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Innate cell-mediated cytotoxicity of CD8$^+$ T cells against the protozoan parasite *Ichthyophthirius multifiliis* in the ginbuna crucian carp *Carassius auratus langsdorfii*

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Abstract

Cytotoxic T cells are known to have the ability to kill microbe-infected host cells, which makes them essential in the adaptive immunity processes of various vertebrates. In this study, we demonstrated innate cell-mediated cytotoxicity of $CD8^+$ T cells against protozoan parasites found in the ginbuna crucian carp. When isolated effector cells such as $CD8^+$, $CD4^+$ ($CD4\text{-}1^+$), or $CD8^-$ $CD4^-$ (double-negative, DN), from naïve ginbuna crucian carp were co-incubated with target parasites (*Ichthyophthirius multifiliis*), $CD8^+$ cells from the kidney and gill showed the highest cytotoxic activity. On the other hand, DN cells, which include macrophages and $CD4^-$ $CD8^-$ lymphocytes, showed the lowest cytotoxic activity against *I. multifiliis*. Additionally, the cytotoxic activity of $CD8^+$ cells was found to significantly decrease in the presence of a membrane separating the effector cells from *I. multifiliis*. Furthermore, the serine protease inhibitor 3,4-dichloroisocoumarin and perforin inhibitor concanamycin A significantly inhibited the cytotoxic activity of $CD8^+$ cells. These results demonstrate that $CD8^+$ T cells of ginbuna crucian carp can kill extracellular parasites in a contact-dependent manner via serine proteases and perforin. Therefore, we conclude that $CD8^+$ T cells play an essential role in anti-parasite innate immunity of teleost fish.

Key words: Cytotoxic activity; $CD8^+$ T cells; *I. multifiliis*; extracellular parasites; ginbuna crucian carp

1. Introduction

In mammals, $CD8^+$ cytotoxic T cells (CTLs) are considered the primary cytotoxic cells in adaptive immunity, and they require antigen pre-sensitization to gain the ability to kill target cells, such as viral or intracellular bacterial-infected cells, tumor cells, and allogeneic cells. It has already been reported that antigen-specific cell-mediated cytotoxicity (CMC) can be induced in teleost $CD8^+$ cells by sensitization with allo-antigen and viral antigen, and they presumably recognize virus-infected cells in a major histocompatibility complex (MHC) class I restricted-manner (Somamoto et al., 2015, 2014; Yamaguchi et al., 2019). These findings show that CTLs play an essential role in the adaptive cell-mediated immunity of teleosts, similar to mammalian CTLs.

Ichthyophthirius multifiliis has been identified as a ciliated protozoan that infects freshwater fishes and causes white spot disease (Buchmann et al., 2001; Cross and Matthews, 1993). *I. multifiliis* has a life cycle that consists of four stages: theront, trophont, tomont, and cyst (Dickerson, 2006; Wang et al., 2019). Because it is easy to obtain these pathogens from diseased fish and to artificially infect recipient fish, immune responses to *I. multifiliis* represent an excellent model in elucidating the immune system of fish against extracellular parasites (Cassidy-Hanley et al., 2011; Coyne et al., 2011; Xu and Klesius, 2013). Innate humoral immune factors such as chemokines and complement components are involved in the protection of several fish species from parasitic infection, including channel catfish (*Ictalurus punctatus*), carp (*Cyprinus carpio*), and rainbow trout (*Oncorhynchus mykiss*) (Cross and Matthews, 1993; Dickerson and Clark, 1998; Olsen et al., 2011). Antibody-secreting cells from fish immunized with *I. multifiliis* produce IgM or IgT that specifically bind trophonts (Von Gersdorff Jørgensen et al., 2011; Yu et al., 2018). Antibodies recognize most glycosylphosphatidylinositol (GPI)-anchored surface

