Generation of virus-specific CD8+ T cells by vaccination with inactivated virus in the intestine of ginbuna crucian carp

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Generation of virus-specific CD8$^+$ T cells by vaccination with inactivated virus in the intestine of ginbuna crucian carp

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Abstract

Although a previous study using ginbuna crucian carp suggested that cell-mediated immunity can be induced by the oral administration of inactivated viruses, which are exogenous antigens, there is no direct evidence that $CD8^+$ cytotoxic T cells (CTLs) in teleost fish are generated by vaccination with exogenous antigens. In the present study, we investigated whether antigen-specific $CD8^+$ CTLs in ginbuna crucian carp can be elicited by intestinal immunization with an exogenous antigen without any adjuvant. The IFNγ-1 and T-bet mRNA expressions were up-regulated in intestinal leukocytes following the administration of formalin-inactivated crucian hematopoietic necrosis virus (FI-CHNV), whereas the down-regulation of these genes was observed in kidney leukocytes. Furthermore, an increase in the percentage of proliferating $CD8^+$ cells was detected in the posterior portion of the hindgut, suggesting that the virus-specific CTLs are locally generated in this site. In addition, cell-mediated cytotoxicity against CHNV-infected syngeneic cells and the *in vivo* inhibition of viral replication were induced by immunization with FI-CHNV. Unexpectedly, intraperitoneal immunization with FI-CHNV induced a type I helper T cell (Th1)-response in the intestine, but not in the kidney; however, its effect was slightly lower than that reported after intestinal immunization. These findings suggest that the posterior portion of the intestine is an important site for generating virus-specific CTLs by vaccination with the inactivated vaccine.

Key words: cytotoxic T cell, inactivated vaccine, virus, cell-mediated immunity, ginbuna crucian carp

Introduction

Cell-mediated immunity (CMI) executed by cytotoxic T-cells (CTLs) is an effective defense mechanism to suppress the propagation and spread of viruses in the host (Zinkernagel and Doherty, 1979). CTLs recognize a viral-antigen presented on MHC class I molecules and kill the virus-infected host cells. The viral peptides on the MHC class I molecules are intracellularly synthesized in the host cells. Therefore, inactivated viral vaccines, which are extracellular antigens, are not capable of efficiently eliciting CMI in hosts because they do not actively invade the cytosol of non-phagocytic host cells (Carbone and Bevan, 1990).

Fish are equipped with T-cell-mediated immunity similar to that present in mammals, and MHC class I-restricted cytotoxicity generated by CTLs is thought to serve an effective anti-viral function in teleost fish (Somamoto *et al*., 2014, Nakanishi *et al*., 2015, Munang'andu *et al*., 2015). Thus, similar to the mammalian system, the generation of viral antigen-specific CTLs in fish should also require stimulation with intracellular antigens. However, the expression profiles of T cell-related genes in several fish species suggest that immunization with exogenous antigens can induce virus-specific CTLs. Oral or bath vaccination with inactivated viruses induced the up-regulation of MHC class I and CD8 mRNA in teleost fishes (Ou-yang *et al*., 2012, Kai *et al*., 2014, Munang'andu *et al*., 2015), suggesting that vaccinations with inactivated viruses can also induce CMI. This finding implies that mucosal vaccination may be an effective method to generate CTLs in teleost fish.

