Non-specific cytotoxic cell receptor protein-1 (NCCRP-1) is involved in anti-parasite innate CD8^+ T cell-mediated cytotoxicity in ginbuna crucian carp

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Non-specific cytotoxic cell receptor protein-1 (NCCRP-1) is involved in anti-parasite innate CD8$^+$ T cell-mediated cytotoxicity in ginbuna crucian carp

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Abstract

$CD8^+$ cytotoxic T cells (CTLs) are a main cellular component of adaptive immunity. Our previous research has shown that $CD8^+$ cells demonstrate spontaneous cytotoxic activity against the parasite *Ichthyophthirius multifiliis* in ginbuna crucian carp, suggesting that $CD8^+$ cells play an important role in innate immunity. Herein, we investigated the molecules and cellular signal pathways involved in the cytotoxic response of ginbuna crucian carp. We considered non-specific cytotoxic receptor protein-1 (NCCRP-1) as candidate molecule for parasite recognition. We detected NCCRP-1 protein in $CD8^+$ cells and the thymus as well as in other cells and tissues. $CD8^+$ cells expressed mRNA for NCCRP-1, Jak2, and T cell-related molecules. In addition, treatment with a peptide containing the presumed antigen recognition site of ginbuna NCCRP-1 significantly inhibited the cytotoxic activity of $CD8^+$ cells against the parasites. The cytotoxic activity of $CD8^+$ cells was significantly inhibited by treatment with the JAK1/2 inhibitor baricitinib. These results suggest that teleost CTLs recognize *I. multifiliis* through NCCRP-1 and are activated by JAK/STAT signaling.

Keywords. $CD8^+$ cytotoxic T cell, cell-mediated cytotoxicity, *Ichthyophthirius multifiliis*, non-specific cytotoxic receptor protein-1 (NCCRP-1), JAK/STAT signaling pathway.

1. Introduction

$CD8^+$ cytotoxic T cells (CTLs), the main cytotoxic cells in adaptive immunity, remove target cells such as viral or intracellular bacterial-infected cells. Teleost $CD8^+$ cells sensitized with either alloantigens or viral antigens show antigen-specific cell-mediated cytotoxicity; they presumably recognize virus-infected cells in a major histocompatibility complex (MHC) class I restricted manner [1,2], suggesting that CTLs play an essential role in the adaptive cell-mediated immunity of teleosts, similar to mammalian CTLs. In addition to its classical function, teleost $CD8^+$ lymphocytes exhibit direct killing ability against the bacteria *Lactococcus garvieae* and *Edwardsiella tarda* [3] and parasites [4] in a non-MHC restricted manner.

Our previous study in clonal ginbuna crucian carp demonstrated that $CD8^+$ lymphocytes had innate cell-mediated cytotoxicity against *Ichthyophthirius multifiliis* [4]. The isolated $CD8^+$ T cells killed the parasites by contacting them without pre-immunization, using multiple cytotoxic factors such as perforin and serine protease, suggesting that $CD8^+$ T cells have the ability to non-specifically recognize some targets in these parasites. However, the parasite recognition molecules in $CD8^+$ cells have not been elucidated so far.

Non-specific cytotoxic receptor protein-1 (NCCRP-1) was first identified as a cell surface protein expressed on non-specific cytotoxic cells (NCCs) in channel catfish [5] , and found in other fishes [6,7]. NCCRP-1 was predicted to be a type III membrane protein with three signature domains, an antigen binding motif, a signaling transduction domain, and a transcriptional activation domain [8]. Like mammalian natural killer cells, NCCs

63 spontaneously kill a variety of target cells, including xenogeneic cells and protozoan
64 parasites recognized via NCCRP-1 [8-10]. Although NCCRP-1 was considered a marker
65 of NCCs in catfish, several studies have demonstrated that NCCRP-1 is expressed not
66 only in NCCs but also in other cells in various fish species [11-14]. Further, NCCRP-1 is
67 expressed in various organs in mice, humans, and fish [12,15]. NCCs express JAK2 and
68 STAT5, suggesting that NCCRP-1 may require the JAK/STAT signaling pathway to
69 activate the NCCs and kill target cells [5,16,17]. The clonal triploid ginbuna crucian carp,
70 a naturally occurring gynogenetic fish, is a useful model for studying T cell-mediated
71 immunity in teleosts [1]. NCCRP-1 has not been identified in this species. In the present
72 study, we hypothesized that NCCRP-1 is the recognition molecule to *I. multifiliis* in $CD8^+$
73 cells and aimed to uncover the signaling pathways involved in $CD8^+$ cell cytotoxicity
74 using ginbuna crucian carp.

