Fish and Shellfish Immunology 86 (2019) 1151–1161

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Glucocorticoid receptor in ayu (*Plecoglossus altivelis*): Genomic and non-genomic effects on monocytes/macrophages function

Ying-Jun Ning[a], Si-Ying Chen[a], Xin-Jiang Lu[a,b,*], Jian-Fei Lu[a], Jiong Chen[a,b,**]

[a] Laboratory of Biochemistry and Molecular Biology, School of Marine Sciences, Ningbo University, Ningbo, 315211, China
[b] Key Laboratory of Applied Marine Biotechnology of Ministry of Education, Ningbo University, Ningbo, 315211, China

ARTICLE INFO

Keywords:
Glucocorticoid receptor
Ayu
Non-genomic pathway
Immune regulation
Monocyte/macrophage

ABSTRACT

The glucocorticoid receptor (GR) is an important feedback regulator of the hypothalamic-pituitary-interrenal (HPI) axis. However, there are a limited number of studies focused on host-pathogen interactions in which an association between GR and immune response has been evaluated in monocytes/macrophages (MO/MΦ) after being challenged with highly pathogenic bacteria. Here, we cloned the cDNA sequence of the glucocorticoid receptor (PaGR) gene from ayu fish. The PaGR transcript was expressed in all tissues, and changes in expression were observed in immune tissues and MO/MΦ after live *Vibrio anguillarum* infection. Subsequently, PaGR was expressed and purified to prepare anti-PaGR antibodies. We analyzed the subcellular localization of PaGR. PaGR was expressed not only in the intracellular space but also in the plasma membrane. PaGR activation decreased the expression of pro-inflammatory cytokines and increased the expression of anti-inflammatory cytokines. However, PaGR activation suppressed the phagocytosis activity of *V. anguillarum*-infected ayu MO/MΦ via a non-genomic pathway. Interestingly, PaGR activation could enhance MO/MΦ bacterial killing capability and apoptosis. Therefore, PaGR may modulate the immune response in ayu MO/MΦ by genomic and non-genomic pathways.

1. Introduction

Glucocorticoids, including cortisol and corticosterone, are secreted by the cortex of the adrenal gland [1,2]. These hormones are widely used as effective anti-inflammatory and immunosuppressive agents in the treatment of many autoimmune, allergic, and inflammatory diseases [3]. In teleosts, cortisol is the primary glucocorticoid in circulation and is produced by the interregnal cells of the head kidney in response to a stressor [4]. The neuroendocrine stress response in fish is mediated by the hypothalamic-pituitary-interrenal (HPI) axis, which is responsible for promoting the synthesis and secretion of cortisol [5,6]. The relationship between the immune system and HPI-axis indicates that hormones are important modulators of this system [7]. Cortisol action is mediated by two corticosteroid receptors (CRs), a glucocorticoid receptor (GR), and a mineralocorticoid receptor (MR); the GR is considered the primary receptor for cortisol action in teleosts [8]. GR is an important feedback regulator of the HPI axis and play a key role in mediating the stress effects of cortisol [6,9].

GR, a nuclear hormone receptor, belongs to the superfamily of ligand-activated transcription factors. Like other members of the family, GR possesses a modular structure consisting of three major domains: the N-terminal activation function-1 domain (AF-1), DNA binding domain (DBD), and a C-terminal ligand binding domain (LBD) [10]. GR signaling interacts with the immune system through two different mechanisms, namely genomic and non-genomic [11]. Upon ligand binding, the GR translocates into the nucleus, where it can regulate the expression of a diverse range of inflammatory and anti-inflammatory genes [12]. However, a recent study has shown non-genomic effects that are mainly mediated through glucocorticoid binding to plasma membrane GR (mGR), which subsequently activates kinase cascades in various tissues [13,14]. GR limits mortality and cytokine production by inducing anti-inflammatory genes [15] and protects macrophages in an LPS-induced shock model [14]. In monocytes, GR signaling is involved in the regulation of apoptosis, adhesion, chemotaxis, phagocytosis, and reactive oxygen metabolism, and can influence monocyte targeting to specific macrophage subpopulations [7,11]. In mice, it has been found that GR signaling in macrophages is involved in cell-and tissue-specific actions of glucocorticoids and plays a crucial role in tissue-repair mechanisms [16].

In teleosts, GR is expressed in almost every cell and regulates genes

* Corresponding author. Laboratory of Biochemistry and Molecular Biology, School of Marine Sciences, Ningbo University, Ningbo, 315211, China.
** Corresponding author. Laboratory of Biochemistry and Molecular Biology, School of Marine Sciences, Ningbo University, Ningbo, 315211, China.
E-mail addresses: lxj711043@163.com (X.-J. Lu), jchen1975@163.com (J. Chen).

https://doi.org/10.1016/j.fsi.2018.12.065
Received 16 November 2018; Received in revised form 25 December 2018; Accepted 27 December 2018
Available online 28 December 2018
1050-4648/

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

Accession number	Species		Gene
	Latin name	English name	
MK086012	*Plecoglossus altivelis*	ayu	GR
XM_011483198	*Oryzias latipes*	Japanese ricefish	GR1
NM_001163133	*Oryzias latipes*	Japanese ricefish	GR2
NM_001124730	*Oncorhynchus mykiss*	rainbow trout	GR1
AY495372	*Oncorhynchus mykiss*	rainbow trout	GR2
AF263738	*Haplochromis burtoni*	Burton's mouthbrooder	GR1
AF263740	*Haplochromis burtoni*	Burton's mouthbrooder	GR2
AJ879149	*Cyprinus carpio*	common carp	GR1
AM183668	*Cyprinus carpio*	common carp	GR2
EF436284	*Danio rerio*	zebrafish	GR
MF766011	*Ictalurus punctatus*	channel catfish	GR1
MF766012	*Ictalurus punctatus*	channel catfish	GR2
XM_010729315	*Larimichthys crocea*	large yellow croaker	GR1
XM_010754714	*Larimichthys crocea*	large yellow croaker	GR2
ENSTRUT00000015714	*Takifugu rubripes*	tiger puffer	GR1
ENSTRUT00000018490	*Takifugu rubripes*	tiger puffer	GR2
ENSONIT00000010671	*Oreochromis niloticus*	Nile tilapia	GR1
ENSONIT00000022590	*Oreochromis niloticus*	Nile tilapia	GR2
XM_010871107	*Esox lucius*	northern pike	GR1
XM_010871111	*Esox lucius*	northern pike	GR2
AY619996	*Dicentrarchus labrax*	European sea bass	GR1
AY549305	*Dicentrarchus labrax*	European sea bass	GR2
XM_018675716	*Lates calcarifer*	barramundi perch	GR
AY863149	*Salmo trutta*	brown trout	GR
DQ486890	*Sparus aurata*	gilthead seabream	GR
AB013444	*Paralichthys olivaceus*	Japanese flounder	GR
DQ227738	*Gallus gallus*	chicken	GR
ENSMUSG00000024431	*Mus musculus*	mouse	GR
ENSG00000113580	*Homo sapiens*	human	GR
EF567113	*Danio rerio*	zebrafish	MR
AJ783704	*Cyprinus carpio*	common carp	MR
XM_019256038	*Larimichthys crocea*	large yellow croaker	MR
XM_011612957	*Takifugu rubripes*	tiger puffer	MR
NM_001163129	*Oryzias latipes*	Japanese ricefish	MR
XM_014209388	*Salmo salar*	Atlantic salmon	MR
NM_001124483	*Oncorhynchus mykiss*	rainbow trout	MR
M16801	*Homo sapiens*	human	MR
NM_001083906	*Mus musculus*	mouse	MR

controlling development, metabolism, and immune response [17,18]. Several teleosts contain two GR genes, GR1 and GR2 [8]. Other groups have found only one GR in some species thus far, such as the Japanese flounder, brown trout, and zebrafish [19]. Furthermore, GR has been identified in a number of teleost species such as rainbow trout (*Oncorhynchus mykiss*) [5], common carp (*Cyprinus carpio L*) [20], and zebrafish (*Danio rerio*) [21]. Currently, there are limited studies focused on host-pathogen interactions in which an association between GR and immune response has been evaluated in macrophages after being challenged with highly pathogenic bacteria.

Plecoglossus altivelis, commonly referred to as ayu, is an economically important fish in East Asia. Bacterial diseases caused by *V. anguillarum* have become widespread in this fish [22]. Innate immune response is considered to be the first line of host defense in opposing pathogenic infection in fish [23]. The innate immune response of teleost fishes occurs primarily in lymphoid organs such as head kidney and spleen, which produce cells and humoral parameters responsible for clearing a pathogen [24]. A deeper understanding of the regulation of the fish's innate immune response is currently required. In teleosts, transcription factors have been found to regulate the expression of immune genes. However, the intracellular signaling mechanisms of ayu monocytes/macrophages (MO/MΦ) are still unknown. Given the important roles of transcription factors in the inflammatory response, studying and investigating their possible roles in pathological processes is a worthwhile endeavor. Therefore, the aim of this study was to evaluate the GR profile and modulation of stress- and innate immune-related genes in macrophages challenged with *Vibrio anguillarum*. Here, we determined the cDNA sequence of GR (PaGR) from ayu and analyzed the association between PaGR mRNA expression and protein levels after *V. anguillarum* infection. The subcellular localization of PaGR was analyzed. Moreover, the effect of PaGR on the expression of inflammatory cytokines, phagocytosis, bacterial killing, and apoptosis of MO/MΦ by genomic or non-genomic pathways during infection were investigated.

2. Materials and methods

2.1. Fish maintenance

Healthy ayu weighing 40–50 g each were purchased from a fishery in Ninghai County, Ningbo City, China. The fish were kept in freshwater tanks at 20–22 °C in a recirculating system using filtered water and held in the laboratory for ≥2 weeks, maintaining a healthy appearance and normal activity prior to use in experiments. All fish used in this study were healthy and without any pathological indications. All experiments were performed according to the Experimental Animal Management Law of China and approved by the Animal Ethics Committee of Ningbo University, and were carried out in compliance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Table 2
Oligonucleotide Primers used in this study.

