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Biology

Characterization and expression analysis of *chemokine-like receptor 3* gene in rainbow trout *Oncorhynchus mykiss*

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Abstract Chemokine-like receptors (CMKLRs) are multifunctional receptors with roles in regulating leukocyte and inflammation. Currently, two members of the CMKLRs, CMKLR1 and CMKLR2, have been found in salmonids, indicating that the CMKLRs had expanded from an ancestor gene in teleost fish. In the present study, the third member of the CMKLRs, defined as CMKLR3, was identified and cloned in rainbow trout. The trout CMKLR3 possessed conserved features of the CMKLR family including seven transmembrane regions, a dynein regulatory complex (DRC) motif, and two cysteine residues, but shared low sequence identities with fish CMKLR1 and CMKLR2 (24–38 %), which was confirmed by phylogenetic tree analysis. Trout *CMKLR3* was highly expressed in body kidney, head kidney, and IgM$^+$ B cells, indicating its functional role in regulating leukocytes. We were able to modulate the expression of trout *CMKLR3* in vivo by bacterial and parasitic infections but it remained apathetic to virus infection, and it was also successfully modulated in vitro by peptidoglycan and cytokines (IFN-γ and IL-6). Our results suggest that trout CMKLR3 is regulated in a complex way and has an important regulatory role in inflammatory responses.

Keywords Rainbow trout · *Oncorhynchus mykiss* · Chemokine-like receptors · CMKLR3 · Expression

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Introduction

Chemokine receptors belong to the family of G-protein-coupled seven-transmembrane receptors, which predominantly express on the surface of leukocytes and play crucial regulatory roles in the movement of leucocytes by binding with their specific ligands (chemokines). According to the chemokine classes that they bind, the various chemokine receptors have been named as follows: CCR, which binds to CC-chemokines, CXCR, which binds to CXC chemokines, XCR, which binds to XC chemokines, and CX2CR, which binds to CX3C chemokines; in all cases, X is any amino acid and C is cysteine [1]. After binding with their ligands, the chemokine/receptor system may trigger multiple intracellular signaling events involving in gene transcription, cytoskeleton rearrangement, and chemotaxis [2, 3].

Except for the classical chemokine receptors, there are some atypical chemokine receptors (ACKRs) that also are seven-transmembrane molecules and can bind to chemokines but cannot induce the classical chemokine receptor signaling pathway [4–6]. Human ACKRs include at least five members such as ACKR1 (Duffy antigen receptor for chemokines, DARC), ACKR2 (D6 and CC chemokine-binding protein 2 [CCBP2]), ACKR3

(CXC-chemokine receptor 7 [CXCR7], and RDC1), ACKR4 (CC-chemokine receptor-like 1 [CCRL1], CCX-CKR and CCR11), and CCRL2 (chemokine receptor on activated macrophages [CRAM]) [7]. ACKRs play important roles in the regulation of classical chemokine/receptor responses. ACKR1 can bind a large number of CXC and CC chemokines, and functions as a chemokine sink [7, 8]. ACKR2 can bind at least 12 inflammatory CC chemokines and plays a role in the regulation of inflammatory responses [5, 6]. CCRL2 can bind chemokine CCL5 and CCL19 to recycle and reduce local concentration of these two chemokines, and functions in the regulation of dendritic cell (DC) trafficking and other immune responses [9–11]. CCRL2 can also bind chemoattractant chemerin and functions in the regulation of inflammatory responses [12, 13].

Competitive binding to the ligand chemerin with CCRL2 brings into focus another seven-transmembrane molecular, chemokine-like receptor (CMKLR1), also termed as ChemR23 [14]. CCRL2 binds chemerin and increases the local chemerin concentration, and then presents it to CMKLR1 on adjacent cells, resulting in development of IgE-dependent, mast cell-dependent cutaneous anaphylaxis [13]. In addition to cooperation with CCRL2, CMKLR1 is also involved in regulating leukocytes and inflammatory processes. Several leukocytes including immature DCs, macrophages, monocytes, and CD4 + T lymphocytes can express it [14]. CMKLR1 expressed on macrophages and DCs can bind the active chemerin and induce cell migration [15, 16]. In-vivo studies using *CMKLR*-deficient mice showed that the CXCL1, interleukin (IL)-6, tumor necrosis factor (TNF), and IL-1β were reduced even under the lipopolysaccharide (LPS) induction [17]. Mammalian CMKLR1 can also serve as a co-receptor for some simian immunodeficiency virus (SIV) clones and human immunodeficiency virus (HIV)-1 strain [18]. These all indicated that CMKLR1 is a multi-functional receptor [19].

In fish, a number of chemokine receptors had been identified and some of them are fish-specific (e.g., CXCR3b, CCR12) [20, 21]. Current studies mainly focus on the classic chemokine receptors of fish but a few have looked at the atypical chemokine receptors. CMKLRs in teleost fish expanded from a common ancestor, as consequently two members (CMKLR1 and CMKLR2) were found in salmonids and three members (CMKLR1-3) in Northern pike *Esox lucius*, which may be the result of genome duplication [22]. To the best of our knowledge, no reports focus on the function of CMKLRs of fish species. Thus, in this study, the third member of the CMKLR family, *CMKLR3*, is identified for the first time from rainbow trout *Oncorhynchus mykiss*, and its expressions in tissues or cells under diseases or stimulation are characterized to broaden our knowledge on CMKLRs in fish, and to provide the basis for clearly understanding their functions.