75

76 **2. Materials and Methods**

77 **2.1. Fish**

78 Fish from the OB1 strain of ginbuna crucian carp (*Carassius auratus langsdorfii*) were
79 hatched in the National Fisheries University and reared at Kyushu University. Fish
80 weighing approximately 20 g were used in these experiments. They were kept in 140 L
81 tanks at 25°C with running water and fed daily with commercial pellets (Nippon Formula
82 Feed Manufacturing Co., Ltd.). No symptom of white spot disease was observed during
83 the experiments. All experiments were performed in accordance with the guidelines of
84 the Animal Experiments Committee at Kyushu University.

2.2. NCCRP-1 identification in *Carassius auratus*

Genomic database (ASM336829v1) of goldfish "*Carassius auratus*", which is thought to be mutant of crucian carp, was used to identified the sequences of ginbuna crucian carp NCCRP-1. Because the sequences in the database show high identity to those of crucian carp, it is useful for ginbuna crucian carp (*C. auratus langsdorfii*). The cDNA sequence encoding *Carassius auratus* NCCRP-1 was obtained from the NCBI nucleotide database under the accession number XM_026212010. To confirm whether the NCCRP-1 gene of the ginbuna crucian carp OB1 strain was identical to it, the predictive functional domain of NCCRP-1 from the ginbuna OB1 strain was sequenced and compared to that of *C. auratus*. The cDNA of ginbuna kidney leukocytes was prepared as previously reported [4,18]. Specific primer sets to amplify NCCRP-1 were designed based on its sequence using Primer 3 (https://bioinfo.ut.ee/primer3-0.4.0/). The polymerase chain reaction (PCR) conditions were as follows: one cycle of 95℃ for 2 min, 35 cycles of 95℃ for 10 s, 60℃ for 10 s, and 72℃ for 30 s. PCR amplification products were collected using FavorPrepTM GEL/PCR Purification Mini Kit (FAVORGEN, Taiwan). GENEWIZE Inc (Japan) performed sequence analysis. The obtained DNA sequences were compared with the NCCRP-1 sequence of *Carassius auratus* (XM_026212010). The sequences of teleost NCCRP-1 (*Cyprinus carpio* [BAD35013.1], *Danio rerio* [AAF19642.1], *Ictalurus punctatus* [ADD84669.1], *Oreochromis niloticus* [AWD77140.1], *Epinephelus coioides* [ADD84669.1]) were aligned with ClustalW. The molecular weight of the *Carassius auratus* NCCRP-1 protein was calculated by the protein molecular weight calculator (https://www.sciencegateway.org/tools/proteinmw.htm).

2.3. Peptide synthesis

Three synthetic peptides (peptide-1, peptide-2, peptide-3) from the region including the presumed antigen recognition site of ginbuna crucian carp NCCRP-1 [5] were synthesized at SCRUM Inc. (Tokyo, Japan) (Fig. S1A). Each synthetic peptide was used for producing an anti-ginbuna NCCRP-1 peptide antibody against peptide-2 or demonstrating in ELISA, Western blot, and inhibition test of cytotoxicity.

2.4. Production of the anti-ginbuna NCCRP-1 peptide antibody

The peptide-2 conjugated with keyhole limpet hemocyanin was emulsified in Freund's complete (primary immunization) or incomplete (second third and fourth) adjuvant for rabbit immunization at SCRUM Co. (Tokyo, Japan). After four subcutaneous injections and a booster at weekly intervals, the rabbits were bled to separate the antisera. The anti-peptide-2 antibody was purified by affinity purification using TOYOPEARL AF-Epoxy-650M (Tosoh Bioscience, Japan) coupled with peptide-2.