Primer	Gene	Accession number	Nucleotide sequence (5′→3′)	Primer Usage
PaGRpF	*GR*	MK086012	CGGATCCCTCATCCCCACCATGCTGTC[a]	Prokaryotic expression
PaGRpR			GGAATTCTCATTTCTGGTGAAAGAGCAGAG[b]	
PaGRF	*GR*	MK086012	TACATCAAGGAGCTGGGCAA	RT-qPCR
PaGRR			ACTGCCAGCCTTGAACTTTG	
PaTNF-αF	*TNF-α*	JP740414	ACATGGGAGCTGTGTTCCTC	RT-qPCR
PaTNF-αR			GCAAACACACCGAAAAAGGT	
PaIL-1βF	*IL-1β*	HF543937	TACCGGTTGGTACATCAGCA	RT-qPCR
PaIL-1βR			TGACGGTAAAGTTGGTGCAA	
PaIL-10F	*IL-10*	JP758157	TGCTGGTGGTGCTGTTTATGTGT	RT-qPCR
PaIL-10R			AAGGAGCAGCAGCGGTCAGAA	
PaTGF-βF	*TGF-β*	JP742920	CTGGAATGCCGAGAACAAAT	RT-qPCR
PaTGF-βR			GATCCAGAACCTGAGGGACA	
V. anguillarum 16SF	*16S*	FM866241	AGAGTTTGATCATGGCTCAG	RT-qPCR
V. anguillarum 16SR	*rRNA*		GGTTACCTTGTTACGACTT	
Pa18SF	*18S rRNA*	FN646593	GAATGTCTGCCCTATCAACT	RT-qPCR
Pa18SR			GATGTGGTAGCCGTTTCT	

[a] Underlined sequence represent the restriction site for *Bam*H I.
[b] Underlined sequence represent the restriction site for *Eco*R I.

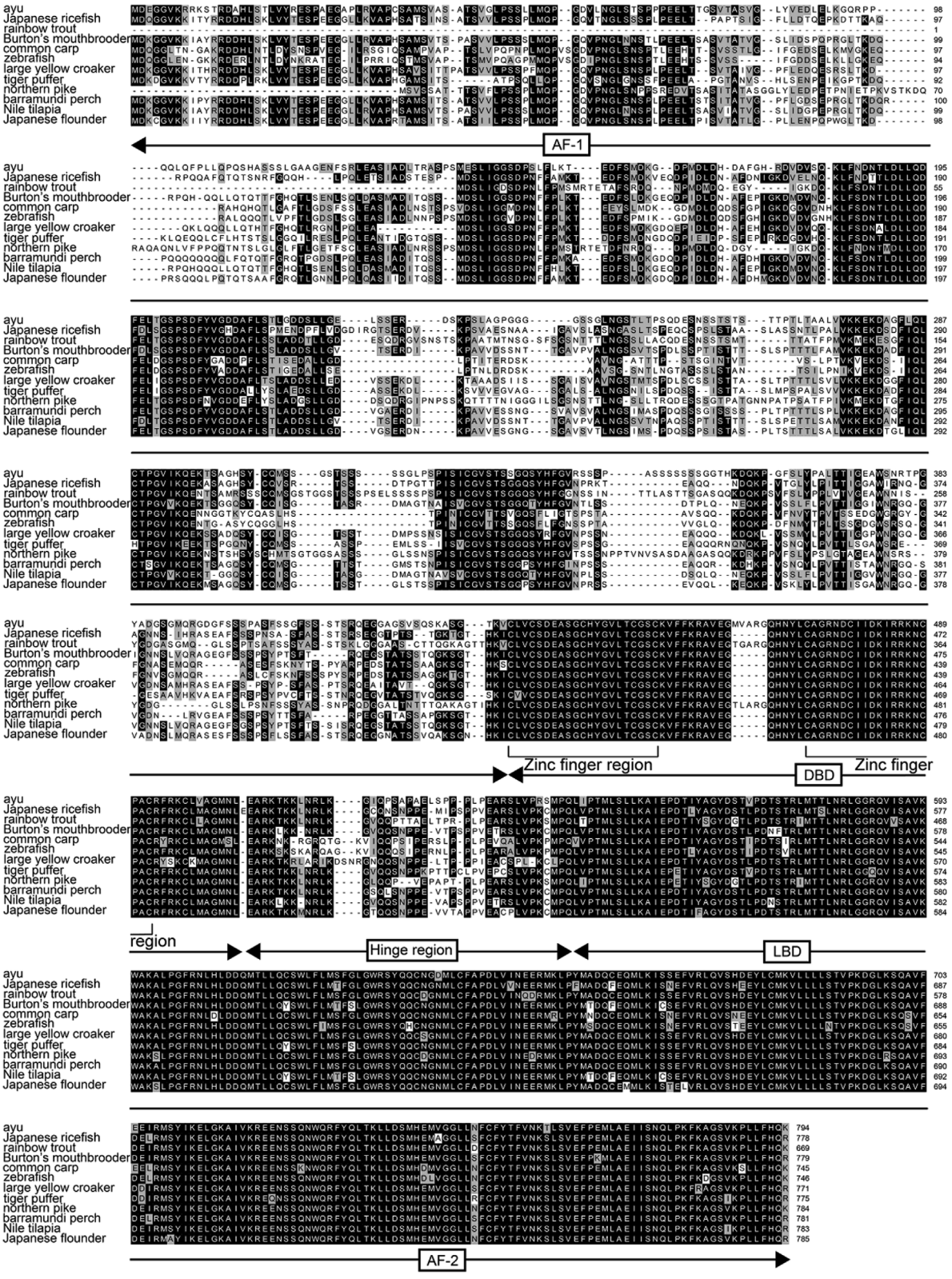

Fig. 1. Multiple alignment of the amino acid sequence of PaGR and other related fish GRs. The threshold for shading was 60%; Similar residues are marked with gray shading, identical residues are marked with black shading, and alignment gaps are marked as "-". The activation function domain (AF-1), DNA-binding domain (DBD), hinge region, ligand-binding domain (LBD), and activation function domain-2 (AF-2) are indicated. The two zinc finger regions are underlined. The GenBank accession numbers of sequences used are listed in Table 1.

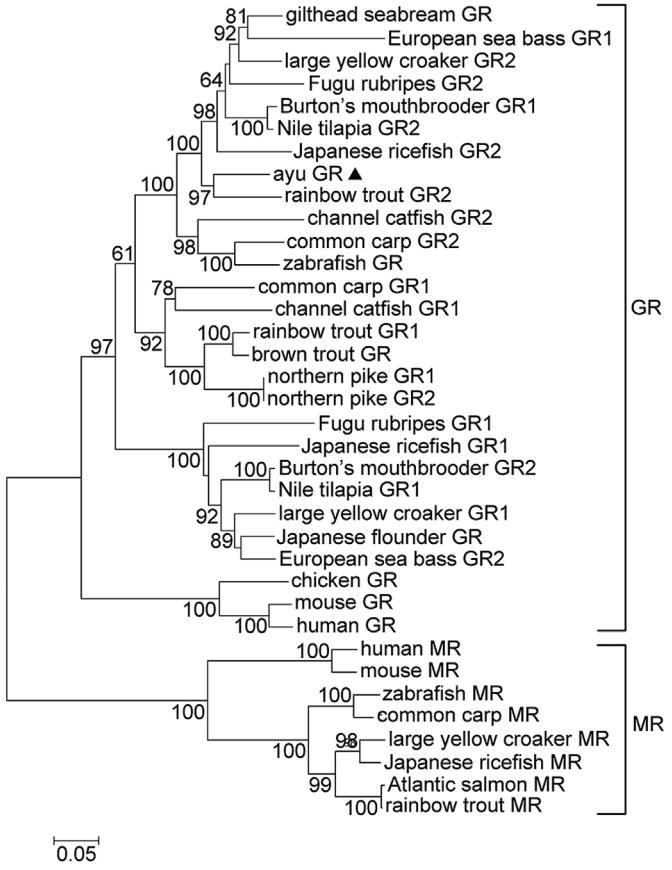

Fig. 2. Phylogenetic (neighbor-joining) analysis of the complete amino acid sequence of PaGR with other related GRs using the MEGA 7 program. The values at the forks indicate the percentage of trees in which this grouping occurred after bootstrapping (1000 replicates; shown only when > 60%). The scale bar shows the number of substitutions per base. The GenBank accession numbers of sequences used are listed in Table 1.

2.2. Cloning and sequence analyses of PaGR

The cDNA sequence of PaGR was identified from transcriptome data of ayu head kidney-derived monocytes/macrophages (MO/MΦ). PCR, cloning, and sequencing were used to confirm the authenticity of the PaGR cDNA. Sequence similarity and identity were analyzed by the BLAST tool (http://www.ncbi.nlm.nih.gov/blast). The prediction of the protein structure was performed by the online tool SMART (http://smart.embl-heidelberg.de/). Additionally, multiple sequence alignments were conducted by the ClustalW program (http://clustalw.ddbj.nig.ac.jp/), and phylogenetic and molecular evolutionary analyses were conducted using the neighbor-joining (NJ) method of MEGA version 7. Sequences used in this study are listed in Table 1.