The intestine is an important immune organ that protects against pathogen invasion and possesses gut-associated lymphoid tissue (GALT) that contains many intraepithelial lymphocytes (IELs) in mammals (Brandtzaeg and Pabst 2004). In teleost fish, $CD8^+$ T

77 cells comprise most of the IEL population found in the intestine, suggesting that the
78 intestine is an important site for the development and activation of T cells in teleost fish
79 (Rombout *et al*., 2011, Salinas 2015, Tafalla *et al*., 2016). Sato *et al*. (2005) reported that
80 the direct administration of allogeneic cells into the intestine could induce systemic
81 adaptive CMI in common carp. In studies on cell-mediated cytotoxicity (CMC) using
82 ginbuna crucian carp, the oral administration of hapten-modified cells also elicited
83 specific CMC activity (Sato and Okamoto 2008). Furthermore, the oral administration of
84 formalin-inactivated crucian carp hematopoietic necrosis virus (CHNV) effectively
85 prevented viral propagation *in vivo* and induced virus-specific CMC in ginbuna crucian
86 carp (Sato and Okamoto 2010). These studies suggest that the fish intestine is an
87 important site for generating CTLs. Based on the above findings, the following hypothesis
88 can be proposed: the teleost fish immune system can easily generate virus-specific CTLs
89 by immunizing the intestine with extracellular antigens. However, these studies failed to
90 show any evidence that Th1-response and T-cell proliferation are induced by intestinal
91 immunization. Therefore, the present study investigated the CTLs function, the
92 transcriptional profile of T-cell-related molecules, the *in vivo* protective effect, and the *in*
93 *vivo* proliferation of $CD8^+$ cells following intestinal immunization with an inactivated
94 virus. These results were then compared with the responses following intraperitoneal
95 (i.p.) immunization.
96
97 **2. Materials and Methods**
98 *2.1. Fish, cell lines, viruses, and formalin-inactivated CHNV*
99 Two strains (OB1 and S3n) of isogeneic ginbuna crucian carp, *Carassius auratus*
100 *langsdorfii*, were maintained at a temperature of 25°C and were fed daily with

commercial food pellets. The fish were anesthetized with 25 mg/L quinaldine when injected or dissected. All experiments using fish were performed in accordance with the guidelines of the Animal Experiments Committee at Kyushu University. CFS (*Carassius* fin from Lake Suwa) cells from the S3n strain of ginbuna crucian carp were used as the syngeneic target cells and for propagation of the CHNV, as described by Somamoto *et al*. (2009, 2013). CHNV was inactivated with formalin by same procedure used in the previous study (Sato and Okamoto 2010).

2.2. Immunization with FI-CHNV or live CHNV

The intestinal immunization procedure used was that described in a previous study (Sato *et al*., 2005). Clonal ginbuna crucian carp (OB1 or S3n strain) were intubated with 0.5 mL/100 g fish weight of a formalin-inactivated CHNV (FI-CHNV) solution (corresponding to 10^8 $TCID_{50}$/mL in PBS) or a sublethal dose of live CHNV (10^6 $TCID_{50}$/100 g weight) into their intestines from the anus via a polyethylene tube (0.5/1.0 mm inner/outer diameter). To prevent the solution from leaking back out, the part of the tube that corresponded to the length of the pelvic fin from the anus was gently inserted, and the solution was slowly administered into the hindgut. The control fish were intubated with PBS, instead of FI-CHNV or live CHNV. To compare intestinal immunization with systemic sensitization, i.p. immunization with FI-CHNV was performed using the same dose as that administered anally. The second immunization was performed one week after the primary sensitization using the same procedure.