An ELISA was performed to confirm the specificity of the purified IgG against the peptide [19]. Each peptide was dissolved in 1 μg/mL of carbonate-bicarbonate buffer and used to coat 100 μL/well on the ELISA plate. No peptide was added to the control wells, however, the immunogenic peptide (peptide-2) and the other peptides (peptide-1 and -3) were included in each test. After plate incubation overnight at 4°C, the plate was washed twice with PBS before blocking with ELISA diluent (BioLegend, San Diego, CA, USA) and incubated at room temperature for 1 h. After washing with PBS, the primary antibody

was diluted in the ELISA diluent at different dilutions (1, 0.5, 0.1 μg/well) and added to each well. After incubating at room temperature for 1 h, the plate was washed twice with 0.1 % Tween and PBS. Then, the secondary antibody, an anti-rabbit IgG (H+L chain) conjugated with HRP (MBL Co. Ltd., Aichi, Japan), was diluted in ELISA diluent at 1:10000 and added to the wells at 100 μL/well. The plate was incubated at room temperature for 45 min and washed twice with 0.1 % Tween 20 PBS. Then, 100 μL of ELISA POD Substrate TMB Solution (Nacalai Tesque, Tokyo, Japan) were added to the wells. Next, the plate was incubated for several minutes until color develops. At that time, the reaction was halted using 100 μL/well of 1 M phosphoric acid. Finally, the absorbance at 450 nm was measured using Multiskan FC (Thermo Fisher Scientific, U.S.A).

Antibody binding to live cells from ginbuna crucian carp was tested by flow cytometry (FCM). Unfortunately, the produced antibody could not recognize the NCCRP-1 protein expressed on the surface of live cells from ginbuna crucian carp.

2.5. Preparation of *I. multifiliis*

Theronts of *I. multifiliis* were prepared as previously described [4]. Briefly, *I. multifiliis* were originally extracted from infected fish obtained from Kyorin Co., Ltd. and ornamental fish shops in Fukuoka city. In addition, live theronts were collected from water in an aquarium with *I. multifiliis*-infected fish. To remove large impurities, theronts were passed through a sieve with a 37 μm pore size (ITOH SEISAKUSHO CO., Tokyo, Japan). To adjust the concentration, the theronts were concentrated by centrifugation at 100 × g for 5 min and suspended in 800 μL of RPMI-1640 medium (Nissui

Pharmaceutical Co., Tokyo, Japan) supplemented with 0.5% heat-inactivated fetal bovine serum (Biowest, France), and counted using an optical plastic plankton counter (MATSUNAMI., Osaka, Japan). Then, they were identified as *I. multifiliis* by microscopy and PCR using *I. multifiliis*-specific primers [20].

2.6. $CD8^+$ cell isolation

$CD8^+$ cells were prepared as described in previous reports [4]. Fish were anesthetized with 2 mL/L 5% 2-methylquinoline in ethanol and dissected to isolate the trunk and head kidneys. Kidney cells were dispensed by aseptically disaggregating the tissue through a sterilized 150-gage mesh stainless steel sieve in RPMI-1640 medium. Cell suspensions were then layered over Percoll (1.08 g/mL, GE Healthcare), followed by centrifugation at 330 × g for 25 min at 4°C. The leukocytes on the Percoll gradient were collected and washed twice with RPMI-1640. $CD8\alpha^+$ cells were then purified by magnetic-activated cell sorting (MACS; Mini MACS, Miltenyi Biotec) using anti-ginbuna CD8α monoclonal antibodies (2C3) according to previous research [4-21]. The purity of $CD8^+$ cells was confirmed by RT-PCR using CD8 primers. In addition, the percentage of lymphocytes among $CD8^+$ cells was investigated by FCM.

2.7. RT-PCR analysis of $CD8^+$ cells

Total RNA was extracted from 5.0×10^5 sorted and unsorted cells using a NucleoSpin® RNA kit (TaKaRa, Japan) and reverse transcribed into cDNA using an M-MLV Reverse Transcriptase (NIPPON GENE) with Oligo d(T)16 primers following manufacturer's

instructions. The specific primer sets for CD8α, NCCRP-1, Zap-70, JAK2, Lck, and EF1-α used for RT-PCR are shown in Table S1 [22-26] (Accession.No, NC_039269.1; Accession.No, XM_026265484). The PCR conditions and electrophoresis of PCR products were as those previously described [4].