Materials and methods

Fish

Rainbow trout (average of 100 g) were obtained from the Mill of Elrich Trout Fishery (Aberdeenshire, UK). Fish were maintained in 1-m-diameter aerated fiberglass tanks with a re-circulating water system at 14 ± 1 °C and fed twice daily with standard commercial pellets (EWOS). Prior to the experiments, fish were acclimated for at least 2 weeks.

Total RNA extraction and cDNA synthesis

Total RNA was independently extracted from 17 selected tissues including caudal fin, adipose fin, thymus, gills, brain, scales, skin, muscle, liver, spleen, head kidney, body kidney, intestine, heart, blood, and adipose tissue using Trizol Reagent (Invitrogen, CA) following the manufacturer's instruction. The cDNA was synthesized from total RNA using the BioScript™ Reverse Transcriptase kit (Bioline, UK).

Cloning of trout *CMKLR3* and sequence analysis

The trout CMKLR1 protein sequence was used as a bait to search the trout expressed sequence tag (EST) database by tBLASTn software, and an EST (704 bp) that contained a 5′-untranslated region (5′-UTR) and an incomplete open reading frame (ORF) were identified. This EST was further used as query to search the trout genome database (http://www.genoscope.cns.fr/trout) using BLASTn software. A scaffold (MMSRT116B_scaff_1281_1) of 4980 base pairs (bp) in length was obtained. This genome sequence was analyzed using GenScan to get the potential 5′-UTR, 3′-UTR, and ORF. Primers were designed within the predicted 5′-UTR and 3′-UTR, and polymerase chain reaction (PCR) amplification was done using head kidney cDNA samples as a template. A single band was obtained and the PCR product was sequenced by Eurofins Biotec. The primers used for gene clone are listed in Table 1.

The predicted amino acid sequences were deduced from the nucleotide sequence using Translate at the ExPASy Molecular Biology Server (http://www.expasy.org/tools). Multiple sequences alignment was conducted using the ClustalW program [23]. Transmembrane domain was predicted by the SMART7 program [24]. A phylogenetic tree was constructed by the neighbor-joining methods using MEGA 6 software and set with a bootstrap of 10,000 times [25].

Tissue distribution of trout *CMKLR3* transcripts

Seventeen tissues as mentioned above were collected from six healthy fish, and RNA preparation, cDNA synthesis, and real-time PCR analysis were performed as described

Table 1 Primers used in this study

Primer	Sequence (5′ to 3′)	Application
CMKLR1-F	CACACACTTCAAGATACTTCA-CAG	Gene clone
CMKLR1-R	GGATGTCAATCATGTTATCAT-AAAG	Gene clone
CMKLR1-QF	TTGAATATACACGTTATTCAA-GGTTCTCAC	Real-time PCR
CMKLR1-QR	TGATGTTGAGGGACTGCCG	Real-time PCR
EF-1α-F	CAAGGATATCCGTCGTGGCA	Real-time PCR
EF-1α-R	ACAGCGAAACGACCAAGAGG	Real-time PCR

previously [26, 27]. The expression levels of trout *CMKLR3* were normalized to that of EF-1α and expressed as arbitrary units. Primers used for real-time PCR are listed in Table 1.

Gene expression of trout *CMKLR3* in IgM$^+$ cells isolated from rainbow trout tissues

In trout, two different subpopulations of B cells have been identified, IgM$^+$/IgD$^+$/IgT$^-$ (IgM$^+$ cells) and IgM$^-$/IgD$^-$/IgT$^+$ (IgT$^+$ cells). Since the expression of many chemokine receptors had been found in IgM$^+$ B cells [28], we chose the IgM + cells isolated from the blood, spleen, head kidney, gills, and intestine of rainbow trout to examine the expression pattern of *CMKLR3* in this study. The IgM$^+$ cells from different tissues were isolated as described previously [28]. The expression of *CMKLR3* in the cells was measured using the same method for those in tissues.

Gene expression of trout *CMKLR3* after bacterial, parasitic, and viral infections

Bacterial infection: Forty-eight fish (average of 100 g) were randomly divided into two groups, a challenge group and a control group. The fish in the challenge group were injected intraperitoneally (i.p) with a 0.5-ml suspension (1×10^6 cfu in PBS) of *Yersinia ruckeri* (strain MT3072), the causative agent of red mouth disease [29], and fish in the control group were injected i.p. with the same amount of PBS followed by rearing at 15 ± 1 °C with a re-circulating water system. Head kidney tissues of six fish from each group were collected at 6, 24, 48, and 72 h post-injection, respectively. Real-time PCR quantification of the expression of *CMKLR3* was done as described previously [26] and expressed as fold change relative to the time-matched controls.

Parasitic infection: The proliferative kidney disease (PKD) caused by the myxozoan parasite, *Tetracapsuloides bryosalmonae*, is a disease with major economic impact on salmonid aquaculture [30]. In the present study, the caudal kidney tissues were sampled in late July at a water temperature of 15-16 °C from fish (average of 100 g) provided by a commercial trout farm in Southern England. The different clinical stages of the fish were determined using the kidney swelling index from Clifton-Hadley et al. [31]. Expression of trout *CMKLR3* in the caudal kidney of the fish in different stages of PKD was studied by real-time PCR quantification.