2.3. Primary culture of MO/MΦ

MO/MΦ were isolated and cultured as previously described [25]. Briefly, ayu were killed by an overdose of anesthetic (0.03% [v/v] ethylene glycol monophenyl ether). Head kidneys were cut into small fragments and dissociated by forcing through a 100 μm nylon mesh. Head kidney leukocyte-enriched fractions were obtained using Ficoll density gradient centrifugation, and seeded into 35-mm culture plates. Non-adherent cells were washed away and the attached cells were incubated with complete RPMI 1640 medium supplemented with 5% fetal calf serum, 5% ayu serum, and 1% penicillin/streptomycin throughout the experiment after overnight incubation at 24 °C. According to Giemsa staining results, over 96% of adherent cells were monocytes/macrophages.

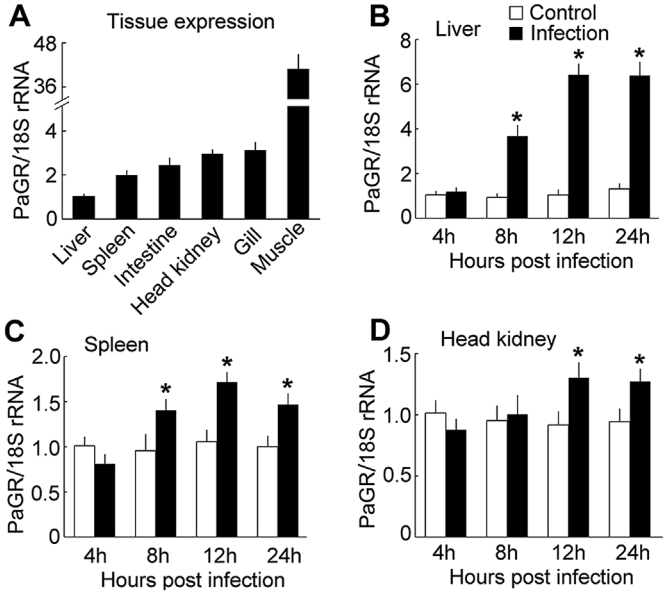

Fig. 3. RT-qPCR analysis of PaGR mRNA expression in various tissues. (A) PaGR mRNA expression in healthy ayu tissues. The PaGR expression level of liver was set to 1. (B–D) PaGR mRNA expression in immune tissues of ayu following live *V. anguillarum* infection. The PaGR expression level of the control at 4 hpi was set to 1 in every graph. Fish were injected intraperitoneally with live *V. anguillarum* for 4, 8, 12, and 24 hpi, PaGR transcript levels were normalized to that of 18S rRNA. Data are expressed as the mean ± SEM. n = 5, $^{*}p < 0.05$.

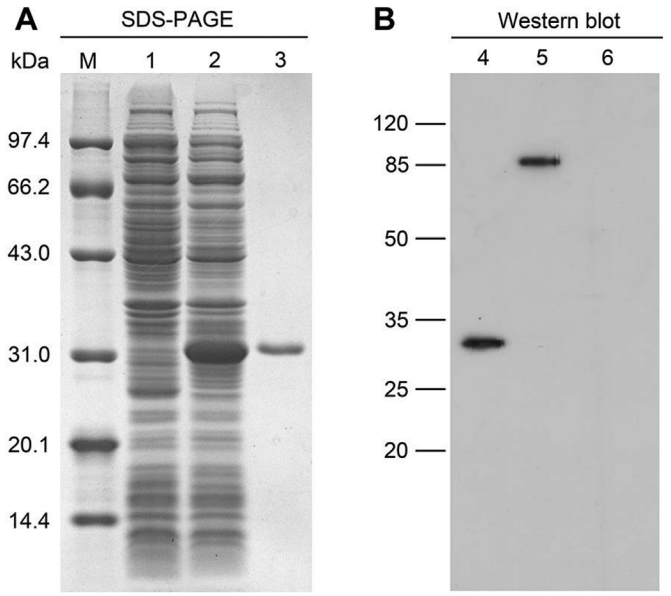

Fig. 4. Prokaryotic expression and western blot analysis of PaGR. (A) The SDS-PAGE analysis of prokaryotically-expressed PaGR. Lane M: protein marker; 1 and 2: protein from BL21 (DE3) transformed with the pET-28a-PaGR plasmid before and after IPTG induction; 3: purified recombinant PaGR. (B) Western blot analysis of the specificity of the antibody for PaGR. Lane 4: purified recombinant PaGR; 5: PaGR in MO/MΦ; 6: negative control (ayu serum).

2.4. Bacterial infection

To investigate the impact of stimuli on PaGR mRNA and protein expression, ayu were infected with live *Vibrio anguillarum* (ayu-H080701, *V. anguillarum* were grown at 28 °C in nutrient broth and

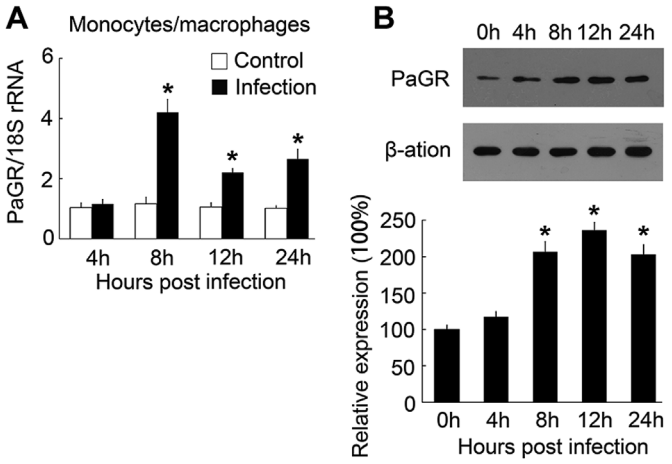

Fig. 5. Alteration of PaGR expression in *V. anguillarum*-infected in MO/MΦ. (A) RT-qPCR was performed to analyze changes in PaGR mRNA in MO/MΦ. PaGR transcript levels were normalized to that of 18S rRNA. Data are expressed as the mean ± SEM. n = 5, $^*p < 0.05$. (B) Western blot analysis was performed to analyze variation in PaGR protein expression in MO/MΦ. Histogram displaying changes in relative band intensity of PaGR in samples collected at 0, 4, 8, 12, and 24 hpi. PaGR protein expression was normalized to that of β-actin. Representative blots of three independent experiments are shown. Data are expressed as the mean ± SEM. $^*p < 0.05$.

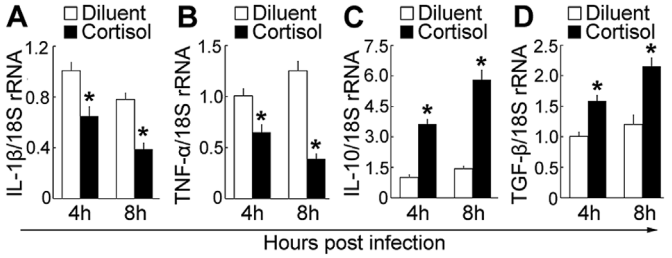

Fig. 6. Effect of PaGR activation by cortisol on inflammatory cytokine mRNA expression in MO/MΦ after live *V. anguillarum* infection. (A–D) MO/MΦ were treated with 100 ng/ml cortisol or diluent after live *V. anguillarum* infection. IL-1β, TNF-α, IL-10, and TGF-β transcripts at different time points were detected. PaGR mRNA transcript levels were normalized to that of 18S rRNA. Diluent as a control group. Data are expressed as the mean ± SEM. n = 5, $^*p < 0.05$.

collected in the logarithmic growth phase). Briefly, infected ayu were injected intraperitoneally with 100 μl bacterial suspension (1.2×10^4 colony-forming units (CFU)/fish), and the control group was challenged with the same volume of PBS. For *in vitro* MO/MΦ treatment, cells were isolated and cultured at a concentration of 2×10^6/ml, and live *V. anguillarum* were added at a multiplicity of infection (MOI) of 2. Fish were sacrificed at 4, 8, 12, and 24 h post-infection (hpi). The tissue and cells were collected and stored at −80 °C until subsequent use.

2.5. Prokaryotic expression and antibody preparation

The cDNA fragment encoding the ligand-binding domain (LBD) of PaGR was amplified with gene-specific primer sets that added *Bam*H I and *Eco*R I (NEB) sites at the 5′ end, respectively (Table 2). Then, the amplicon was inserted into the multiple cloning site of vector pET28a and transformed into *Escherichia coli* BL21 (DE3). The recombinant His-tagged PaGR protein (rPaGR) was induced by isopropyl-b-Dthiogalactopyranoside (IPTG). rPaGR was then purified using a nickel-nitrilotriacetic acid (Ni-NTA) column (QIAGEN, Shanghai, China), and resolved by SDS-PAGE. The endotoxin level in the recombinant protein was less than 0.1 EU/mg after toxin removal with an endotoxin-removal column (Pierce, Rockford, IL). In addition, a polyclonal antiserum against rPaGR was generated by subcutaneously injecting the purified protein into ICR mice as described previously [26]. Protein G HP SpinTrap columns (GE healthcare, New Jersey, USA) were used to purify polyclonal anti-rPaGR IgG from antiserum according to the manufacturer's instructions. The concentration of anti-rPaGR antibody was 2.5 mg/ml as quantified by the Bradford method. For the analysis of the specificity of PaGR antibody, ayu serum was used as a negative control.

2.6. Phagocytosis assay and bacterial killing

An *in vitro* phagocytosis assay of ayu MO/MΦ was performed as previously described [27]. Briefly, *Escherichia coli* DH5α in the logarithmic phase of growth were labeled with fluorescein isothiocyanate (FITC) (Sigma, St. Louis, MO, USA) and designated as DH5α-FITC. MO/MΦ were treated with cortisol (100 ng/ml, Sigma) or cortisol-BSA (10^{-4} M, Cusabio, Wuhan, Hubei, China) for 1 h. To inhibit the activity of PaGR, MO/MΦ were pre-treated with GR antagonist RU486 (10^{-4} M, Sigma) for 30 min before treatment with cortisol or cortisol-BSA. MO/MΦ were treated with diluent as a control group. DH5α-FITC cells were added at a MOI of 10 and incubated for another 30 min. Cells were washed extensively with sterile PBS to remove extracellular particles. Trypan blue (0.4%) was used to quench the fluorescence that resulted from non-specific adhesion of *E. coli*-FITC. Bacterial uptake was measured using a Gallios flow cytometer (Beckman Coulter, Miami, USA) and FlowJo software (TreeStar Inc).