2.8. Western blotting analysis of isolated cells and organs

Fish were anesthetized and dissected to isolate the thymus, kidney, spleen, gills, and ovary. The isolation method of $CD8^+$ cells is described above. Then, cells and organs were lysed in SDS-sample buffer. The lysates were electrophoresed in 10% SDS gels and transferred to nitrocellulose membrane filters using standard techniques. Then, the membranes were blocked overnight at room temperature with 5% non-fat dry milk in PBS and incubated with primary antibody (NCCRP-1, 1 μg/mL; β-actin, 1:5000 dilution) diluted in 5% non-fat dry milk in PBS for 1 h at room temperature. The membranes were washed with PBS for 3 times (5 min between each wash) and incubated with secondary antibody (Anti-rabbit IgG with HRP against NCCRP-1 0.5 μg/mL, Anti-mouse IgG with HRP against β-actin; 1:5000 dilution) diluted in 5% non-fat dry milk in PBS for 1 h RT. Next, the membranes were treated with a mixture of ECLTM prime western blotting detection regents (solution A: solution B = 1:1) (GE Healthcare, USA), illuminated using Multi Imager II ChemiBox (IEDA TRADING Corporation, Japan), and taken a photograph. The blots were quantified using ImageJ; NCCRP-1 expression is normalized to that of β-actin.

2.9. Effect of the NCCRP-1 synthetic peptide on cytotoxicity inhibition

The effect of NCCRP-1 on the cytotoxic activity of $CD8^+$ cells was examined using synthetic peptides derived from ginbuna NCCRP-1. The effector cells were extracted from the kidney of ginbuna carp as described above. *I. multifiliis* were treated with each peptide at 200 μg/mL for 1 h at 22°C; untreated *I. multifiliis* were used as a control. The concentration of each peptide was determined according to previous research [5]. The cytotoxicity assay was performed as previously described [4]. Briefly, $CD8^+$ cells were mixed with 300 *I. multifiliis* cells in RPMI-1640 medium in 96-well plates at effector/target cell ratios (E:T) of 300:1. Next, they were incubated at 20°C for 2 h. After incubation, dead *I. multifiliis* were stained with 0.2% trypan blue (Wako, Japan). The stained *I. multifiliis* were counted on plankton number count boards (MATSUNAMI, Japan, Osaka) under a microscope to calculate the cytotoxic activity [4, 27]. Parasites that spontaneously died in the medium of the negative control were also counted. The cytotoxic activity was calculated using the following formula and indicated as percentage: Cytotoxic activity (%) = {100 × [(Number of dead *I. multifiliis* in test group) − (Number of dead *I. multifiliis* in negative control)]/[(Total number of *I. multifiliis* in test group) − (Number of dead *I. multifiliis* in negative control)]}

To further investigate the effect of the NCCRP-1 synthetic peptide in cytotoxicity inhibition, two concentrations (100, 200 μg/mL) of peptide-1 was tested. $CD8^+$ cells were separated from the kidney of ginbuna carp as described above and *I. multifiliis* were treated with peptide 1 at two concentrations for 1 h at 22°C. The subsequent experimental process was described above.

2.10. Effect of JAK1/2 and Src family kinase inhibitors on cytotoxicity

The effect of JAK1/2 or Src family kinase inhibitors on the cytotoxic activity of $CD8^+$ cells was examined using baricitinib (Sigma, St. Louis MO) or dasatinib (Sigma, St. Louis MO, USA), respectively. $CD8^+$ cells were separated from the kidney of ginbuna carp as described above. Effector cells were treated with baricitinib at two concentrations (200 and 50 ng/mL) or dasatinib at two concentrations (100 and 10 μM) for 30 min at 22℃; untreated effector cells were used as control. The concentrations of these inhibitors were determined according to previous studies [28–30]. The cytotoxic assay was performed as described above.