Virus infection: Fish weighing 14.7 g were bath challenged with the viral hemorrhagic septicemia virus (VHSV) at 14 °C in an aquarium facility [32]. The infection of VHSV was confirmed by real-time PCR using primers of the VHSV N gene as described previously [33]. The kidney was sampled from six of the challenged fish or from phosphate buffered saline (PBS) mock-challenged fish at 1 and 3 days post-challenge. Expression of trout *CMKLR3* was analyzed by real-time PCR and expressed in terms of fold change relative to the time-matched controls.

Gene expression of trout *CMKLR3* in head kidney primary macrophages

Since *CMKLR1* could be expressed and regulated by several cytokines in human macrophages, the primary trout head kidney macrophages were isolated to study the expression modulation of trout *CMKLR3*. The primary head kidney macrophages were prepared as in a previous study [34], and stimulated with peptidoglycan (PGN, 5 μg/ml, Invivogen) and recombinant cytokines including rIFN-γ (20 ng/ml) [26], rIL-6 (100 ng/ml) [34], and rTNF-α (10 ng/ml) [35] for 2, 4, 8, and 24 h. RNA extraction and real-time PCR were performed according to the method described above.

Statistical analysis

All data were expressed as mean ± SEM. Statistical analysis was analyzed using SPSS Statistics package 19.0 (SPSS Inc., Chicago, Illinois). The data from the infection studies were analyzed using one-way analysis of variance (ANOVA) and the least significant difference (LSD) post-hoc test, and the data from in-vitro experiments were analyzed by a paired-sample *t* test. Differences with $p < 0.05$ were considered statistically significant.

Results

Cloning and sequence analysis of trout *CMKLR3*

The amplified trout *CMKLR3* cDNA (GenBank accession No. KM516349) was 1381 bp in length, which contained

Table 2 Identities of predicted amino acid sequences between trout CMKLRs and other vertebrates' CMKLRs

Molecules	1	2	*3*	4	5	*6*	7	8	9	10	11	12	13	14	*15*
1. Trout CMKLR3		93.5	75.8	38.4	39.7	38.4	33.9	38.0	32.8	24.0	36.7	41.1	40.6	40.5	41.8
2. Salmon CMKLR3			76.7	39.5	39.9	39.5	34.2	39.8	33.9	25.0	38.4	41.9	40.6	40.6	41.7
3. Pike CMKLR3				35.2	36.3	35.2	33.9	38.7	31.4	21.7	35.2	41.5	40.9	41.5	40.6
4. Salmon CMKLR2a					87.4	76.4	32.7	34.9	34.6	30.0	37.2	34.9	39.0	32.1	34.4
5. Trout query CMKLR2						73.7	33.0	33.7	35.2	31.4	36.8	33.5	36.1	32.1	34.5
6. Pike CMKLR2							34.5	36.2	34.1	32.8	54.0	37.7	40.6	34.9	36.1
7. Trout CMKLR1								54.4	77.2	39.9	54.4	41.4	39.3	39.0	37.8
8. Zebrafish CMKLR1									53.5	42.1	67.7	42.4	39.3	41.1	39.5
9. Pike CMKLR1.1										39.3	54.0	38.8	37.5	37.0	37.5
10. Pike CMKLR1.2											46.8	29.2	27.4	27.4	27.6
11. Pike CMKLR1.3												39.0	38.0	37.5	38.5
12. Lizard CMKLR1													51.7	55.6	54.5
13. Frog CMKLR1														49.9	50.6
14. Mouse CMKLR1															80.9
15. Human CMKLR1															

a 5′-UTR of 150 bp, an ORF of 1146 bp encoding for 381 amino acids, and a 3′-UTR of 85 bp. The trout CMKLR3 gene was perfectly mapped to the genomic scaffold 1281, which contained a single intron of 1221 bp in the 5′-UTR. There was a stop codon (TGA) in the 5′-UTR upstream of the start codon (ATG) (Fig. S1), indicating that the complete ORF of this gene had been obtained. In addition, the *CMKLR3* of Atlantic salmon was obtained by using trout CMKLR3 as a query to search the salmon genome database (http://salmondb.cmm.uchile.cl), whose ORF was 1098 bp in length encoding for 365 amino acids and located on genomic contig AGKD00000000.4 (Fig. S2).

Trout CMKLR3 shared respectively 93.5 and 75.8 % amino acid sequence identities with Atlantic salmon and Northern pike CMKLR3, which were higher than those with fish CMKLR1 and CMKLR2 (24–38 %), and mammalian CMKLR1 (40.5–41.8 %) (Table 2). Multiple sequence alignment revealed that trout CMKLR3 contained several conserved CMKLRs family features. Firstly, trout CMKLR3 possessed the G-coupled protein seven-transmembrane regions (TM), which divided the molecular into an extracellular amino-terminal, three extracellular regions (ECL), three intracellular regions (ICL), and a cytoplasmic carboxyl region (Figs. 1, 2). Secondly, unlike the classical chemokine receptors, which contain four cysteine residues (one in the N-terminal and three in the ECLs) [1, 4, 6], trout CMKLR3 only possessed two cysteine residues in the ECL1 and ECL2 region. The same case was also found in other CMKLRs family members. Thirdly, all CMKLRs shared a dynein regulatory complex (DRC) motif at the beginning of ICL2 instead of DRY motif at the same position in the classical chemokine receptors. Lastly, the N-terminal or C-terminal of CMKLRs contained several residues known to be important for activation or function of chemokine receptors, e.g., N-glycosylation sites and tyrosine O-sulfation sites at the N-terminal (Fig. 1).