73 membrane proteins to resemble the immobilization antigen (i-antigen) of the free-living
74 ciliates and interfere with ciliary beat, leading to immobilization of the parasite (Clark
75 and Forney, 2003). This suggests that antibodies contribute to the elimination of parasites.
76 These studies on immune responses to *I. multifiliis* mainly focus on the humoral immune
77 factors, while information on cell-mediated immunity against parasites is limited.
78 Nonspecific cytotoxic cells (NCCs), which were first identified in channel catfish, are the
79 only known anti-parasitic cytotoxic cells in teleost fishes (Graves et al., 1985; Jaso-
80 Friedmann et al., 1997). The anti-parasitic abilities of NCCs suggest that cell-mediated
81 immunity is an effective defense mechanism against parasites in teleost fish.
82 In mammals, $CD4^+$ and $CD8^+$ T cells have innate cytotoxic activity against various types
83 of pathogens, including bacteria, fungi, and parasites. CTLs have direct antibacterial
84 activity that can kill extracellular bacteria in mice (Mody and Oykhman, 2010).
85 Furthermore, murine CTLs were shown to exhibit fungicidal activity against the fungus
86 *Candida albicans* and inhibited its growth (Beno et al., 1995; Beno and Mathews, 1992).
87 In addition, human CTLs stimulated with phytohemagglutinin (PHA) killed the
88 multicellular parasites *Schistosoma mansoni* and *Entamoeba histolytica*, and the
89 cytotoxic activity was dependent on contact with the target pathogens (Ellner et al., 1982;
90 Salata et al., 1987). We have previously shown that $CD8^+$ cells from ginbuna crucian carp
91 exhibit direct cytotoxic ability against the bacteria *Lactococcus garvieae* and
92 *Edwardsiella tarda*, suggesting that fish CTLs have the ability to recognize target cells in
93 a manner independent of the T cell receptor (TCR) (Nayak and Nakanishi, 2013). These
94 findings further suggest a possibility that fish CTLs possess innate cytotoxic activity
95 toward various types of pathogens, in addition to self-infected and allogeneic cells.
96 In the present study, we aimed to demonstrate that $CD8^+$ T cells possess natural cytotoxic

ability against theronts of *I. multifiliis* and describe the cytolytic mechanisms involved. Our findings provide an important insight into anti-parasite immunity and the evolution of cell-mediated immunity.

2. Materials and Methods

2.1. Fish

Ginbuna crucian carps (*Carassius auratus langsdorfii* [OB1 clone]) were hatched from the National Fisheries University and reared at Kyushu University. Adult ginbuna crucian carps weighing approximately 23.2 g were used in this study. Fish were kept in 140-L tanks at 25□ with running water; they were fed daily with commercial pellets (Nippon Formula Feed Manufacturing Co., Ltd.). Spontaneous occurrence of white spot disease was not observed in these experimental fish. All experiments involving fish were performed in accordance with the guidelines of the Animal Experiments Committee at Kyushu University.

2.2. Preparation of *I. multifiliis*

It has been reported that only few of artificially exposed theronts can transform into mature trophonts, which suggests that most invading theronts are eliminated before they become mature trophonts (Xu et al., 2004; unpublished observation). Therefore, we focused on the immune response at this stage of *I. multifiliis* and used theronts as the target in this study. The parasite strains used in the present study originated from infected fish obtained from Kyorin Co., Ltd. and ornamental fish shops in Fukuoka city. They were identified as *I. multifiliis* by microscopy and polymerase chain reaction (PCR) using

I. multifiliis-specific primers (data not shown) (Sun et al., 2006). The fish infected with mature parasitic trophonts were anesthetized with 5% 2-methylquinoline in EtOH and rinsed with water. The anesthetized fish were placed on petri dishes with pure water, and the skin was gently scraped with a cell scraper to collect the trophonts (Zellschaber; Techno Plastic Products AG., Trasadingen, Switzerland). To collect live theronts, the isolated trophonts were transferred to a beaker filled with water. To avoid contaminating the mucus, trophonts were replaced in a beaker with fresh pure water and were then incubated for 18 h at 20°C (Xu et al., 2009). Alternatively, 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 pore size of 37 μm (ITOH SEISAKUSHO CO., Tokyo, Japan). 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). Theronts were counted using an optical plastic plankton counter (MATSUNAMI., Osaka, Japan).