2.11. Statistical analysis

Steel's multiple comparison test was used to analyze the effects of baricitinib, dasatinib, and synthetic peptides on cytotoxicity. *P*-values <0.05 were considered statistically significant.

3. Results

3.1. NCCRP-1 sequence

The sequence of predictive functional domain from ginbuna crucian carp NCCRP-1 was a complete match with that of *C. auratus* (XM_026212010). Multiple alignments of teleost NCCRP-1 showed that *C. auratus* NCCRP-1 has a sequence homology between

239 62.0% and 95.7% with other species. The proline-rich sequence (PRM), which is "box-
240 2" (PxP) is conserved in all fish species. Although "box-1" is conserved in *I. punctatus*,
241 *O. niloticus*, and *E. coioides*, last proline in "box-1" (PxPxxxP) is replaced to serine in
242 cyprinoid fishes (zebrafish, common and crucian carps). The presumed antigen
243 recognition site (LPxxxWFxxEQxVxLKA) is conserved in *C. auratus* NCCRP-1 (Fig. 1).

244

245 **3.2. NCCRP-1 detection in T cells using an anti-ginbuna NCCRP-1 antibody**

246 The ELISA absorbance value (A_{450}) for peptide 2 was significantly higher than that for
247 peptides 1 and 3 (Fig. S2). There were no significant differences in absorbance between
248 peptide 1, peptide 3, and the negative control. This indicated the specificity of the anti-
249 ginbuna NCCRP-1 peptide antibody against the immunogenic peptide. Bands were
250 detected at about 27 and 42 kDa, consistent with the predicted size of the ginbuna
251 NCCRP-1 protein and β-actin, respectively (Figs. 2 and 3).

252 The NCCRP-1 protein was detected in all examined organs: the thymus, kidney, spleen,
253 gill, and ovary, indicating that ginbuna NCCRP-1 is expressed in various organs (Fig. 2).
254 Detection in the thymus provides one evidence that T cells express NCCRP-1 in ginbuna
255 crucian carp. In addition, NCCRP-1 protein was detected not only in $CD8^-$ cells but also
256 in $CD8^+$ cells (Fig. 3A, B). Since most $CD8\alpha^+$ cells were lymphocytes in the kidney of
257 ginbuna crucian carp, NCCRP-1 protein appears to be mostly expressed on $CD8\alpha^+$ T cells.
258 Similar to findings in other fish [13], ginbuna NCCR-1 was expressed in both cell
259 membrane and cytoplasmic fractions, indicating that NCCR-1 is present in the cell
260 membrane (Fig. 3C, D).

3.3. Expression of T cell-related molecules in isolated cells

To characterize the isolated cells in the kidney, the expression profile of T cell-related molecules was examined as well (Fig. 4). $CD8^+$ cells expressed CD8α, Lck, Zap-70, and JAK2, indicating that they primarily included $CD8^+$ T cells. Similar to the results on protein expression, NCCRP-1 was expressed in $CD8^+$ cells. $CD8^-$ cells showed high expression of NCCRP-1, Lck, Zap-70, and JAK2, but not CD8α. Unsorted leukocytes exhibited higher CD8α, NCCRP-1, Lck, Zap-70, and JAK2 expression.

3.4. Effect of JAK1/2 and Src family kinase inhibitors on cytotoxicity

The cytotoxic activity of $CD8^+$ cells from the kidney was significantly inhibited by the addition of 50 or 200 μg/mL baricitinib (Fig. 5A). This suggested that cytotoxicity of $CD8^+$ cells against *I. multifiliis* involves the JAK1/2 pathway. However, there was no significant difference in the activity of untreated and dasatinib-treated effector cells (Fig. 5B). These results indicate that cytotoxicity of $CD8^+$ cells against *I. multifiliis* is dependent on the JAK1/2 pathway but do not involve the TCR/ Src family kinase pathway.