To confirm the sequence identities, a phylogenetic tree was constructed based on a multiple alignment of vertebrates' CMKLRs (Fig. 3). Clearly, the tree was divided into four main clades. The CMKLR1 of mammals, birds, amphibians, and reptiles formed one clade, while fish CMKLR1 and CMKLR2 formed respective separate clades. Trout CMKLR3 was well clustered with the clade of fish CMKLR3 with a convincing bootstrap value (100 %).

Tissue distribution of trout *CMKLR3* expression

CMKLR3 expressions in 17 tissues of healthy trout were examined using real-time PCR. Results showed that trout *CMKLR3* was ubiquitous, with the highest expression in caudal kidney and head kidney ($p < 0.05$) and the lowest in the liver and tail fins ($p < 0.05$). A high expression level of trout *CMKLR3* was also found in spleen, blood, muscle, gills, intestines, gonad, and scales (Fig. 4).

Gene expression of trout *CMKLR3* in IgM$^+$ cells isolated from rainbow trout tissues

Since CMKLRs are involved in cell migration, the expression of *CMKLR3* in cells will characterize the cell

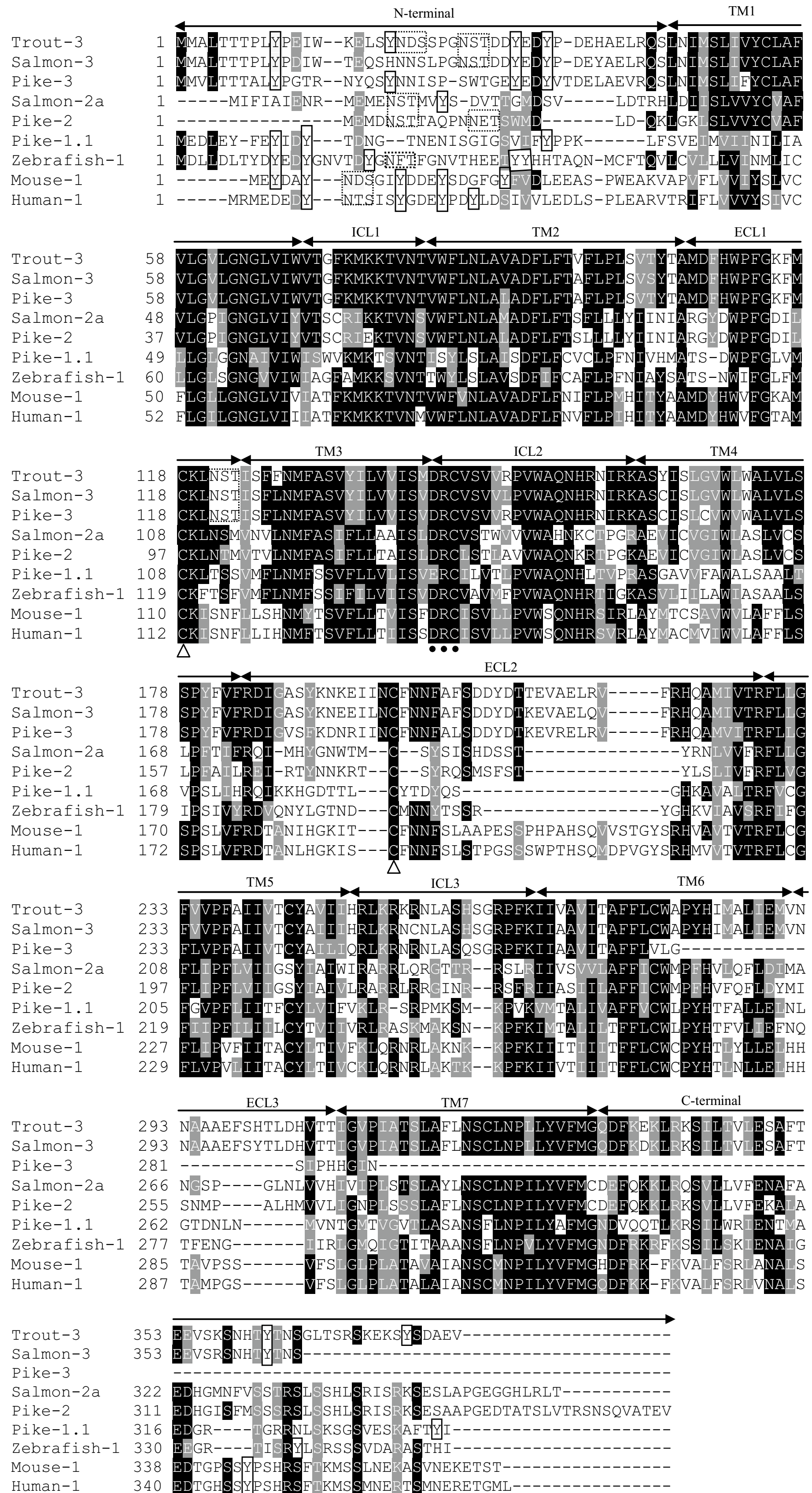

Fig. 1 Multiple alignment of CMKLRs. The multiple alignment of CMKLRs was produced using ClustalW and the conserved amino acids were shaded using BOXSHADE (version 3.21). The N-terminus, seven-transmembrane domains (TM1-7), three extracellular loops (ECL1-3), three intracellular loops (ICL1-3) and C-terminus are marked above the alignment. The two conserved cysteine residues in ECL1 and ECL2 are indicated by a white triangle below the alignment. The potential N-liked glycosylation site at the N-terminal and conserved tyrosine residues are marked in the black box and the dotted box, respectively. The DRC motif in the ICL2 region is marked by a black dot under the sequences. The accession numbers for sequences used in this alignment are given in Fig. 3