2.3. Preparation of the effector cells

Fish were anesthetized with 5% 2-methylquinoline in EtOH and dissected to isolate the trunk and head kidneys and gills. Leukocytes from both the kidneys and the gills of ginbuna carp were prepared by aseptically disaggregating the tissue through a sterilized 150-gauge mesh stainless steel sieve in RPMI-1640 medium. Leukocyte suspensions were then layered over a Percoll density gradient (1.08 g/ml, GE Healthcare), followed by centrifugation at 330 × g for 25 min at 4°C. Leukocytes on the Percoll gradient were collected and washed twice with RPMI-1640. T cell subsets ($CD8\alpha^+$ and $CD4^+$ cells) were

then separated by magnetic-activated cell sorting (MACS; Mini MACS, Miltenyi Biotec) using anti-ginbuna CD8α and CD4 (CD4-1) monoclonal antibodies (MAbs) according to the method described by Toda et al. (2011b). In brief, a suspension of kidney and gill cells in RPMI-1640 medium was incubated with 1:10000 rat anti-ginbuna CD8α MAb (2C3: mouse ascites) for 40 min on ice. The cells were then washed thrice with RPMI-1640, incubated for 20 min at 4°C with 100 µl of a 1:5 dilution of magnetic bead-conjugated goat anti-rat IgG antibody (Miltenyi Biotec GmbH, Germany), and washed three times. CD8α-positive and CD8α-negative cells were then separated using MACS by applying the cell suspension to a plastic column equipped with an external magnet. $CD8\alpha^+$ cells were retained in the column, whereas the $CD8\alpha^-$ cells were not. $CD8\alpha^-$ cells were further separated into $CD4^+$ and DN fractions using rat-anti-ginbuna CD4-1 MAb (6D1) according to the method described above.

2.4. RT-PCR analysis of MACS-sorted cells

Total RNA was extracted from 5.0×10^5 sorted cells of each cell population using a NucleoSpin® RNA kit (TaKaRa, Japan) and reverse transcribed into cDNA using M-MLV Reverse Transcriptase (NIPPON GENE) with Oligo $d(T)_{16}$ primers. Specific primer sets for CD4-1, CD8α, TCRβ, TCRγ, Lck, and EF1-α were used for RT-PCR, as shown in Table 1 (Barreda et al., 2005; Matsuura et al., 2014; Miyazawa et al., 2018; Nonaka et al., 2008; Toda et al., 2011b, 2011a; Accession.No, NC_039269.1.). The PCR conditions were as follows: one cycle of 95°C for 2 min, 30–37 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 30 s. The PCR reactions were performed in 24.5-µl reaction mixtures containing 10× Ex Taq Buffer, dNTP Mixture (2.5 mM each), 1.0 µM of each primer, TaKaRa Ex Taq (5 units/µl) (TaKaRa, Japan), and 0.5 µl of cDNA template. The PCR

products were electrophoresed in 1.3% agarose gels and visualized by staining the gel with 0.5 μg/ml ethidium bromide (Wako, Japan). Images of the PCR products were photographed using Gel Scene Tablet (ASTEC, Japan).

2.5. Cytotoxic activity of unsorted leukocytes, $CD8^+$, $CD4^+$, and DN cells against *I. multifiliis*

Unsorted leukocytes were mixed with 300 *I. multifiliis* cells in RPMI-1640 medium in 96-well plates at effector/target cell ratios (E:T) of 2000:1, 1000:1, 300:1, or 100:1. Next, they were incubated at 20°C for 2, 4, and 6 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 of leukocytes against *I. multifiliis* (Ahmadpour et al., 2019). Parasites that spontaneously died in the medium were also counted in the negative control. The cytotoxic activity was calculated using the following formula and indicated by percentage (Ellner et al., 1982):

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 identify the anti-parasite effector cell population, unsorted or $CD8^+$, $CD4^+$, or DN cells were adjusted at effector/target cell ratios (E:T) of 1000:1 and then incubated for 2 h. The cytotoxic activity was measured and calculated as described above.

2.6. Binding of $CD8^+$ cells to *I. multifiliis* observed by fluorescence microscopy

Leukocytes from the kidney and gill were prepared according to the method described above. Leukocytes in RPMI-1640 medium were incubated with 1:10^4 rat anti-ginbuna CD8α MAb (2C3) for 40 min on ice. To label $CD8^+$ cells, the cells were washed three times with medium and were further incubated for 30 min at 4°C with anti-rat IgG conjugated with PE (BioLegend, USA, State of California, San Diego). *I. multifiliis* that were incubated with 1 mg/mL 3',6'-di(O-acetyl)-4',5'-bis[N,N-bis(carboxymethyl)aminomethyl] fluorescein tetraacetoxymethylester (DOJINDO, Japan) diluted 1:50 for 30 min were washed three times with water and suspended in RPMI-1640 medium. Leukocytes (3.0×10^5 cells) and *I. multifiliis* (300 cells) were added to RPMI-1640 medium in 96-well plates at E:T ratios of 1000:1 and then incubated at 20°C for 2 h. After incubation, each sample was observed using fluorescence microscopy (Leica).