3.5. Effect of the NCCRP-1 synthetic peptide on cytotoxicity inhibition

The cytotoxic activity of $CD8^+$ cells from the kidney treated with 200 μg/mL peptide 1 was significantly lower than that of untreated cells (Fig. 6). On the other hand, there were no significant differences in the activity of peptides 2 and 3-treated effector cells. This

282 indicated that the peptide 1 region involves in the recognition of *I. multifiliis* by $CD8^+$
283 cells. The cytotoxic activity of $CD8^+$ cells from the kidney treated with 100 or 200 μg
284 /mL peptide 1 was significantly lower than that of untreated cells (Fig. 6A). Our findings
285 that the cytotoxic activity of $CD8^+$ cells from the kidney and gill was inhibited by the
286 NCCRP-1 synthetic peptide suggest that $CD8^+$ cells recognize and kill *I. multifiliis*
287 through a pathway involving NCCRP-1.

288

289 **4. Discussion**

290 NCCRP-1 was considered as an NCC marker, which binds foreign antigens in the
291 membrane of NCCs [5–7]. However, NCCRP-1 mRNA is expressed in various organs or
292 cells in human, mouse, and fish [12,31]. In addition, a recent study suggests that common
293 carp express NCCRP-1 on γδT and mucosal associated invariant cells (Shimon-Hophy et
294 al., 2020). Adding to previous evidence, the present study first demonstrated that
295 NCCRP-1 is expressed in the thymus and $CD8^+$ cells at the protein level of ginbuna fish.

296 The structure of NCCRP-1 includes an PRM at its N-terminal region called "box-1"
297 (PxPxxxP) and "box-2" (PxP) [6,12,32].In particular, box-1 is important for
298 transcriptional activation through the JAK/STAT pathway; in particular, studies in catfish
299 indicated that the JAK/STAT pathway is involved in NCC activation [11,33,34].
300 Accordingly, the JAK/STAT pathway may contribute to cell activation through NCCRP-
301 1 antigen recognition in the innate cell-mediated cytotoxicity response of fish $CD8^+$ T
302 cells against parasites. The current study showed that the cytotoxic activity of $CD8^+$ cells
303 against parasites was significantly suppressed by an inhibitor for JAK/STAT signaling.

Furthermore, the teleost NCCRP-1 possesses a presumed antigen recognition site (LPxxxWFxEQxVxLKA) for pathogens' antigens [5,11,13,35,36]. In this study, a peptide including the sequence of the presumed antigen recognition site inhibited the antiparasitic cytotoxic activity of $CD8^+$ cells, suggesting that this NCCRP-1 site recognizes the parasites. Accordingly, fish $CD8^+$ T cells as well as NCCs would have an innate cytotoxic mechanism that recognizes foreign antigens via NCCRP-1 and transduces cell activation signal using the JAK/STAT pathway.

Although catfish NCCRP-1 was predicted to be a type III membrane protein with three signature domains [5], a bioinformatics analysis showed that teleost NCCRP-1 has no signal peptide or transmembrane helices. Moreover, the present and previous studies have shown that NCCRP-1 is located in the cell membrane [12,35], suggesting that teleost NCCRP-1 is a membrane protein. However, human NCCRP-1 is an intracellular ubiquitin ligase protein, not a cell surface protein, and does not contribute to innate cell-mediated cytotoxicity [31]. This may be because over evolution NCCRP-1, like other FBXO proteins, may have changed its role to cell cycle regulation. In fact, human NCCRP-1 has been reported to contribute to cell proliferation and is considered a putative tumor suppressor in esophageal squamous cell carcinoma carcinogenesis [31,37].

Studies on anti-protozoan parasite immunity in mammals suggest that TCRγ/δ is the candidate receptor in innate cell-mediated cytotoxic activity against parasites [38] . In mammals, γδ T cells are important players in protection and recognize protozoan parasite, malaria and TCR γ/δ recognize malaria antigens [39,40]. T cells recognize antigens via TCRγ/δ, getting activated by signal transduction via TCR signaling. To that end, Src family tyrosine kinases such as Lck and Zap-70 are needed to transmit the signal into the

nucleus [41–46]. Therefore, TCR signaling and Src family tyrosine kinases may have an important role in T cell activation in the cytotoxic response against parasites. However, no significant difference in the activity between untreated and dasatinib-treated effector cells was observed in our study. This suggests that $CD8^+$ cells are not activated against *I. multifiliis* in a TCR/Src family kinase dependent manner. Thus, we conclude that the TCR is not involved in the recognition and activation of $CD8^+$ cells against parasites; however, whether effector cells are TCRγ/δ or TCRα/β positive remains unclear and needs to be further investigated in the future.