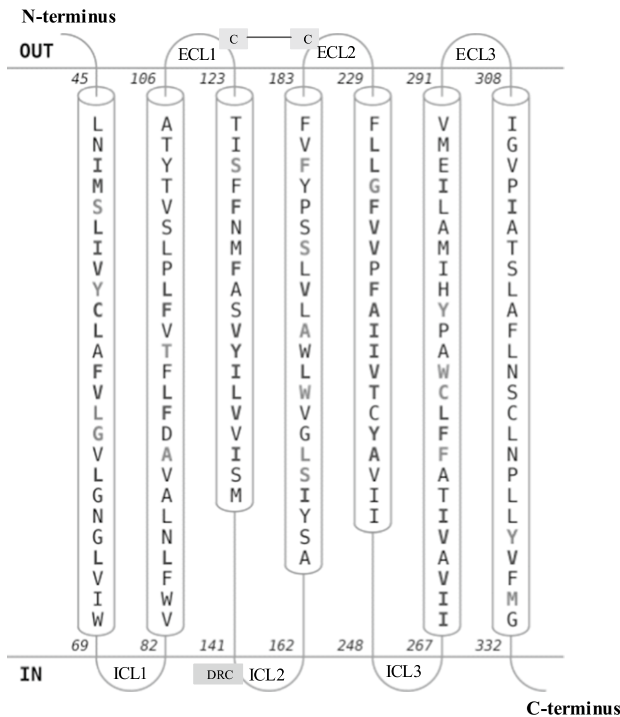

Fig. 2 Secondary structure of rainbow trout *O. mykiss* CMKLR3. The N-terminus, seven-transmembrane domains (TM1-7), three extracellular loops (ECL1-3), three intracellular loops (ICL1-3) and C-terminus are shown. The DRC motif in the ICL2 region are boxed. Potential cysteine bond is shown as a black line

migration pattern. Thus, we analyzed the transcription of trout *CMKLR3* in sorted IgM + cells by real-time PCR. Results showed that trout *CMKLR3* was expressed in IgM$^+$ cells derived from kidney, blood, spleen, gills, and intestine: it was lowest in blood IgM$^+$ cells, moderate in head kidney and gills IgM$^+$ cells, and highest in intestine IgM$^+$ cells (Fig. 5).

Gene expression of trout *CMKLR3* after bacterial, parasitic, and viral infections

The expression of trout *CMKLR3* after infection with bacteria (*Y. ruckeri*), a parasite (*T. bryosalmonae*), and a virus (VHSV) were also investigated by real-time PCR. After infection of trout with *Y. ruckeri*, the expression of trout *CMKLR3* in the head kidney of challenge group fish was significantly increased at 24 and 48 h post-infection when compared with that in the control group ($p < 0.05$) (Fig. 6a). In fish with clinical PKD, *CMKLR3* expression was significantly decreased in the kidney of the fish with a PKD swelling grade of 1-2, but not changed in the fish with a swelling grade of 1, 2, and 3 (Fig. 6b), compared with those in the uninfected fish (the swelling grade 0). Following VHSV bath exposure, trout *CMKLR3* remained apathetic at 1 and 3 days post-infection (Fig. 6c).

