]. In mammals, SAP has been reported to be involved in macrophage polarization in a manner dependent on the micro-environment [15]. For example, in mouse models of renal fibrosis, SAP can decrease the expression of M1 markers and increase the expression of M2 markers of kidney macrophages [55], while in pulmonary macrophages, SAP administration has been shown to promote polarization of the M1 type [56]. Proteomic analyses have indicated that CRP can also regulate the phenotype of

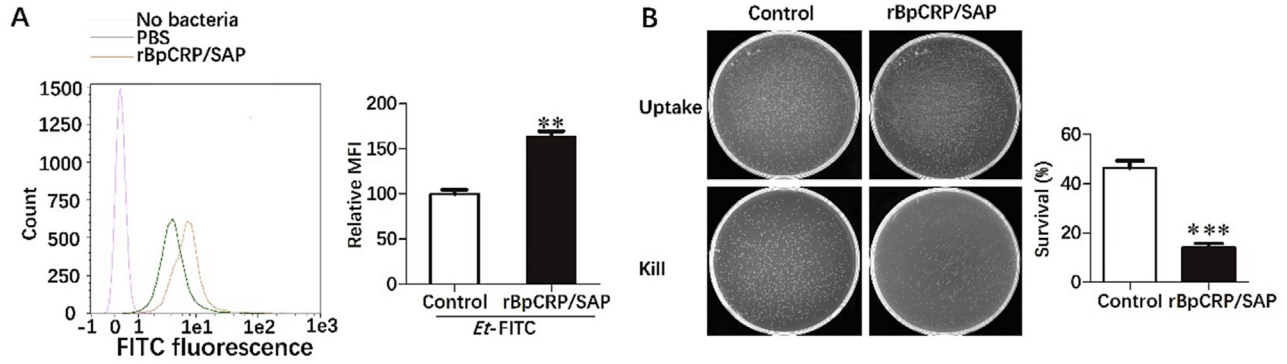

Fig. 6. Effect of rBpCRP/SAP on phagocytosis and bacterial killing of mudskipper MO/MΦ. (A) rBpCRP/SAP promotes phagocytosis of *E. tarda* by mudskipper MO/MΦ. MO/MΦ were pre-incubated with rBpCRP/SAP or PBS for 8 h. *Et*-FITC was then added at a MOI of 10 and cells were incubated for an additional 30 min. Phagocytosis of *Et*-FITC by rBpCRP/SAP- or PBS-treated MO/MΦ was determined by flow cytometry, and MFI was presented as fold-change compared with the PBS-treated group, which was assigned a value of 100. (B) Effect of rBpCRP/SAP on bacterial killing of mudskipper MO/MΦ. MO/MΦ were infected with live *E. tarda* following treatment with PBS or rBpCRP/SAP. The viability of *E. tarda* was examined using CFU assays, and the effect of rBpCRP/SAP on MO/MΦ bactericidal activity was determined using a plate-counting method. The plates presented the survival of *E. tarda* under the different treatments. Percent bacterial survival was calculated by dividing the CFU counts of the kill group by those of the uptake group. Data are expressed as the mean ± SEM of the results from three replicates. $^{**}p < 0.01$.