$CD8^+$ lymphocytes are more abundant than neutrophils and macrophages in the skin, gill epithelium, and fin cavity of ginbuna crucian carp [4,18] (unpublished data). Intra-fin administration of *I. multifiliis* induced $CD8^+$ and $CD4^+$ T cell migration, suggesting that T cells are the first responders against the parasites [18]. Moreover, the gene expression levels of chemokines or chemokine receptors CXCa, CXCR1, CK10, CK12, and CXCR2 increase in the epidermis of common carp infected with *I. multifiliis* [47]. These observations indicate that local $CD8^+$ T cells present in mucosal tissues first contact the parasite and kill them before the theronts transform to mature trophonts. Thus, systemic $CD8^+$ T cells may be attracted to the infection sites by chemokines secreted from other leukocytes. Thus, further studies would be required to identify the factors which induce T cells to mucosal sites.

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Figure legends

Figure 1. Multiple alignment of teleost NCCRP-1 amino acid sequences. Alignment gaps are indicated by dashes (-). Conserved or similar amino acids are shown with an asterisk (*) or dot and colon (. and:), respectively. The putative antigen recognition site (LPxxxWFxxEQxVxLKA), Box-1, and Box-2 are indicated by lines on top of the alignment.

548 **Figure 2.** Tissue distribution of NCCRP-1 protein in ginbuna crucian carp. NCCRP and
549 β-actin as detected by Western blotting (A). Data from one representative fish among the
550 three analyzed. Arrows indicate the predicted size of NCCRP-1 (27 kDa) and β-actin (42
551 kDa). NCCRP-1 protein expression indicated as relative value to that of β-actin (B). Data
552 are shown as means of three individual fish. Error bars indicate SD.

553

554 **Figure 3.** NCCRP-1 protein expression in leukocytes. NCCRP and β-actin expression in
555 $CD8^+$ and other cells ($CD8^-$ cells) or in the membrane and cytoplasm from kidney cells
556 (A and C, respectively) as detected by Western blotting. Data from one representative
557 fish among the three analyzed. Arrows indicate the predicted size of NCCRP-1 (27 kDa)
558 and β-actin (42 kDa). NCCRP-1 protein expression indicated as relative value to that of
559 β-actin (B). Data are shown as means of three individual fish. Error bars indicate SD.

560

561 **Figure 4.** mRNA expression profile of T cell markers and NCCRP-1 in MACS sorted
562 leukocytes from ginbuna crucian carp kidney cells. Other indicates CD8-negative
563 lymphocytes; NC indicates non-template control. EF1α was used as internal control. The
564 numbers on the right indicate PCR cycles. Data from one representative fish among the
565 three analyzed.

566

567 **Figure 5.** Effect of baricitinib (A) and dasatinib (B) on the cytotoxic activity of $CD8^+$
568 cells in the kidney. $CD8^+$ cells were pre-incubated with various concentrations of
569 baricitinib (0, 50, 200 ng/mL) and dasatinib (0, 10, 100 nM). $CD8^+$ cells were co-cultured

570 with *I. multifiliis* at an E:T ratio of 300:1. Data are shown as the mean activity of three
571 individual fish. Error bars indicate SD. Asterisks indicate significant differences from the
572 control group (0 ng/mL; $P < 0.05$).

573

574 **Figure 6.** Effect of each NCCRP-1 synthetic peptide on the cytotoxic activity of $CD8^+$
575 cells from the kidney. *I. multifiliis* were pre-incubated with peptides 1, 2, and 3 (200
576 μg/mL) (A) and two concentrations (100, 200 μg/mL) of peptide 1 (B). $CD8^+$ cells were
577 co-cultured with *I. multifiliis* at an E:T ratio of 300:1. Control indicates the activity
578 observed without peptide. Data are shown as the mean activity from three individual fish.
579 Error bars indicate SD. The asterisk indicates significant differences from the control
580 group (0 μg/well; $P < 0.05$).

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