The bacterial killing assay was performed by analyzing CFU as previously described [28] with modifications. A standard curve was generated with the real-time quantitative PCR (RT-qPCR) results to assess the bacterial number. RNA obtained from *V. anguillarum* culture at a concentration of 10^9 CFU/ml was serially diluted 10-fold. Each RNA dilution was used to construct a standard curve. After pretreatment with RU486, cortisol, or cortisol-BSA as previously described, MO/MΦ were infected with live *V. anguillarum* at a MOI of 10 for 30 min and then washed extensively with PBS. One set of samples (uptake group) were collected until RNA extraction. The samples of the kill group were further incubated for 1.5 h to allow for bacterial killing before cell lysis. Cells or bacteria were subjected to RNA template preparation and RT-qPCR using primers 16SF and 16SR (Table 2) for the *V. anguillarum* 16S gene [29]. The Ct values obtained from RT-qPCR were used to calculate the total number of CFU/ml present in all samples, based on the standard curve previously generated. Bacterial survival was determined by dividing the number of CFU in the kill group by those in the uptake group.

2.7. Immunofluorescence microscopy

Immunofluorescence assays were performed as previously described [30]. Briefly, MO/MΦ were fixed in 4% paraformaldehyde in PBS for 30 min and air-dried. Following three washes with PBS, cells were blocked with PBS containing 5% BSA for 1 h at room temperature. The slides were incubated with polyclonal mouse antibody to PaGR (1:50 dilution) at 4 °C overnight. Primary antibodies were removed, and cells were washed three times in PBS before addition of secondary antibodies. Cells were stained with FITC-labeled Goat Anti-Mouse IgG (H + L) (1:500 dilution, Beyotime, Shanghai, China) for 2 h. DAPI (10 μg/ml, Sigma) was used to stain the cell nucleus for 5 min, and rhodamine phalloidin (66 nM, Invitrogen, Carlsbad, CA) was used to stain the filamentous cell membrane for 30 min. Cells were visualized using a laser confocal microscope IX81-FV1000 (Olympus, Tokyo, Japan).

2.8. Detection of apoptosis by flow cytometry

Detection of apoptosis was conducted using a FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's protocol. Briefly, after pretreatment with

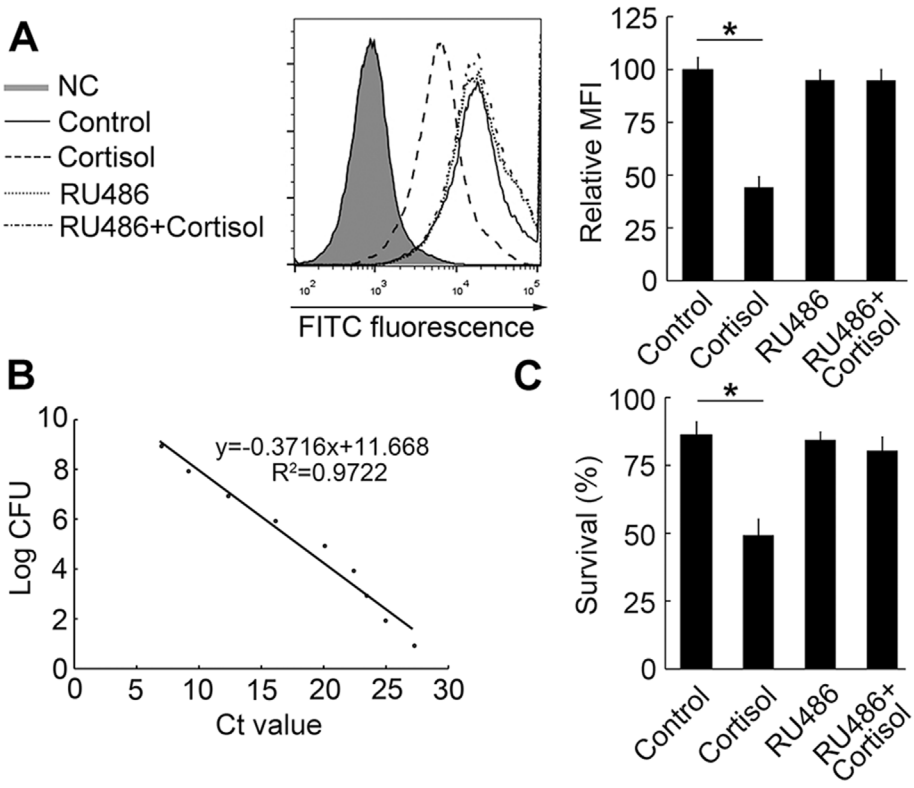

Fig. 7. Effect of PaGR activation by cortisol on the phagocytosis and bacterial killing abilities of ayu MO/MΦ. (A) MO/MΦ were pretreated with RU486 for 30 min and then exposed to 100 ng/ml cortisol for 1 h before FITC-DH5α cells were added at a MOI of 10, followed by incubation for an additional 30 min. Phagocytosis of FITC-DH5α was determined by flow cytometry analysis. The MFI is presented as fold change over the diluent-treated control group, which was assigned an arbitrary unit of 100. (B) A standard curve was generated from 10-fold serial dilutions of live *V. anguillarum*, as detected by RT-qPCR with *V. anguillarum* 16S rRNA. The Ct values are plotted against the corresponding number of *V. anguillarum*. (C) MO/MΦ were pre-treated with RU486 for 30 min and then exposed to 100 ng/ml cortisol for 1 h before live *V. anguillarum* were added at a MOI of 10. The kill group was further incubated for 1.5 h to allow for the killing of bacteria. The effect of cortisol on MO/MΦ bactericidal activity was determined using RT-qPCR. Data are expressed as the mean ± SEM. n = 5, $^*p < 0.05$.

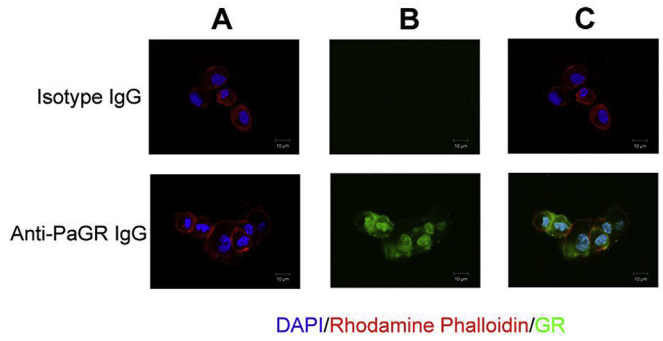

Fig. 8. Expression and co-localization of PaGR in MO/MΦ. (A–C) Immunofluorescent staining of MO/MΦ incubated with isotype IgG or anti-PaGR IgG shows co-localization of PaGR to the cells. Nuclei (blue) were stained with DAPI; plasma membranes (red) were detected with rhodamine phalloidin. Green fluorescence indicates presence of PaGR. Scale bar, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

RU486, cortisol or cortisol-BSA as previously described, MO/MΦ were infected with live *V. anguillarum* at a MOI of 2 for 8 h. MO/MΦ were treated with diluent as a negative control group, and the *V. anguillarum*-infected group without pretreatment served as a positive control. Then the cells were collected, washed with PBS twice, and stained for 30 min in the dark at room temperature with Annexin-V-FITC and PI. Finally, the stained cells were analyzed by flow cytometry using a Gallios flow cytometer (Beckman Coulter). Ten thousand events were recorded for each treatment group and analyzed using FlowJo software (TreeStar Inc).

2.9. Western blotting

The cells were pelleted and lysed in a buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 100 mM NaCl, 0.2 mM DTT, 0.5 mM sodium orthovanadate, and 0.4 mM PMSF [pH 7.4]) containing phosphatase inhibitors (Phosphatase Inhibitor Cocktail; Sigma). The protein concentration was measured in each soluble fraction using the Bradford method. For Western blot analysis, the proteins were resolved using SDS-PAGE, transferred to membranes, incubated with specific antibodies, and visualized using ECL as previously described [25]. The optical density of the bands was quantified using NIH ImageJ software.

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

To assess the level of gene expression of PaGR, fish were anesthetized and sacrificed and tissues of the infected and control fish were collected and preserved at −80 °C until examination. RT-qPCR was performed as previously described [31]. Briefly, total RNA of ayu tissues or MO/MΦ was extracted using an RNeasy® Mini Kit (Qiagen, Maryland, USA) and reverse-transcribed into cDNA using AMV reverse transcriptase (TaKaRa, Dalian, China). The primer sequences for the target genes are listed in Table 2. RT-qPCR were performed on an ABI StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA) using SYBR Premix Ex Taq II (Takara). Amplifications were carried out in a 25 μl reaction volume containing the sample cDNA, primers, and SYBR Premix Ex Taq II. The reaction mixture was incubated for 300 s at 95 °C, followed by 40 amplification cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. The cycle threshold (Ct) values of PaGR and other targets were normalized to housekeeping gene Pa18S rRNA expression for all samples using the $2^{-\Delta\Delta Ct}$ method. All RT-qPCR experiments were carried out in quintuplicate and each assay was repeated five times.

2.11. Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). All data were subjected to one-way ANOVA with SPSS (version 13.0, Chicago, IL, USA). *P* values < 0.05 were considered statistically significant.