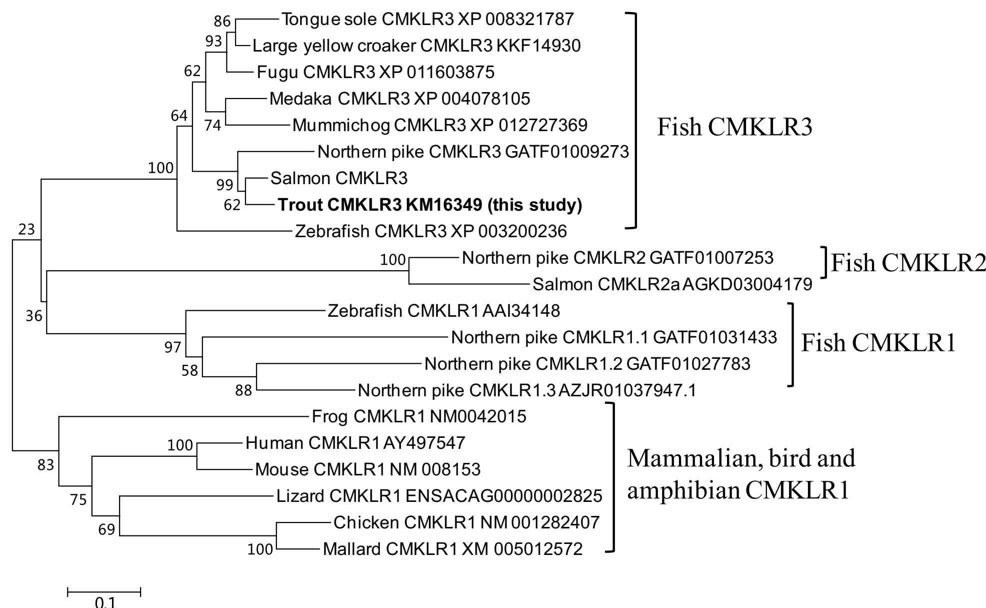

Fig. 3 An unrooted phylogenetic tree of CMKLRs. The tree was constructed using the neighbor-joining method by MEGA 6 software. The evolutionary distances were computed using the JTT matrix-based method and pairwise deletion option. The GenBank accession number of each sequence was given after the species name and molecular type

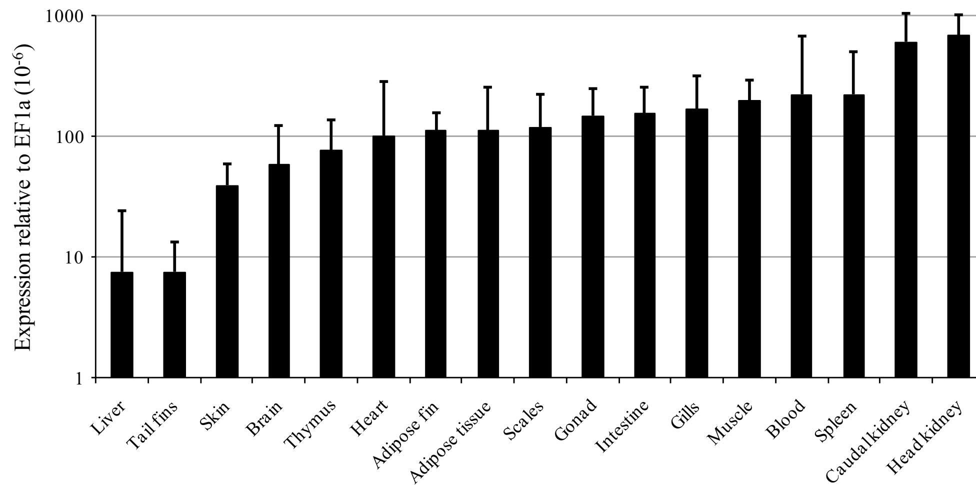

Fig. 4 Tissue distribution of rainbow trout *O. mykiss CMKLR3*. The expression of trout *CMKLR3* was analyzed by real-time PCR in 17 tissues from six fish. Transcript levels of trout *CMKLR3* are presented relative to that of EF-1α as means + SEM

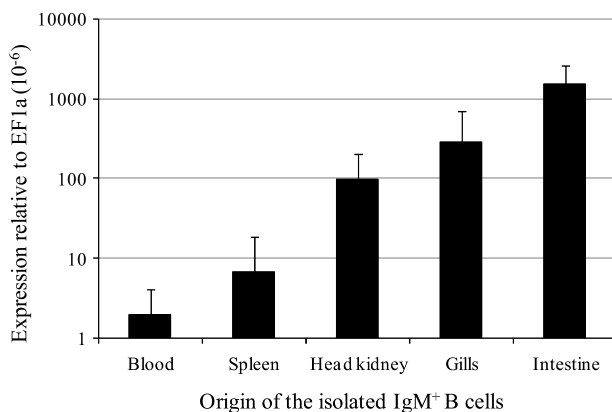

Fig. 5 Expression level of rainbow trout *O. mykiss CMKLR3* in isolated IgM$^+$ cells. Constitutive expression level of trout *CMKLR3* in IgM$^+$ cells isolated from different tissues were measured by real-time PCR. Transcript levels of trout *CMKLR3* are presented relative to that of EF-1α as means + SEM

Gene expression of trout *CMKLR3* in head kidney primary macrophage

The expression of trout *CMKLR3* in the head kidney primary macrophages stimulated with several known macrophage stimulants including PGN and recombinant cytokines (rIL-6, rIFN-γ, and rTNF-α) were analyzed by real-time PCR (Fig. 7). No changes were observed for trout *CMKLR3* after rTNF-α stimulation. PGN upregulated *CMKLR3* expression at 4 h, and the level remained high at 8 h and 24 h post-stimulation. Both rIL-6 and rIFN-γ downregulated *CMKLR3* expression from 2 to 8 h post-stimulation, but no effect on the expression was observed at 24 h post-stimulation.