2.7. Cytotoxicity assay of effector cells using Transwell inserts

To determine whether the cytotoxic activity of each population of effector cells ($CD8^+$ T cells, $CD8^-$ cells) required contact to the target parasites, 12-mm Transwell®-COL Collagen-Coated 0.4 μm Pore PTFE Membrane Inserts (Corning, USA, State of New York, Corning) were used, as described by Haitham et al. (Tartor et al., 2014). Briefly, a 100 μL aliquot containing 300 *I. multifiliis* cells was added to the upper compartment of the tissue culture inserts, and a 200 μL aliquot containing 1.5×10^5 effector cells was added to the lower compartment of a 12-well plate well. The two compartments were separated by a membrane with a pore size of 0.4 μm. The plates were then incubated at 22°C for 2 h. After incubation, 100 μL of the culture suspension was collected from the upper compartment. Dead *I. multifiliis* were counted to determine the percentage of

killing activity as described above. As a negative control, a 100 μL aliquot containing 300 *I. multifiliis* cells was added to the upper compartment of the tissue culture inserts, and 200 μL of cell-free RPMI-1640 medium was added to the lower compartment of a 12-well plate.

2.8. Effect of perforin and serine protease inhibitors on cytotoxicity

The effect of perforin or serine protease inhibitors on the cytotoxic activity of each effector cell population ($CD8^+$ cells and $CD8^-$ cells) was examined using concanamycin A (CMA, Sigma, St. Louis MO) or 3,4-dichloroisocoumarin (DCI, Sigma, St. Louis MO, USA), respectively. Each effector cell was separated from the gill and kidney of ginbuna carp as described above. Effector cells were treated with CMA at two concentrations (1 and 0.5 μM) for 2 h or DCI at two concentrations (40 and 20 μM) for 3 h at 22°C. The concentrations of these inhibitors were determined according to previous studies, and untreated effector cells were used as a control (Toda et al., 2011a, 2011c). The inhibitor-treated effector cells (9.0×10^4 cells) were then co-cultured with 300 *I. multifiliis* cells per well in 96-well plates in the presence of inhibitors for 2 h at 22°C. Dead parasites were then counted in each treated group at all inhibitor concentrations, and the percentage of dead cells was then calculated using the above formula, accounting for negative controls. The percentage of killing activity was calculated as described.

2.9. Statistics

Differences among the four groups were compared using the Kruskal–Wallis test. The Mann–Whitney U test was used to determine significant differences between the two groups. Steel's multiple comparison test was used to analyze the effects of perforin and

serine proteases inhibitors on cytotoxicity. P values less than 0.05 were considered statistically significant.

3. Results

3.1. Expression of T cell-related molecules in effector cells

To characterize the effector cells in the kidney and gills, we examined the expression profile of T cell-related molecules (Fig. 1). In both the kidney and gill, $2C3^+$ lymphocytes strongly expressed CD8α, TCRβ, and Lck, indicating that they were primarily composed of $CD8^+$ T cells. Although $6D1^+$ lymphocytes from the kidney showed high expressions of CD4-1 and Lck, the same level of expression of CD8 mRNA was detected in this cell population from gills. This tendency was also observed in $CD4^+$ cells from the gills of each individual fish. Thus, although the possibility that $CD8^+$ cells were contaminated in this cell population cannot be excluded, a small population of CD8 and CD4 double-positive cells may exist in the gill. In the kidney cell population, DN leukocytes exhibited higher expression of TCRɤ than $CD8^+$ and $CD4^+$ cells. In contrast, $CD8^+$ cells from gills showed the highest expression of TCRɤ, suggesting that $CD8^+$TCRɤδ T cells are present in the gills of ginbuna crucian carp.