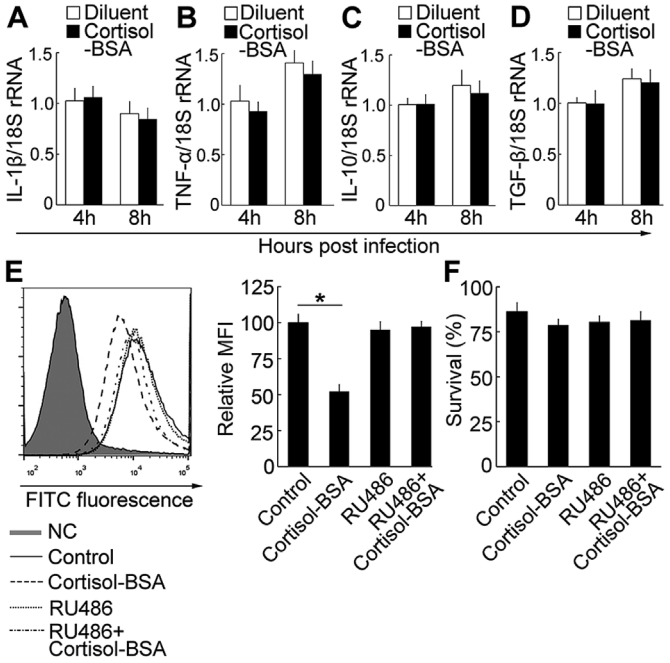

Fig. 9. The effect of PaGR activation by cortisol-BSA on inflammatory cytokine expression, phagocytosis, and bacterial killing abilities of ayu MO/MΦ. (A–D) MO/MΦ were treated with 10^{-4} M cortisol-BSA or diluent after live *V. anguillarum* infection. IL-1β, TNF-α, IL-10, and TGF-β transcripts at different time points were detected. PaGR mRNA transcript levels were normalized to that of 18S rRNA. (E) MO/MΦ were pre-treated with RU486 for 30 min and then exposed to 10^{-4} M cortisol-BSA for 1 h before FITC-DH5α cells were added at a MOI of 10, followed by incubation for an additional 30 min. Phagocytosis of FITC-DH5α was determined by flow cytometry analysis. MFI was presented as fold change over the diluent-treated control, which was assigned an arbitrary unit of 100. (F) The kill group was further incubated for 1.5 h to allow for the killing of bacteria. The effect of cortisol-BSA on MO/MΦ bactericidal activity was determined using RT-qPCR. Data are expressed as the mean ± SEM. n = 5, $^{*}p < 0.05$.

3. Results

3.1. Molecular characterization and sequence analysis of PaGR

The PaGR sequence was deposited in the GenBank Data Library under accession number MK086012. The cDNA was 4236 nucleotides (nt) in length and comprised a large open reading frame (ORF) of 2385 nt, which formed a 794 amino acid (aa) polypeptide. The 794 aa predicted amino acids of the PaGR polypeptide resulted in a calculated molecular weight (MW) of 85.42 kDa and a theoretical isoelectric point (*pI*) of 6.64. PaGR comprises an N-terminal activation function-1 domain (AF-1) at aa position 1–431, a central DNA-binding domain (DBD) at aa position 432–504, a D region (hinge domain) at aa position 505–544, and a C-terminal ligand-binding domain (LBD) at aa position 545–794, with a ligand-dependent activation function domain-2 (AF-2). Multiple alignment of PaGR amino acid sequence with other known fish GR sequences revealed that the DBD and LBD was highly conserved in teleosts, and contained two conserved zinc finger regions in the DBD which are critical for receptor dimerization and target binding (Fig. 1).

Phylogenetic tree analysis grouped teleost GR and mammalian GR together in a cluster that was distinct from the MR cluster. The fish GR clusters were distinct from the mammalian GR. PaGR was most closely related to GR2 from rainbow trout (Fig. 2). The GenBank accession numbers of sequences used are listed in Table 1.

3.2. Spatiotemporal expression of PaGR following live V. anguillarum infection

To illustrate the relationship between *V. anguillarum* infection and PaGR expression, RT-qPCR was performed to analyze the mRNA expression levels of PaGR in normal and infected tissues. As shown in Fig. 3A, PaGR mRNA expression was detected in all tested tissues of healthy ayu, including liver, spleen, intestine, head kidney, gill, and muscle, with the highest expression being found in the muscle. Following infection with live *V. anguillarum*, PaGR mRNA expression was shown to be altered in the tested immune tissues. In the liver and spleen, PaGR mRNA expression was significantly up-regulated at 8, 12, and 24 hpi (Fig. 3B and C). In the head kidney, PaGR mRNA expression was up-regulated at 12 and 24 hpi (Fig. 3D).

3.3. PaGR prokaryotic expression and antibody preparation

The sequence comprising the LBD of PaGR (PaGR-LBD) was selected for prokaryotic expression. The recombinant PaGR-LBD protein was over-expressed after isopropyl-β-d-thiogalactopyranoside (IPTG) induction and was subsequently purified using a Ni-NTA column and used to immunize mice to produce antiserum (Fig. 4A). SDS-PAGE analysis showed that the molecular weight of the purified PaGR-LBD was approximately 33 kDa including the 6 X His-tag (Fig. 4A), which was in accordance with the western blot results (Fig. 4B lane 4). Moreover, The PaGR-LBD antiserum was able to detect PaGR protein in MO/MΦ (Fig. 4B lane 5).

3.4. Altered PaGR expression after V. anguillarum infection in MO/MΦ

In order to ascertain if PaGR expression was altered in MO/MΦ after infection, RT-qPCR and western blotting were performed to analyze the expression levels of PaGR in normal and infected MO/MΦ. RT-qPCR showed that live *V. anguillarum* infection led to significantly higher PaGR mRNA expression at 8 hpi (4.20 fold), 12 hpi (2.19 fold), and 24 hpi (2.65 fold) than that observed for the controls (Fig. 5A). Western blot analysis showed that PaGR protein in MO/MΦ was up-regulated at 8, 12, and 24 hpi (Fig. 5B).

3.5. Effect of PaGR activation by cortisol on inflammatory cytokine mRNA expression in MO/MΦ after live V. anguillarum infection

To investigate whether cortisol influences the expression of inflammatory cytokines IL-1β, TNF-α, IL-10, and TGF-β, we determined the mRNA expression of these cytokines in live *V. anguillarum*-infected MO/MΦ after treatment with either cortisol or diluent. RT-qPCR analysis revealed significantly lower mRNA expression of IL-1β and TNF-α at all time points in MO/MΦ treated with cortisol than in the diluent control group (Fig. 6A and B), while significantly higher mRNA expression of IL-10 and TGF-β was observed at all time points in MO/MΦ treated with cortisol than that observed in the diluent control group (Fig. 6C and D).

3.6. Effect of PaGR activation by cortisol on the phagocytosis and bacterial killing abilities of ayu MO/MΦ

As cortisol was shown to alter cytokine mRNA expression of MO/MΦ, we next determined whether GR mediates the effect of cortisol on the phagocytosis and bactericidal activity of ayu MO/MΦ. In the phagocytosis assay, treatment with cortisol resulted in a significant suppression of MO/MΦ phagocytosis, which was 0.44-fold compared to that of the diluent-treated control group. However, pretreatment with RU486 antagonized and nearly reversed the suppressive effect of cortisol on the MO/MΦ phagocytosis (Fig. 7A). In addition, to confirm the CFU of viable *V. anguillarum*, RT-qPCR was performed by diluting purified RNA from the bacteria and constructing a general standard