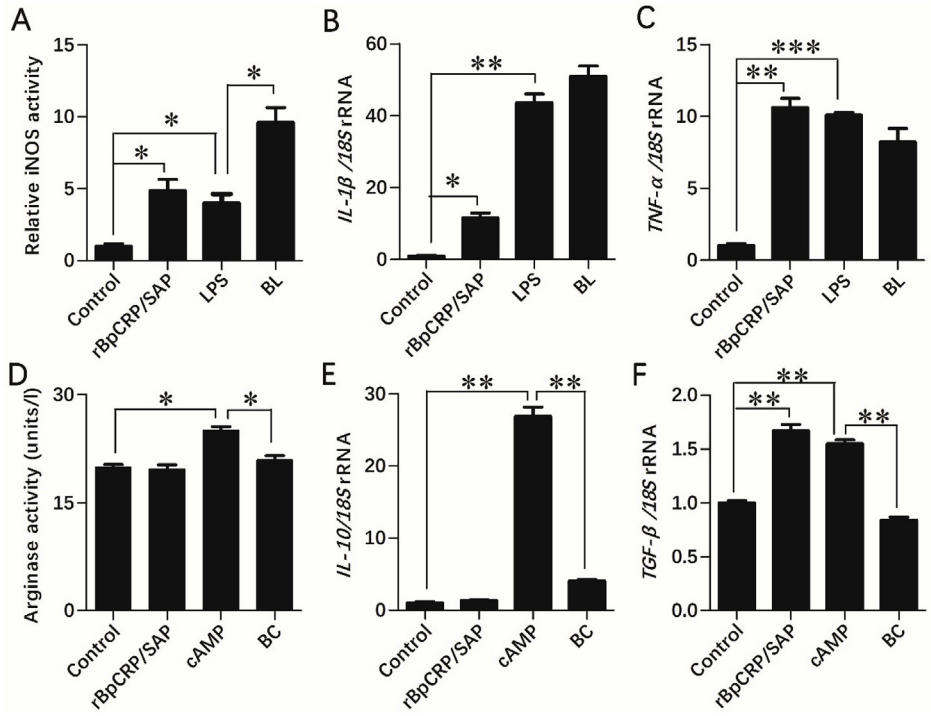

Fig. 7. rBpCRP/SAP promotes the polarization of M1-type mudskipper MO/MΦ. Mudskipper MO/MΦ were pre-stimulated with rBpCRP/SAP or PBS for 8 h before being further treated with rBpCRP/SAP, LPS (*E. coli*), or cAMP for 6 h (to evaluate cytokine expression) or 18 h (to evaluate iNOS/arginase activity). The group treated with PBS only was used as a negative control. (A–C) rBpCRP/SAP administration promoted the LPS-induced polarization of M1-type mudskipper MO/MΦ. iNOS activity (A) and mRNA expression of pro-inflammatory cytokine genes *IL-1β* (B) and *TNF-α* (C) were determined. (D–F) rBpCRP/SAP administration inhibited the cAMP-induced polarization of M2-type mudskipper MO/MΦ. Arginase activity (D) and mRNA expression of anti-inflammatory cytokine genes *IL-10* (E) and *TGF-β* (F) were determined. BL represents the co-administration of rBpCRP/SAP and LPS. BC represents the co-administration of rBpCRP/SAP and cAMP. Data represent the means ± SEM of three replicates. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

monocytes [17] Also, CRP with ligands can promote M2 macrophage differentiation to fibroblasts through FcγR activation, which may result in an anti-inflammatory effect despite a proinflammatory T cell environment caused by oxidized lipids [34]. In the present study, rBpCRP/SAP treatment promoted LPS-induced M1-type polarization, characterized by increased expression of pro-inflammatory cytokines and enhanced iNOS activity (BL-treated and LPS-treated groups). Alternatively, cAMP-induced M2-type polarization was inhibited by rBpCRP/SAP, as shown by the decreased expression of anti-inflammatory cytokines and decreased arginase activity (BC-treated and cAMP-treated groups). These data indicate that BpCRP/SAP may play a positive role in the M1-type polarization of mudskipper kidney-derived MO/MΦ and inhibit M2-type polarization.

In summary, this study identified a CRP/SAP homolog from mudskipper and determined its expression pattern upon *E. tarda* infection. BpCRP/SAP was found to be involved in the regulation of pro-inflammatory responses of mudskipper MO/MΦ, as indicated by phagocytosis, bacterial killing, and cytokine expression. BpCRP/SAP also played a role in M1-type MO/MΦ polarization. Hence, further study on how BpCRP/SAP or teleost CRP/SAP initiate signaling pathways is required.

Acknowledgements

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