3.2. Time course of cytotoxic activity of leukocytes against *I. multifiliis* at different effector and target ratios

Kidney and gill leukocytes demonstrated cytotoxic activity against *I. multifiliis* (Fig. 2). The cytotoxic activities at E:T ratios of 2000:1, 1000:1, and 300:1 were detected at 2 h and increased with incubation time. The activities at the E:T ratio of 100:1 were lower than 20% at all incubation times. The cytotoxic activity was increased in an effector/target

ratio-dependent manner, and the highest activities were detected at E:T ratios of 2000:1 and 1000:1. No notable difference was observed in cytotoxic activity between leukocytes isolated from the kidney and those isolated from the gill.

3.3. Characterization of dominant effector cells against *I. multifiliis*

$CD8^+$ cells from the kidney and gill exhibited the highest cytotoxic activity against *I. multifiliis* among all analyzed cells (Fig. 3). Meanwhile, DN cells from the kidney and gill showed the lowest cytotoxic activity. The cytotoxic activity of $CD8^+$ cells from both organs was significantly higher than that of the unsorted cells, $CD4^+$ cells, and DN cells (Figs. 3A and B). $CD4^+$ cells from the gill showed significantly higher activity than DN cells (Fig. 3B). The effector cells from both kidney and gill showed similar levels of cytotoxic activity. These results show that $CD8^+$ cells can directly recognize and kill *I. multifiliis*.

3.4. $CD8^+$ cells bound to *I. multifiliis*

Kidney and gill $CD8^+$ cells physically interacted with the surface of *I. multifiliis* cells (Fig. 4). $CD8^+$ cells from the kidney, which were bound to the parasites, exhibited lymphocyte morphology. The parasites that came into contact with $CD8^+$ cells ceased movement activity, but other parasites moved vigorously during our observation. The activities of parasites that interacted with $CD8^+$ cells gradually decreased during incubation. These results indicate that $CD8^+$ T cells can directly recognize and kill *I. multifiliis*.

3.5. Requirement of effector cell-target contact for killing of *I. multififiliis*

In the presence of a membrane separating effector cells from *I. multifiliis*, the cytotoxic

activity of $CD8^+$ cells from the kidneys and gills against the target parasite was significantly reduced, whereas that of $CD8^-$ cells from the kidneys and gills was not decreased (Fig. 5). These results suggest that $CD8^+$ effector cells require direct contact with the parasite surface to exhibit cytotoxic effects.

3.6. Effect of perforin and serine protease inhibitors on cytotoxicity against *I. multifiliis*

The cytotoxic activities of $CD8^+$ cells from kidney and gill were inhibited by the addition of 20 or 40 μM DCI (Fig. 6). This suggested that $CD8^+$ cells kill *I. multifiliis* using serine proteases such as granzymes.

The cytotoxic activity of $CD8^+$ cells from the kidney treated with 1 μM of CMA was significantly lower than that of the untreated cells (Fig. 7). There was no significant difference in the activity of $CD8^+$ cells from the gills, whereas the mean percentage of CMA-treated effector cells was lower than that of untreated cells. These results indicate that $CD8^+$ cells kill *I. multifiliis* in a manner dependent on the activity of perforin and serine proteases.

4. Discussion

CMC is an important defense mechanism for eliminating targets such as host cells infected with virus or intracellular bacteria and tumor cells in both mammals and teleost fish. Although previous studies identifying cytotoxic cells using clonal ginbuna crucian carp have demonstrated antigen-specific CMC of $CD8^+$ cells against allogeneic and virus- and bacteria-infected syngeneic cells (Somamoto et al., 2006; Toda et al., 2009; Yamasaki et al., 2014), no studies have reported anti-parasite cytotoxicity in this species. Several

types of natural killer (NK)-like cells including NCC have already been identified in teleost fish (Fischer et al., 2013). However, the innate CMC of $CD8^+$ cells remains to be reported in any fish species. In the present study, we showed that $CD8^+$ cells from non-sensitized effectors are the primary cytotoxic effector cells against protozoan parasites, suggesting that $CD8^+$ T cells are important effector cells in anti-parasite innate immunity of teleost fish. This finding provides a novel insight into the evolution of cytotoxic cells and further contributes to the understanding of anti-parasite immunity of teleost fish.