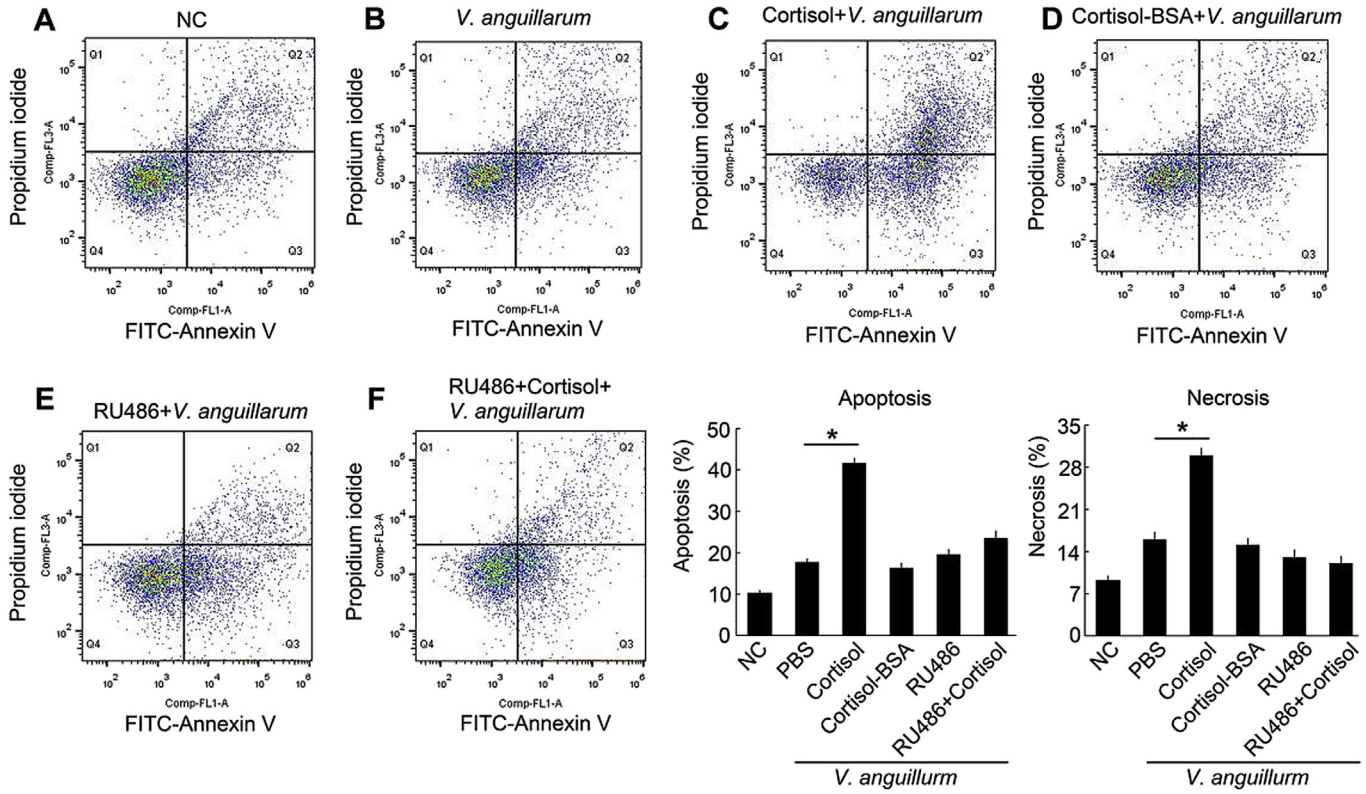

Fig. 10. Effect of GR activation on the apoptosis of ayu MO/MΦ challenged with live *V. anguillarum*. (A–F) MO/MΦ were pre-treated with RU486 for 30 min and then exposed to 100 ng/ml cortisol, 10^{-4} M cortisol-BSA, or diluent for 1 h before live *V. anguillarum* infection at a MOI of 2 for 8 h. Diluent-treated group was used as the negative control, *V. anguillarum*-infected alone group was used as the positive control. Levels of apoptosis (Annexin V^+PI^-) and necrosis (Annexin V^+PI^+) were analyzed by flow cytometry. Histograms represent the percentage of apoptosis and necrosis before and after incubation. Data are expressed as the mean ± SEM. Scale bar, 10 μm, n = 5. *$p < 0.05$.

curve (Fig. 7B). After MO/MΦ was treated with cortisol, RU486, or diluents, the survival rate of the bacteria was determined by quantifying intracellular *V. anguillarum* CFU number in ayu MO/MΦ. In MO/MΦ treated with cortisol, the survival rate of *V. anguillarum* was significantly reduced (49.33 ± 5.85%) compared to the diluent-treated control group (86.19 ± 4.99%) (Fig. 7C). Moreover, after RU486 treatment, no significant effect was seen in the survival of *V. anguillarum* in MO/MΦ compared to that of the diluent-treated control group (84.42 ± 2.83%), but RU486 treatment reversed the effects of cortisol on the survival rate of the *V. anguillarum*-infected MO/MΦ (80.48 ± 4.97%) (Fig. 7C).

3.7. The expression and co-localization of PaGR in MO/MΦ

Immunofluorescence microscopy was used to determine the localization of PaGR in MO/MΦ. Ayu MO/MΦ was stained with PaGR antibody to determine the cellular localization of PaGR. In addition, we used rhodamine phalloidin and DAPI to mark the plasma membrane and nucleus (Fig. 8A), respectively. The intracellular location of GR was found in ayu MO/MΦ (Fig. 8B). Interestingly, we also found that PaGR was also localized on the plasma membrane (Fig. 8B). The result showed that PaGR was expressed not only in the intracellular area but also in the plasma membrane of ayu MO/MΦ (Fig. 8C).

3.8. Cortisol-BSA-mediated PaGR effect on the inflammatory cytokine, phagocytosis and bacterial killing abilities of MO/MΦ

To explore whether PaGR could affect inflammatory cytokine expression, phagocytosis, or bacterial killing via non-genomic pathways in MO/MΦ after *V. anguillarum* infection, MO/MΦ were treated with cortisol-BSA (a membrane-impermeable agent), RU486, or diluent-treated control before live *V. anguillarum* infection. There was no significant change of cytokine expression between the cortisol-BSA and diluent-treated control group for all tested incubation time points (Fig. 9A–D). In addition, treatment with cortisol-BSA also resulted in a significant suppression of MO/MΦ phagocytosis, which was 0.52-fold compared to that of the diluent-treated control group (Fig. 9 E). With PaGR blockage using RU486, the phagocytosis activity of RU486-treated MO/MΦ was similar to that of the diluent-treated control group. Interestingly, RU486 significantly almost antagonized cortisol-BSA-suppressed MO/MΦ phagocytosis (Fig. 9E). For the bacterial killing assay, the result showed that cortisol-BSA had no significant effect on the bacterial killing ability of MO/MΦ (Fig. 9F).

3.9. Effect of GR activation on the apoptosis of ayu MO/MΦ challenged with live V. anguillarum

We used flow cytometry with Annexin V-FITC and PI staining to measure the rate of apoptosis. As shown in Fig. 10 A-C, the percentage of MO/MΦ undergoing apoptosis (Annexin V^+PI^-) increased from 17.92 ± 1.5% to 41.62 ± 2.41%, and the percentage undergoing necrosis (Annexin V^+PI^+) increased from 16.04 ± 2.48% to 29.96 ± 2.59% in the cortisol-treated group when compared with those in the live *V. anguillarum* infected alone-treated group. Meanwhile, cortisol-BSA had no significant effect on apoptosis and necrosis of MO/MΦ challenged with live *V. anguillarum* compared with the live *V. anguillarum* infected alone-treated group (Fig. 10D). By contrast, pretreatment with RU486 suppressed the apoptotic and necrotic rates of the cortisol-treated group to 23.53 ± 3.64% and 12.05 ± 2.49% (Fig. 10E and F).

4. Discussion

GR are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors. Previous studies have shown that GR are involved in the immune response [8,17]. However, the function of GR in an immune response after being challenged with pathogenic bacteria remains unclear. In the present study, we identified the cDNA sequence of a PaGR protein from ayu, a teleost fish. Multiple alignment of ayu GR with other teleost GR sequences revealed that the DBD, zinc finger regions and LBD were highly conserved. Phylogenetic analysis showed that PaGR was most closely related to rainbow trout GR2. In addition, GR gene and functions have been characterized in several teleosts, including zebrafish, rainbow trout, and Japanese ricefish. A study in zebrafish showed that activation of GR inhibits the expression of pro-inflammatory cytokines and decreases the migration of neutrophils [32]. In rainbow trout, GR signaling induced the expression of reactive oxygen species (ROS) via a non-genomic pathway [33]. In the present work, we identified a novel GR gene in ayu, and analyzed the subcellular localization of PaGR. Moreover, PaGR regulated inflammatory cytokine production, suppressed phagocytosis, promoted bacterial killing and apoptosis in MO/MΦ after live *V. anguillarum* infection. Taking together, these results suggest teleost GR play a crucial role in the immune system and can provide valuable insights into the role of nuclear hormone receptors in innate immunity from teleosts to mammals.

In humans, GR cDNA was isolated by expression cloning in 1985, and GR is expressed virtually in all organs and tissue of mammals [3]. In the present study, PaGR was detected in all the tested tissues, including the main immune organs, the liver, head kidney, and spleen; the highest mRNA expression was detected in the muscle. Unlike PaGR, gilthead seabream GR mRNA is highly expressed in the heart and brain [34]. In channel catfish, expression of GR transcript is found in a number of immune tissues and is widely distributed in the gill and intestine [35]. Estuarine tapertail anchovy GR mRNA is strongly expressed in the liver and muscle [36]. The discrepancies in GR expression might result from both species variation and differences in immunological status, growth stage, genetic background, and environment. After *V. anguillarum* infection, PaGR expression was up-regulated in the liver, spleen, and head kidney, suggesting that PaGR expression was induced by infection. In gilthead seabream, GR transcripts were up-regulated in spleen, brain, and head kidney after LPS injection [34]. These tissues are important immune organs in teleosts and play a role in the immune response upon pathogen invasion [37]. Previous reports have shown that GR expression is up-regulated in neutrophils and B lymphocytes in both blood and spleen after LPS treatment [38]. Hence, both mRNA and protein levels of PaGR were also significantly up-regulated in MO/MΦ after infection with live *V. anguillarum*. Together, these results suggested that PaGR expression is closely related to the ayu immune response against pathogenic infections.

The glucocorticoid-GR complex exerts wide-spread cellular effects through two distinct mechanisms categorized as genomic and non-genomic pathways [3,39]. Most non-genomic pathways originate from activation of mGR [40]. These actions of the GR appear to all play an important role in the regulation of the immune system. Although some studies have previously alluded to the existence of mGR in other cell types, including lymphoma cells and peripheral blood mononuclear cells [41], their precise location is still unclear in teleosts. In the present study, we found ayu mGR (mPaGR) by immunofluorescence detection, and PaGR was expressed not only in the intracellular area but also in the plasma membrane. However, there is a limit to the immune regulation of mGR via non-genomic pathways in teleost fish. Here, we used cortisol-BSA, a membrane-impermeable agent, to verify the PaGR-non-genomic effect. Macrophages across all vertebrate species are typically one of the first cell types to encounter intruding pathogens and play critical roles in host protection and tissue homeostasis [42–44]. Cytokines are major regulators of macrophage activation [7,45,46]. Therefore, the highly-regulated balance between pro-inflammatory cytokines and anti-inflammatory cytokines plays an important role in immune responses against pathogens [8]. In fish macrophages, treatment with cortisol results in heightened anti-inflammatory cytokine expression (IL-10 and TGF-β) and decreased pro-inflammatory cytokine expression (IL-1β, TNF, and IL-6) [4,32]. In rainbow trout, Co-stimulation of cortisol with the inflammatory agents resulted in the up-regulated of IL-10, and the down-regulated of IL-6 and IL-8 in macrophage cell line [7]. Furthermore, cortisol does not show significant modulatory effects on cytokine expression induced by *V. anguillarum* bacteria in the rainbow trout macrophage cell line, while in sea bream cortisol did produce a clear inhibitory effect on both pro-inflammatory and anti-inflammatory cytokines by *V. anguillarum* bacterial infection in head kidney cells [11]. These results indicate that there are species differences in GR activity during immune regulation. A previous study has found that the teleost immune system is very diverse in different species [47]. In the present study, cortisol-induced activation of PaGR resulted in the down-regulation of IL-1β and TNF-α, and up-regulation of IL-10 and TGF-β mRNA expression after *V. anguillarum* infection in ayu MO/MΦ. However, there were no significant changes in cytokine expression between the cortisol-BSA treatment and the control. These data suggest that activation of PaGR is involved in the regulation of cytokine expression via a genomic pathway in ayu MO/MΦ during infection.