Discussion

Two members of the CMKLR family had been characterized in salmonids in a previous study [22]. In the present study, the third member of the CMKLRs, which was termed CMKLR3, was identified and functionally analyzed in rainbow trout. The newly identified CMKLR3 possessed conserved structure features of the CMKLRs family that were revealed by sequence alignment analysis, including seven-transmembrane structure, DRC motif, and two cystine residues in the ECL1 and ECL2 regions (Figs. 1, 2). Meanwhile, trout CMKLR3 shared more than 75.8 % sequence identities with reported fish CMKLR3, which were higher than that with CMKLR1 (40.5–41.8 %) and CMKLR2 (24–38 %) (Table 2). In addition, phylogenetic tree analysis revealed that trout CMKLR3 clustered together with fish CMKLR3 into a clade with a convincing bootstrap value (100 %), which was clearly separated from the clade of fish CMKLR1 and CMKLR2 (Fig. 3). We also identified CMKLR3 in Atlantic salmon, which was also well clustered into the CMKLR3 clade (Fig. S2). Thus, it was reasonable to name this newly identified gene as CMKLR3. The finding of CMKLR3 in fish revealed that CMKLRs had been greatly expanded in teleost fish.

Mammalian CMKLR1 was an orphan receptor involved in signaling pathway regulation, cell migration, and inflammation [19]. The information on fish CMKLRs was scarce. In Atlantic salmon, CMKLR1, CMKLR2a, and ΨCMKLR2b had been identified, which might be the result of genome duplication [22]. Atlantic salmon *CMKLR1* and *CMKLR2a* were highly expressed in head kidney, kidney, and spleen [22]. In the present study, we found that trout *CMKLR3* was ubiquitously expressed in 17 tested tissues

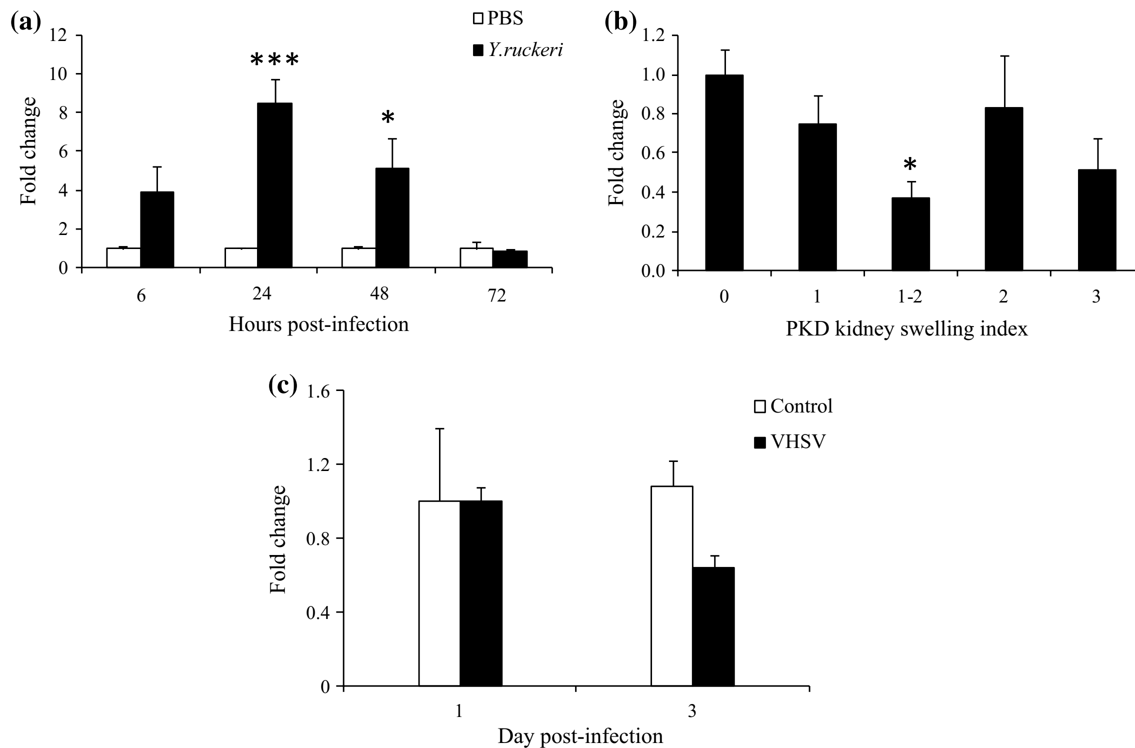

Fig. 6 Modulation of rainbow trout *O. mykiss CMKLR3* expression by bacterial **(a),** parasitic **(b),** and VHSV **(c)** infection. **a** Rainbow trout were i.p. injected with *Y. ruckeri* or PBS. The gene expression in the head kidney of the challenge group fish is expressed as a fold change relative to those of the control fish (means + SEM of five fish). **b** Kidneys from rainbow trout infected with *Tetracapsuloides bryosalmonae* (kidney swelling index 1, 1–2, 2, and 3) or from uninfected (index 0) fish were collected during a spontaneous infection. Results are presented as a fold change relative to the control fish (means + SEM). The numbers of fish analyzed were 11, 5, 9, 10, and 9, representing the index 0 (uninfected control), 1, 1–2, 2, and 3, respectively. **c** The kidney was sampled from six VHSV-challenged fish or PBS mock-challenged fish at 1 and 3 days post-challenge. Expression of trout *CMKLR3* is expressed as a fold change relative to the time-matched controls (means + SEM). The significance of LSD post-hoc tests after one-way ANOVA between infected and control fish is shown above the bars: *$p < 0.05$ and **$p < 0.01$