$CD8^+$ and $CD4^+$ cells showed significant cytotoxic activity against *I. multifiliis*, whereas DN cells showed the lowest cytotoxic activity. In particular, $CD8^+$ T cells were identified as the main effector cells against *I. multifiliis* in ginbuna crucian carp. Studies have demonstrated that $CD8^+$ and $CD4^+$ cells from fish immunized with bacteria can kill the bacteria without the need for presentation of target antigens (Nayak and Nakanishi, 2013). The present study showed that $CD8^+$ cells from naïve fish possess cytotoxic activity against parasites and can also recognize and kill target cells without any stimulation. These findings suggest that $CD8^+$ T cells may play a role in the first line of defense against protozoan parasites. On the other hand, DN cells, which include NK-like cells and macrophages, did not possess significant cytotoxicity against *I. multifiliis*, even though these cells are known to possess innate cytotoxic capabilities (Fischer et al., 2013). Theronts have been shown to express a protein similar to chlamydial polymorphic repeat-containing outer membrane protein, which can suppress the innate immune response and avoid the host immune system (Cassidy-Hanley et al., 2011; Mukura et al., 2017; Tanzer et al., 2001). Thus, the possibility that cytotoxic activities of DN cells are inhibited by *I. multifiliis* protein cannot be excluded. Further studies are warranted to identify additional factors that may be involved in the interaction between hosts and *I. multifiliis*.

Recent studies have characterized $CD8^+$ dendritic cells in rainbow trout mucosal organs, indicating that $CD8^+$ macrophage/DC cell lineages exist in teleost fish (Soleto et al., 2018). We observed that a few gill effector cells, which were bound to *I. multifiliis*, were larger than normal lymphocytes; these were, however, not observed in the kidneys. Approximately 5% of $CD8^+$ cells were detected in the gating of gill macrophages by flow cytometry analysis, while the percentage of $CD8^+$ cells was less than 1.5% in the kidney macrophage gating (data not shown). These findings suggest that $CD8^+$ macrophage/DC cells in the gills of ginbuna crucian carp may contribute to the elimination of *I. multifiliis*, whereas the main effectors were concluded to be $CD8^+$ lymphocytes.

$CD8^+$ T cells required direct contact with *I. multifiliis* to exhibit direct cytotoxic activity. In human, anti-parasitic cytotoxic activity of human CTLs is contact-dependent, and supernatants from PHA-stimulated T cells were shown to be incapable of killing the multicellular parasite, *S. mansoni* (Markham et al., 2020; Mody and Oykhman, 2010). In teleost fish, $CD8^+$ T cells of ginbuna crucian carp previously demonstrated cytotoxic activity against bacteria without contacting the target cells (Tartor et al., 2014). These findings suggest that the cytotoxic mechanism of $CD8^+$ T cells of ginbuna carp differs between bacteria and parasites. In the present study, it remains to be determined which type of receptor $CD8^+$ T cells are utilized to recognize parasites. On the other hand, NCCs in catfish have the ability to bind to and kill parasites (Graves et al., 1985). Moreover, nonspecific cytotoxic cell receptor protein (NCCRP) in catfish is involved in the recognition of antigens in the mechanism of cytotoxic activity of NCCs (Evans et al., 1998), implying that NCCRP is involved in the recognition of parasites in teleost fish. However, no TCRs for parasites have been found in any fish species.

$CD8^+$ T cells from both kidney and gills have been identified as anti-parasitic cytotoxic