Phagocytosis is a cellular process, a well-conserved innate defense mechanism that is important to the induction of antimicrobial responses and the regulation of adaptive immunity [45,48]. Phagocyte capacity has been described as an indicator of the health status of fish [49,50]. A previous study has shown that GR signaling is involved in regulating apoptosis and proliferation of B lymphocytes, inhibiting phagocytosis of tilapia leukocytes, and respiratory bursts in striped bass [51]. In addition, GR signaling promotes phagocytosis and bacteria killing in mammal monocytes [52,53]. In this study, we found that both cortisol and cortisol-BSA treatment could suppress phagocytosis in ayu MO/MΦ, but only cortisol treatment could enhance its bacterial killing capability. Furthermore, RU486 antagonized and nearly reversed the effects of cortisol and cortisol-BSA on ayu MO/MΦ. The results indicate that non-genomic pathway are involved in mediating the rapid suppression of phagocytosis by PaGR activation. The bacterial killing effect may be regulated by a genomic pathway. Our data suggested that PaGR could increase bacterial killing and reduce inflammatory responses to protect MO/MΦ during live *V. anguillarum* infection, and further support the GR's ability to modulate macrophage function via non-genomic pathways in an antagonistic way. However, further investigation is needed to determine the specific signaling pathways underlying the effect of PaGR on phagocytosis and bacterial killing.

Apoptosis, a form of programmed cell death, is a critical component in maintaining homeostasis and growth in all tissues and plays a significant role in immunity and cytotoxicity [54,55]. Although GR are expressed in all immune cells, the physiological outcomes of GR activation are highly cell type-specific: for example, glucocorticoids are anti-apoptotic in neutrophils, but pro-apoptotic in eosinophils, dendrites and some T-cells [56]. In addition, cortisol treatment could increase apoptosis of B cells and inhibit apoptosis of neutrophils in carp [57]. Previous studies have shown that apoptosis can be induced in fish by exposure to environmental stressors during different stages of the fish life cycle [58]. One of the interesting observations of this study is that cortisol treatment promoted apoptosis by PaGR activation under the condition of *V. anguillarum* infection, rather than in control MO/MΦ, while RU486 antagonized and nearly reversed the effects of cortisol. This result indicated that PaGR was also involved in MO/MΦ apoptosis. The main role of apoptosis is to prevent inflammation by removal of damaged cells [58]. Further investigations are needed on the regulation of apoptosis and PaGR activation, which together contribute to improving the understanding of the ayu immune system and

help to elucidate the innate immune mechanisms of other teleosts.

In summary, we have identified a novel GR protein from ayu, and the presence of PaGR was found not only in the intracellular area, but also in the plasma membrane. PaGR transcripts were significantly up-regulated in immune tissues and cells after bacterial infection. We demonstrate that the activation of PaGR can mediate cytokine expression, promote bacterial killing and apoptosis. Moreover, the activation of PaGR suppressed the phagocytosis activity of ayu MO/MΦ via a non-genomic pathway. This study establishes a basis for further examination of disease prevention and treatment in ayu. Further studies are required to determine the detailed mechanisms underlying the role of GR in the regulation of the immune response in fish.

Acknowledgments

This project was supported by the Program for the Natural Science Foundation of China (31772876; 31472300; 41776151), the Zhejiang Provincial Natural Science Foundation of China (LZ18C190001; LR18C040001), the Natural Science Foundation of Ningbo City of China (2017A610284; 2017A610174), the Scientific Innovation Team Project of Ningbo (2015C110018), Young Talent Cultivation Project of Zhejiang Association for Science and Technology (2016YCGC003) and the KC Wong Magna Fund in Ningbo University.

References

[1] M. Vazzana, A. Vizzini, M.A. Sanfratello, M. Celi, G. Salerno, N. Parrinello, Differential expression of two glucocorticoid receptors in seabass (teleost fish) head kidney after exogeneous cortisol inoculation, Comp. Biochem. Physiol. Mol. Integr. Physiol. 157 (2010) 49–54.

[2] P. Suarez-Bregua, P.M. Guerreiro, J. Rotllant, Stress, glucocorticoids and bone: a review from mammals and fish, Front. Endocrinol. 9 (2018) 526.

[3] T. Kino, E. Charmandari, G.P. Chrousos, Glucocorticoid receptor: implications for rheumatic diseases, Clin. Exp. Rheumatol. 29 (2011) S32–S41.

[4] A.R. Khansari, D. Parra, F.E. Reyes-Lopez, L. Tort, Cytokine modulation by stress hormones and antagonist specific hormonal inhibition in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) head kidney primary cell culture, Gen. Comp. Endocrinol. 250 (2017) 122–135.

[5] S.L. Alderman, A. McGuire, N.J. Bernier, M.M. Vijayan, Central and peripheral glucocorticoid receptors are involved in the plasma cortisol response to an acute stressor in rainbow trout, Gen. Comp. Endocrinol. 176 (2012) 79–85.

[6] X.H. Liu, B.W. Xie, Z.J. Wang, L. Jin, Y.G. Zhang, The secretion, synthesis, and metabolism of cortisol and its downstream genes in the H-P-I axis of rare minnows (*Gobiocypris rarus*) are disrupted by acute waterborne cadmium exposure, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 185 (2016) 112–121.

[7] R. Castro, J. Zou, C.J. Secombes, S.A. Martin, Cortisol modulates the induction of inflammatory gene expression in a rainbow trout macrophage cell line, Fish Shellfish Immunol. 30 (2011) 215–223.

[8] F.E. Reyes-Lopez, J. Aerts, E. Vallejos-Vidal, B. Ampe, K. Dierckens, L. Tort, et al., Modulation of innate immune-related genes and glucocorticoid synthesis in gnotobiotic full-sibling European sea bass (*Dicentrarchus labrax*) larvae challenged with *Vibrio anguillarum*, Front. Immunol. 9 (2018) 914.

[9] N.R. Bury, A. Sturm, P. Le Rouzic, C. Lethimonier, B. Ducouret, Y. Guiguen, et al., Evidence for two distinct functional glucocorticoid receptors in teleost fish, J. Mol. Endocrinol. 31 (2003) 141–156.

[10] D.M. Presman, G.L. Hager, More than meets the dimer: what is the quaternary structure of the glucocorticoid receptor? Transcription 8 (2017) 32–39.

[11] A.R. Khansari, D. Parra, F.E. Reyes-Lopez, L. Tort, Modulatory in vitro effect of stress hormones on the cytokine response of rainbow trout and gilthead sea bream head kidney stimulated with *Vibrio anguillarum bacterin*, Fish Shellfish Immunol. 70 (2017) 736–749.

[12] Y. Lu, H. Liu, Y. Bi, H. Yang, Y. Li, J. Wang, et al., Glucocorticoid receptor promotes the function of myeloid-derived suppressor cells by suppressing HIF1alpha-dependent glycolysis, Cell. Mol. Immunol. 15 (2018) 618–629.

[13] O. Stojadinovic, A. Sawaya, I. Pastar, M. Tomic-Canic, Glucocorticoid receptor localizes to adherens junctions at the plasma membrane of keratinocytes, PLoS One 8 (2013) e63453.

[14] D.W. Cain, J.A. Cidlowski, Immune regulation by glucocorticoids, Nat. Rev. Immunol. 17 (2017) 233–247.

[15] S. Bhattacharyya, C.K. Ratajczak, S.K. Vogt, C. Kelley, M. Colonna, R.D. Schreiber, et al., TAK1 targeting by glucocorticoids determines JNK and IkappaB regulation in Toll-like receptor-stimulated macrophages, Blood 115 (2010) 1921–1931.

[16] S. Vettorazzi Galuppo, J. Hovelmann, C.J. Scholz, J.P. Tuckermann, J. Bauersachs, et al., The glucocorticoid receptor in monocyte-derived macrophages is critical for cardiac infarct repair and remodeling, Faseb. J. 31 (2017) 5122–5132.

[17] J.W. Koper, E.F. van Rossum, E.L. van den Akker, Glucocorticoid receptor polymorphisms and haplotypes and their expression in health and disease, Steroids 92 (2014) 62–73.

[18] E. Faught, M.M. Vijayan, Mechanisms of cortisol action in fish hepatocytes, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 199 (2016) 136–145.

[19] M.A. Kim, D.S. Kim, Y.C. Sohn, Characterization of two functional glucocorticoid receptors in the marine medaka *Oryzias dancena*, Gen. Comp. Endocrinol. 171 (2011) 341–349.

[20] E.H. Stolte, M. Chadzinska, D. Przybylska, G. Flik, H.F. Savelkoul, B.M. Verburg-van Kemenade, The immune response differentially regulates Hsp70 and glucocorticoid receptor expression in vitro and in vivo in common carp (*Cyprinus carpio L.*), Fish Shellfish Immunol. 27 (2009) 9–16.

[21] A. Chatzopoulou, U. Roy, A.H. Meijer, A. Alia, H.P. Spaink, M.J. Schaaf, Transcriptional and metabolic effects of glucocorticoid receptor alpha and beta signaling in zebrafish, Endocrinology 156 (2015) 1757–1769.