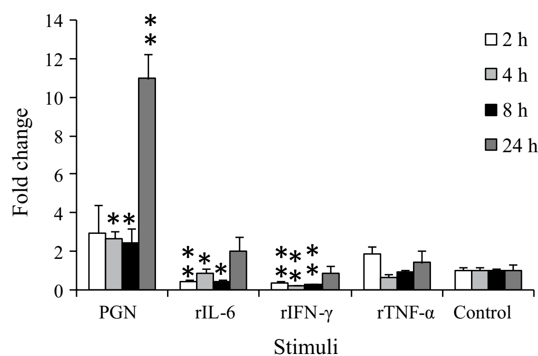

Fig. 7 Modulation of rainbow trout *O. mykiss CMKLR3* expression in primary head kidney macrophage. Four-day-old primary head kidney macrophages were stimulated with PGN or recombinant trout cytokines (rIL-6, rTNF-α and rIFN-γ) for 2, 4, 8, and 24 h. The gene expression of *CMKLR3* is expressed as a fold change calculated as the mean expression levels in stimulated cells normalized to that of time-matched controls. The means + SEM of the cells isolated from four fish are shown. The paired sample *t*-tests between stimulated and time-matched control samples were analyzed with SPSS package 19.0: *$p < 0.05$ and **$p < 0.01$

(Fig. 4), indicating that this receptor might play role(s) in a broad range of tissues. The high expression of trout *CMKLR3* in the head kidney and spleen suggested its functional role(s) in regulating leukocytes [14]. Further expression analysis of trout *CMKLR3* in IgM + B cells isolated from the intestine, gills, head kidney, and spleen revealed that it might play roles in the regulation of B lymphocytes.

We further examined whether trout *CMKLR3* was modulated in bacterial, parasite, and viral infection to confirm its role in inflammatory responses. *Y. ruckeri* is the causative agent of enteric redmouth disease in rainbow trout and other fish species [29]. It has been found that several pro-inflammatory cytokines, e.g., *IL-1β*, *IL-6*, *IL-8*, *TNF-α*, and *IFN-γ* [36, 37], and some chemokine receptors, e.g., *CXCR2-3* [27] and *CCBP2* [38], were upregulated in immune organs of trout after *Y. ruckeri* infection. In the present study, we found that trout *CMKLR3* was upregulated at 4, 8, and 24 h post-infection (Fig. 6a), suggesting its role in the regulation of the inflammatory response during bacterial infection. PKD of salmonid fish is caused by

myxozoan *Tetracapsuloides bryosalmonae*, which targets the kidney of infected fish where it causes a chronic lymphoid immunopathology and induces an anti-inflammatory phenotype [30]. The downregulation of trout *CMKLR3* at early stages of PKD disease (grade 1–2) (Fig. 6b) indicated its negative role in regulating anti-inflammatory responses in the kidney. Interestingly, although it is known that a number of chemokine receptors, e.g., *CCR7*, *CCR9*, *CXCR3B* and *CXCR4*, are upregulated during VHSV infection [39], trout *CMKLR3* was refractory to VHSV infection in this study (Fig. 6c). Mammalian CMKLR1 seemed restrictedly to serve as a co-receptor for some SIV clones and HIV-1 strain [18, 22]. Whether the unchanged *CMKLR3* expression in trout is due to the strain of the virus or without function in response to virus infection need further investigation.

Similar to mammalian CMKLRs, trout *CMKLR3* was also expressed in macrophages and could be regulated by several cytokines and pathogen-associated molecular patterns (PAMPs) (Fig. 7). In primary head kidney macrophages, trout *CMKLR3* was upregulated by PGN stimulation, confirming its regulating role in a host inflammatory response to bacterial infection. Furthermore, trout *CMKLR3* was downregulated by IFN-γ stimulation. Similar results were also observed in mammalian *CMKLR1*. IFN-γ is produced by activated T cells, NK cells, and NKT cells, which is the hallmark of Th1 responses and can regulate both innate and cell-mediated immune responses [40]. The downregulation of *CMKLR3* by IFN-γ stimulation demonstrated that CMKLR3 might be involved in the regulation of T cell development and activation in fish. In fish, IL-6 had been proved to be an anti-inflammatory cytokine that can modulate a number of cytokines and chemokine receptors [41]. The downregulation of trout *CMKLR3* following IL-6 stimulation suggested the negative role of CMKLR3 in regulating anti-inflammatory response. In addition, trout *CMKLR3* remained unchanged following TNF-α stimulation. TNF-α had a different role in the regulation of CMKLRs in different cell types. Mammalian *CMKLR1* was upregulated by TNF-α in macrophages but was downregulated in monocytes [42]. The unchanged status of *CMKLR3* after TNF-α revealed that fish CMKLRs might have a fish-specific regulated pattern under TNF-α stimulation. The different effects of cytokines on the expression of *CMKLR3* also suggest that CMKLR3 can be regulated in a complex way. Further studies on the expression of CMKLRs in other cell types are needed to elucidate the regulation of the CMKLR network.

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