362 cells, indicating that systemic and local $CD8^+$ T cells possess CMC against parasites.
363 Theronts attach to and penetrate the host epithelium layer of skin or gills (Dickerson,
364 2014). $CD8^+$ lymphocytes are more abundant than neutrophils and macrophages in the
365 skin and gill epithelium and fin cavity of ginbuna crucian carp (unpublished observation).
366 Thus, $CD8^+$ cells in mucosal tissues first contact the parasite and kill them before the
367 theronts transform to mature trophonts. In contrast, because kidney $CD8^+$ cells also
368 possess anti-parasite cytotoxicity, they are considered to migrate to infectious sites and
369 eliminate the parasites following mucosal resident $CD8^+$ cells. The gene expression levels
370 of chemokines or chemokine receptors CXCa, CXCR1, CK10, CK12, and CXCR2 were
371 increased in the epidermis of common carp infected with *I. multifiliis* (Gonzalez et al.,
372 2007; Syahputra et al., 2019). Although it remains unclear whether the chemokines or
373 chemokine receptors act on $CD8^+$ cells or are expressed on $CD8^+$ cells in ginbuna crucian
374 carp, systemic $CD8^+$ cells may be attracted by chemokines secreted from other leukocytes.
375 Further studies are required to reveal which cells or factors attract $CD8^+$ T cells to the
376 infectious site.
377 The present study suggests that serine proteases and perforin are involved in direct
378 cytotoxic activity against *I. multifiliis*. Serine proteases are expressed by several
379 hematopoietic cell lineages, including mast cells, neutrophils, CTLs, and NK cells (Akula
380 et al., 2015; Voskoboinik et al., 2015). Perforin is a pore-forming protein that functions
381 in CMC and delivers serine proteases to the intracellular portion of pathogen-infected
382 cells (Osińska et al., 2014; Voskoboinik et al., 2015). It has been shown that perforin
383 breaks down the mycobacterial cell wall structure directly and triggers direct cytotoxic
384 activity against bacteria (Lu et al., 2014). Furthermore, perforin and granulysin (GNLY)
385 bind phosphoethanolamine, which is a natural compound essential for transportation and

386 localization of the cell wall GPI-anchored proteins in protozoan parasites (Dotiwala and
387 Lieberman, 2019, Ouyang et al., 2019). GPI-anchored proteins play important roles in a
388 variety of biological processes, and deficiency of GPI-anchored proteins results in cell
389 death of trypanosomes in the bloodstream (Simon Lillico et al., 2003). GPI-anchored
390 proteins in the surface membrane of *I. multifiliis* cells are known as immobilization
391 antigens (i-antigens) (Clark et al., 2001). Antibodies against i-antigens inhibit ciliary beat
392 and cause rapid immobilization of cells *in vitro* (Dickerson and Findly, 2014). According
393 to these studies, perforin appears to kill *I. multifiliis* by binding to and deforming GPI-
394 anchored proteins. GNLY and NK-lysin (NKL) are potential candidates that may be
395 associated with cytotoxic activity against parasites. GNLY and NKL are member of the
396 saponin-like protein family, are secreted by $CD8^+$ T cells and NK cells, and have functions
397 in pore formation and granzyme transfer into pathogen-infected cells (Clayberger and
398 Krensky, 2003; Davis et al., 2005; Voskoboinik et al., 2015). Human GNLY demonstrated
399 cytotoxic activity against parasites (Dotiwala et al., 2016; Farouk et al., 2004; Stenger et
400 al., 1998). Similarly, NKL possessed anti-parasitic activity (Hong et al., 2008; Lama et
401 al., 2018). In brief, it is possible that GNLY and/or NKL-dependent pathways are directly
402 involved in the cytotoxic activity against parasites in addition to the perforin-dependent
403 pathway. The results of inhibition assays suggest that kidney $CD8^+$ T cells use a perforin-
404 dependent pathway, while gill $CD8^+$ T cells may utilize a GNLY and/or NKL-dependent
405 pathway to eliminate parasites. Thus, $CD8^+$ T cells in teleost fish may use several
406 cytotoxic pathways to kill parasites.
407 The innate cytotoxicity of $CD8^+$ T cells in the present study demonstrates that the function
408 of innate-like T cells in teleost fish is similar to these cells in other organisms. In mammals,
409 $CD8^+$ T cells and γδ T cells demonstrate an immune response against plasmodium