[22] C. Li, J. Chen, Y. Shi, M. Li, Characterization of *Listonella anguillarum* as the aetiological agent of vibriosis occurred in cultured ayu (*Plecoglossus altivelis*) in Ninghai country, China, Zool. Res. 49 (2009) 931–937.

[23] X. Zhang, Y. Mu, P. Mu, J. Ao, X. Chen, Transcriptome analysis reveals comprehensive insights into the early immune response of large yellow croaker (*Larimichthys crocea*) induced by trivalent bacterial vaccine, PLoS One 12 (2017) e0170958.

[24] D.R. Causey, M.A. Pohl, D.A. Stead, S.A. Martin, C.J. Secombes, D.J. Macqueen, High-throughput proteomic profiling of the fish liver following bacterial infection, BMC Genomics 19 (2018) 719.

[25] Q. Chen, X.J. Lu, M.Y. Li, J. Chen, Molecular cloning, pathologically-correlated expression and functional characterization of the colonystimulating factor 1 receptor (CSF-1R) gene from a teleost, *Plecoglossus altivelis*, Zool. Res. 37 (2016) 96–102.

[26] Y.J. Ning, X.J. Lu, J. Chen, Molecular characterization of a tissue factor gene from ayu: a pro-inflammatory mediator via regulating monocytes/macrophages, Dev. Comp. Immunol. 84 (2018) 37–47.

[27] X.H. Zhang, Y.H. Shi, J. Chen, Molecular characterization of a transmembrane C-type lectin receptor gene from ayu (*Plecoglossus altivelis*) and its effect on the recognition of different bacteria by monocytes/macrophages, Mol. Immunol. 66 (2015) 439–450.

[28] F. Crisafi, R. Denaro, M. Genovese, S. Cappello, M. Mancuso, L. Genovese, Comparison of 16SrDNA and toxR genes as targets for detection of *Vibrio anguillarum* in *Dicentrarchus labrax* kidney and liver, Res. Microbiol. 162 (2011) 223–230.

[29] J. Yu, J. Chen, Y. Li, W. Gou, W. Ji, H. Xu, Detection of *vibrio anguillarum* from lateolabrax japonicus by using polymerase chain reaction (PCR), J. Oceanogr. Huanghai Bohai Seas 20 (2002) 60–64.

[30] Y. Ren, S.F. Liu, L. Nie, S.Y. Cai, J. Chen, Involvement of ayu NOD2 in NF-kappaB and MAPK signaling pathways: insights into functional conservation of NOD2 in antibacterial innate immunity, Zool. Res. (2018), https://doi.org/10.24272/j.issn.2095-8137.598 2018.066.

[31] Q. Chen, J. Chen, J. Chen, X.J. Lu, Molecular and functional characterization of liver X receptor in ayu, *Plecoglossus altivelis*: regulator of inflammation and efferocytosis, Dev. Comp. Immunol. 65 (2016) 358–368.

[32] A. Chatzopoulou, J.P. Heijmans, E. Burgerhout, N. Oskam, H.P. Spaink, A.H. Meijer, et al., Glucocorticoid-induced attenuation of the inflammatory response in zebrafish, Endocrinology 157 (2016) 2772–2784.

[33] M.B. Espinoza, J.E. Aedo, R. Zuloaga, C. Valenzuela, A. Molina, J.A. Valdes, Cortisol induces reactive oxygen species through a membrane glucocorticoid receptor in rainbow trout myotubes, J. Cell. Biochem. 118 (2017) 718–725.

[34] L. Acerete, J.C. Balasch, B. Castellana, B. Redruello, N. Roher, A.V. Canario, et al., Cloning of the glucocorticoid receptor (GR) in gilthead seabream (*Sparus aurata*). Differential expression of GR and immune genes in gilthead seabream after an immune challenge, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 148 (2007) 32–43.

[35] B.C. Small, S.M.A. Quiniou, Characterization of two channel catfish, Ictalurus punctatus, glucocorticoid receptors and expression following an acute stressor, Comp. Biochem. Physiol. Mol. Integr. Physiol. 216 (2018) 42–51.

[36] F.K. Du, G.C. Xu, Y. Li, Z.J. Nie, P. Xu, Molecular cloning and differential expression of the glucocorticoid receptor gene in the estuarine tapertail anchovy *Coilia nasus*, Genet. Mol. Res. 16 (2017).

[37] 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.

[38] M. Bergquist, M. Nurkkala, C. Rylander, E. Kristiansson, G. Hedenstierna, C. Lindholm, Expression of the glucocorticoid receptor is decreased in experimental *Staphylococcus aureus* sepsis, J. Infect. 67 (2013) 574–583.

[39] C. Das, M. Thraya, M.M. Vijayan, Nongenomic cortisol signaling in fish, Gen. Comp. Endocrinol. 265 (2018) 121–127.

[40] J. Haller, E. Mikics, G.B. Makara, The effects of non-genomic glucocorticoid mechanisms on bodily functions and the central neural system. A critical evaluation of findings, Front. Neuroendocrinol. 29 (2008) 273–291.

[41] S. Boncompagni, L. Arthurton, E. Akujuru, T. Pearson, D. Steverding, F. Protasi, et al., Membrane glucocorticoid receptors are localised in the extracellular matrix and signal through the MAPK pathway in mammalian skeletal muscle fibres, J. Physiol. 593 (2015) 2679–2692.

[42] J.W. Hodgkinson, L. Grayfer, M. Belosevic, Biology of bony fish macrophages, Biology 4 (2015) 881–906.

[43] G.F. Wiegertjes, A.S. Wentzel, H.P. Spaink, P.M. Elks, I.R. Fink, Polarization of immune responses in fish: the 'macrophages first' point of view, Mol. Immunol. 69 (2016) 146–156.

[44] E.D. Abarike, F.K. Kuebutornye, J. Jian, J. Tang, Y. Lu, J. Cai, Influences of immunostimulants on phagocytes in cultured fish: a mini review, Rev. Aquacult.

(2018), https://doi.org/10.1111/raq.12288.
[45] L. Grayfer, B. Kerimoglu, A. Yaparla, J.W. Hodgkinson, J. Xie, M. Belosevic, Mechanisms of fish macrophage antimicrobial immunity, Front. Immunol. 9 (2018) 1105.
[46] J. Zou, C.J. Secombes, The function of fish cytokines, Biology 5 (2016) 23.
[47] T. Nakanishi, J.-i. Hikima, T. Yada, Osteichthyes: immune systems of teleosts (*Actinopterygii*), Advances in Comparative Immunology, Springer, Cham, 2018, pp. 687–749.
[48] A.M. Rieger, J.D. Konowalchuk, L. Grayfer, B.A. Katzenback, J.J. Havixbeck, M.D. Kiemele, et al., Fish and mammalian phagocytes differentially regulate pro-inflammatory and homeostatic responses in vivo, PLoS One 7 (2012) e47070.
[49] C.E. Covantes-Rosales, A.M. Trujillo-Lepe, K.J.G. Diaz-Resendiz, G.A. Toledo-Ibarra, G.H. Ventura-Ramon, P.C. Ortiz-Lazareno, et al., Phagocytosis and ROS production as biomarkers in Nile tilapia (*Oreochromis niloticus*) leukocytes by exposure to organophosphorus pesticides, Fish Shellfish Immunol. 84 (2018) 189–195.
[50] A.O. Kordon, H. Abdelhamed, H. Ahmed, J.Y. Park, A. Karsi, L.M. Pinchuk, Phagocytic and bactericidal properties of channel catfish peritoneal macrophages exposed to *Edwardsiella ictaluri* live attenuated vaccine and wild-type strains, Front. Microbiol. 8 (2017) 2638.
[51] A. Vizzini, M. Vazzana, M. Cammarata, N. Parrinello, Peritoneal cavity phagocytes from the teleost sea bass express a glucocorticoid receptor (cloned and sequenced) involved in genomic modulation of the in *vitro* chemiluminescence response to zymosan, Gen. Comp. Endocrinol. 150 (2007) 114–123.
[52] A. van der Goes, K. Hoekstra, T.K. van den Berg, C.D. Dijkstra, Dexamethasone promotes phagocytosis and bacterial killing by human monocytes/macrophages in *vitro*, J. Leukoc. Biol. 67 (2000) 801–807.
[53] M.J. Olivares-Morales, M.K. De La Fuente, K. Dubois-Camacho, D. Parada, D. Diaz-Jimenez, A. Torres-Riquelme, et al., Glucocorticoids impair phagocytosis and inflammatory response against crohn's disease-associated adherent-invasive *Escherichia coli*, Front. Immunol. 9 (2018) 1026.
[54] J.W. Kim, J.M. Jeong, J.S. Bae, D.H. Cho, S.H. Jung, J.Y. Hwang, et al., First description of programmed cell death10 (PDCD10) in rock bream (*Oplegnathus fasciatus*): potential relations to the regulation of apoptosis by several pathogens, Dev. Comp. Immunol. 55 (2016) 51–55.
[55] J. Lu, Y. Li, Z. Shen, C. Lu, L. Lu, TNF-alpha is involved in apoptosis triggered by grass carp reovirus infection in *vitro*, Fish Shellfish Immunol. 55 (2016) 559–567.
[56] L.C. Meagher, J.M. Cousin, J.R. Seckl, C. Haslett, Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes, J. Immunol. 156 (1996) 4422–4428.
[57] K.J. Laing, J. Holland, S. Bonilla, C. Cunningham, C.J. Secombes, Cloning and sequencing of caspase 6 in rainbow trout, *Oncorhynchus mykiss*, and analysis of its expression under conditions known to induce apoptosis, Dev. Comp. Immunol. 25 (2001) 303–312.
[58] H. AnvariFar, A.K. Amirkolaie, H.K. Miandare, H. Ouraji, M.A. Jalali, S.I. Ucuncu, Apoptosis in fish: environmental factors and programmed cell death, Cell Tissue Res. 368 (2017) 425–439.