2.3. Gene expression analysis of T-cell-related genes

The OB1 strain of ginbuna crucian carp, which weighed 21-24 g, underwent a gene

125 expression assay using real-time PCR. Three fish in each group were sampled at 4, 8, and
126 12 days post-second sensitization with PBS and FI-CHNV or live CHNV. The trunk
127 kidney and posterior intestine (the posterior one-third of the intestine) were excised from
128 the fish. The trunk kidney was disaggregated by passing it through a 150-gauge mesh
129 stainless steel sieve in RPMI-1640 (Nissui Pharmaceutical Co., Tokyo, Japan)
130 supplemented with 1% heat-inactivated (56°C for 30 min) fetal bovine serum (FBS:
131 Biowest, France). The posterior intestines were homogenized using scissors and then
132 disaggregated by being passed through a 150-gauge mesh stainless steel sieve in RPIM-
133 1640 containing 1% FBS. The suspended cells were then applied to a discontinuous
134 Percoll (GE healthcare) density gradient of 1.04 and 1.08 g/ml, and then centrifuged at
135 350 × g for 30 min at 4°C. The cells present at the 1.04-1.08 interface were collected and
136 washed twice with RPMI-1640. Total RNA was extracted from the isolated cells of the
137 intestine and kidney using ISOGEN (Nippon gene, Tokyo, Japan), according to the
138 manufacturer's protocol. First-strand cDNA was synthesized from 2 μg of total RNA using
139 the Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Life
140 Technologies, Carlsbad, CA, USA) with an oligo (dT) primer, according to the
141 manufacturer's instructions. Primers used for the real-time PCR had been previously
142 designed to amplify cDNA fragments encoding the following T-cell related genes: T-bet,
143 GATA-3, IFN-γ1, IFN-γ2, IFN-rel2 (Yamasaki *et al*., 2014), IL-10, and T cell receptor-β
144 (TCRβ) (Somamoto *et al*., 2009, 2015). EF-1α served as an internal control for the
145 normalization (Toda *et al*., 2011). The sequences or primer sets are indicated in the
146 reference articles. Quantitative real-time PCR was performed in duplicate on a M × 3000P
147 System (Stratagene, La Jolla, CA) with 16 μL reaction mixtures containing 2 μl of
148 template cDNA, 0.5 μM primers, and other reagent components from the Fast Start DNA
149 Master SYBR_Green (Roche Applied Science, Mannheim, Germany). Thermal cycling
150 was performed using a two-step thermal cycling mode composed of an initial denaturation
151 for 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 60 sec at 61°C (EF1α,

IFNγ-1, IFNγ-2, IFNγ-rel2, GATA-3, T-bet, and TCRβ) or 40 sec at 60°C (IL-10). For all the quantitative real-time PCR assays, melting curve analyses were performed and single specific melting peaks were observed, which indicated amplification specificity. The relative quantitative value of each gene was calculated according to the standard curve from a serial dilution of a reference cDNA in the same plate and normalized with the level of EF1α. Data from the three individual fish are shown as the mean fold change in mRNA expression relative to the PBS-administered fish.

2.4 Detection of proliferating cell nucleic antigens (PCNAs) in CD8α⁺ cells by flow cytometry (FCM)

To determine whether CD8α⁺ cells proliferated in the kidney and intestine *in vivo* after FI-CHNV-sensitization, the PCNA protein, which is expressed in cells in the later stages of the G1 and S-phases, was detected in CD8α⁺ cells by FCM. The OB1 strain of ginbuna crucian carp, weighing 4.8-7.9 g, was used in this experiment. Intestinal or i.p. immunization with FI-CHNV was followed according to the method described above. To reveal a more detailed site for generating virus-specific CTLs in the posterior intestine, the posterior intestine was divided into two parts as shown in Fig. S1. The leukocytes from the tissues were collected at 2 and 4 dpi from the immunized or control fish (four fish in each group). The number of collected cells was counted on a hemocytometer. The collected cells from the tissues were incubated in a 1:10,000 dilution of rat anti-ginbuna CD8α mAbs (mouse ascites) for 40 min on ice (Toda *et al*., 2011). The cells were then washed twice with OPTI-MEM (Invitrogen, Carlsbad, CA) and incubated for 30 min at 4°C with a 1:100 dilution of anti-rat donkey IgG (H+L) antibodies conjugated with Alexa Fluor 488 (Invitrogen, Carlsbad, CA). The control samples were stained with just the secondary antibody (no primary antibody). After two washes with the medium, the cells