410 infection (Dantzler and Jagannathan, 2018; Hviid et al., 2019; Kurup et al., 2019).
411 Furthermore, innate-like $CD8^+$ αβ T cells have been defined in mammals and are
412 identified to be involved in the protection against *Staphylococcus aureus* and cancer
413 (Barbarin et al., 2017; St. Leger et al., 2018). The existence of innate-like T cells has been
414 suggested in fish, and their features were often found to resemble mammalian innate-like
415 γδ T cells (Scapigliati et al., 2018). However, although innate-like $CD8^+$ αβ T cells have
416 not yet been identified in teleost fish, $CD8^+$ γδ T cells in zebrafish are known to possess
417 innate immune functions (Wan et al., 2017). These studies support our hypothesis that
418 teleost innate-like $CD8^+$ T cells play an important role in the initiation of cytotoxic
419 activity against parasites to eliminate them. In addition, innate-like T cells may induce
420 the secretion of inflammatory cytokines and demonstrate phagocytic activity against
421 pathogens. Furthermore, γδ T cells also possess antigen-presenting cell functions to
422 activate $CD4^+$ and $CD8^+$ T cells (Dantzler and Jagannathan, 2018). Therefore, it is
423 conceivable that teleost innate-like $CD8^+$ T cells also present parasitic antigens to
424 conventional $CD4^+$ and $CD8^+$ T cells to establish secondary immunity. If teleost innate-
425 like $CD8^+$ T cells can generate a secondary response to parasites, understanding their
426 functions might provide a novel insight into developing fish vaccines against parasites.
427
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434

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Figure legends

Figure 1. Expression profile of T cell markers in MACS-sorted leukocytes from the kidney and gill cells of ginbuna crucian carp. DN indicates CD8 and CD4 double-negative lymphocytes; NC indicates non-template control. EF1α was used as an internal control. Numbers to the right indicate PCR cycles. Data from one fish are shown as representative

698 of the three fish analyzed.

699

700 **Figure 2.** Time course of cytotoxic activity of kidney (A) and gill (B) leukocytes against
701 *I. multifiliis*. Kidney and gill leukocytes from naïve ginbuna crucian carp were incubated
702 with *I. multifiliis* at various E:T ratios (2000:1, 1000:1, 300:1, and 100:1). Data from one
703 fish are shown as representative of the three fish analyzed.

704

705 **Figure 3.** Cytotoxic activity of unsorted and sorted effector cells from the kidneys (A)
706 and gills (B) against *I. multifiliis*. The mean activities of $CD4^+$ cells, $CD8^+$ cells, DN cells,
707 and unsorted cells are shown (gill, n = 3; kidney, n = 4). The effector cells were incubated
708 for 2 h with *I. multifiliis* at E:T ratio of 1000:1. Error bars indicate SD. Different letters
709 on each bar indicate significant differences among the groups ($P < 0.05$).

710

711 **Figure 4.** Contact of $CD8^+$ cells from the kidneys and gills to the surface of *I. multifiliis*.
712 Arrows indicate $CD8^+$ cells (red) and *I. multifiliis* (green). Kidney leukocytes and *I.*
713 *multifiliis* are shown in the upper panels (A, B, C). Scale bars indicate 10 μm.

714

715 **Figure 5.** Cytotoxic activity of the effector cells from the kidney (A and B) and the gill
716 (C and D) against *I. multifiliis* with or without the culture insert. $CD8^+$ cells (A and C)
717 and $CD8^-$ cells (B and D) were used as the effector cells. $CD8^+$ cells were co-cultured
718 with *I. multifiliis* at E:T ratio of 500:1. The results from the three individual fish are shown
719 as the mean of cell killing activities. White and black bars indicate the activities of the
720 effector cells in the absence (contact) and presence (non-contact) of the insert,
721 respectively. The asterisks indicate significant differences of the activities between non-

722 contact and contact (*P < 0.05).

723

724 **Figure 6.** Effect of DCI on the cytotoxic activity of $CD8^+$ cells from the kidney (A) and
725 the gill (B). $CD8^+$ cells were pre-incubated with various concentrations (0, 20, 40 μM) of
726 DCI. $CD8^+$ cells were co-cultured with *I. multifiliis* at E:T ratio of 300:1. Data are shown
727 as the means of the activities from three (kidney) or four (gill) individual fish, respectively.
728 Error bars indicate SD. Asterisks indicate significant differences from control group (0
729 μM) (P < 0.05).

730

731 **Figure 7.** Effect of CMA on the cytotoxic activity of $CD8^+$ cells from the kidney (A) and
732 the gill (B). $CD8^+$ cells were pre-incubated with various concentrations (0, 0.5, 1.0 μM)
733 of CMA. $CD8^+$ cells were co-cultured with *I. multifiliis* at E:T ratio of 300:1. Data are
734 shown as means of the activities from three (kidney) or four (gill) individual fish. Error
735 bars indicate SD. Asterisks indicate significant differences from control group (0 μM) (P
736 < 0.05).