177 suspended in OPTI-MEM supplemented with 1% FBS (OPTI-1) were mixed with the
178 same volume of 4% paraformaldehyde (PFA) PBS solution (Nacalai Tesque Inc., Kyoto,
179 Japan) (final conc. 2% PFA) and were incubated for 20 min at room temperature. The
180 fixed cells were washed three times and suspended in PBS containing 0.2% saponin
181 (Nacalai Tesque Inc., Kyoto, Japan) and 3% bovine serum albumin (BSA) (PBS-Sapo-
182 BSA); they were then incubated for one hour at room temperature. The membrane-
183 permeabilized cells were incubated with a 1:100 dilution of anti-PCNA mouse mAbs
184 (PC10; Santa Cruz Biotechnology), which have been shown to react with cells from
185 various species including *Drosophila*, zebrafish, and ginbuna crucian carp (Nakanishi and
186 Ototake 1999), or the same concentration of normal mouse IgG (IgG2a) as a control, for
187 40 min at room temperature. After being washed twice with OPTI-1, the cells were
188 resuspended in PBS-Sapo-BSA and incubated with a 1:100 dilution of anti-mouse IgG
189 goat antibodies conjugated with PerCP/Cy5.5 (Biolegend, San Diego, CA). The labeled
190 cells were washed twice and suspended in OPTI-1. The fluorescent stained cells were
191 analyzed with an Attune NxT Flow Cytometer (Thermo Fisher Scientific, Waltham, MA,
192 USA). The region including $CD8\alpha^+$ cells was gated and classified as the lymphocyte
193 fraction. Alexa Fluor 488 or PerCP/Cy5.5 fluorescence was detected in the BL1 channel
194 using a 530/30 nm bandpass filter or the BL3 channel using a 695/40 nm bandpass filter
195 with 488 nm excitation, respectively.
196
197 *2.5. CMC against CHNV-infected syngeneic cells*
198 The S3n strain of ginbuna crucian carp, weighing 35-72 g, was used in the cytotoxic
199 assays. The fish were sensitized twice via anal administration or i.p. injection with FI-
200 CHNV as described above. Blood was collected from the fish at 4 and 8 dpi (four fish per

sample). Peripheral blood leukocytes (PBLs) were used as effector cells to evaluate the systemic response in the CMC assay because the spontaneous release of LDH from PBLs was low. PBLs were isolated using a Percoll gradient method as previously described (Somamoto *et al*., 2015). The PBLs were then suspended in DMEM/F-12 medium (Invitrogen, Carlsbad, CA) without phenol red and with 1% heat-inactivated FBS (DMEM/F12-1); they were then used as effector cells for a cytotoxicity assay. The cytotoxicity was assayed as previously described (Somamoto *et al*., 2015). In brief, CFS cells were used as syngeneic target cells, seeded in 96-well, flat-bottom microtiter plates (Nunc, Roskilde, Denmark) at a concentration of 10^4 cells/well, and allowed to settle for 6 h. Half of the wells with the target cells were infected with CHNV at 25°C for 3–4 h (MOI = 10). Effector: target ratios were adjusted to 80:1 and 40:1. At least duplicate wells were analyzed for each test. The maximum amount of lactate dehydrogenase (LDH) released by the infected and uninfected targets was determined by damaging the cells with 1% Triton X-100 (high control). Spontaneous LDH release from the effector and target cells was determined with untreated cells that were cultured with only medium (low control). Cytotoxic activity against the infected and uninfected targets was detected by LDH release using a Cytotoxicity Detection Kit-LDH (Takara Bio Inc., Shiga, Japan), according to the manufacturer's protocol. The absorbance of plates containing the supernatants from the test wells and the substrate mix were determined with an ELISA reader at 490 nm. The value was subtracted from the background (only medium or medium + Triton X-100). There was no significant difference in spontaneous release between the uninfected and infected target cells.

The percentage of specific cytotoxicity was calculated using the following formula: Cytotoxicity (%) = A − low control/high control − low control × 100 (A: [effector - target

225 cell mix] − [effector cell control]).

226 CD8α$^+$ and CD8α$^-$ cells were separated from PBLs using magnetic cell separation as
227 previously described (Somamoto *et al*., 2013). The cytotoxic activity of CD8α$^+$ and
228 CD8α$^-$ cells from fish sampled at 8 dpi was measured as described above. Effector:
229 target ratios were adjusted to 20:1 and 10:1.

230

231 *2.6 Virus titers in organs of vaccinated fish after CHNV infection*

232 The organ virus titers were measured following the protocols of previous studies
233 (Somamoto *et al*., 2013; Sato and Okamoto 2010). Ginbuna crucian carp (OB1 strain),
234 weighing 34-58 g, were used in this experiment. The fish (four fish per group) were anally
235 administered FI-CHNV or PBS into the hindgut and were intraperitoneally injected with
236 FI-CHNV as described above. At 14 dpi, the sensitized fish were intraperitoneally
237 immunized with CHNV at 10^6 $TCID_{50}$/50 g body weight. The spleen and trunk kidney
238 were collected at 3 dpi and placed in MEM (Nissui Pharmaceutical Co., Tokyo, Japan)
239 (1:10, weight: volume) containing 10% heat-inactivated FBS, homogenized, and then
240 centrifuged at 2000 × g for 20 min. The supernatants were collected, passed through a
241 0.45-μm membrane filter, and stored at −80°C until required. The virus titers were
242 determined according to a $TCID_{50}$ endpoint titration in CFS cells incubated for 21 days
243 at 25°C. The results were expressed as the $TCID_{50}$ per gram of organ.

244

245 *2.7. Statistical analysis*

246 The results were expressed as the mean ± standard deviation. Statistical differences
247 were assessed for more than two groups using an analysis of variance (one-way ANOVA)
248 followed by Tukey’s comparison test or analyzed by student's *t*-test for comparison

between two groups. The significance level was set at 0.05.

3. Results

3.1 Expression of T-cell-related molecules after FI-CHNV and live CHNV sensitization

The expressions of TCRβ as well as the transcription factors GATA-3 and T-bet are shown in Fig. 1A. The intestinal immunization with FI-CHNV induced up-regulations of T-bet in intestinal leukocytes at 4 and 8 dpi, whereas the expression in kidney leukocytes significantly decreased at 4 dpi and showed no up-regulation at 8 and 12 dpi. On most sampling days, the expression level of TCRβ mRNA in the kidney and intestine was significantly higher than that in the control fish, although there was no difference in the kidney leukocytes sampled at 12 dpi. Conversely, there was no significant difference in GATA-3 mRNA expression at any time point. The expression profiles induced by i.p. immunization were largely similar to those induced by intestinal immunization; however, the expression of TCRβ mRNA was not enhanced by immunization.

The expression profiles of cytokine expression in the FI-CHNV-immunized fish are shown in Fig. 1B. Intestinal immunization significantly enhanced IFNγ1 expression in intestinal leukocytes sampled at 4 and 8 dpi, whereas the expression in kidney leukocytes was significantly lower than that in the control fish. Systemic immunization also induced the enhancement in intestinal leukocytes, but not in kidney leukocytes. Although significant up-regulation of IFN-γ2 was not observed in the intestinal immunized fish, the expression of IFNγ-*rel* mRNA was increased in intestinal leukocytes at 8 and 12 dpi. In both organs, the expression profiles of IL-10 mRNA were enhanced following the immunizations.

The expressions of most of the genes in the fish immunized with live CHNV showed a

similar pattern as those in the fish immunized with FI-CHNV via the intestine, whereas the expression of IFNγ1 in the intestine was not enhanced at any time point by the immunization.

3.2. Proliferation of CD8$^+$cells in the intestine and kidney by FI-CHNV-sensitization

Whether virus-specific CD8$^+$ cells were generated in the intestine or kidney after FI-CHNV administration was investigated by employing an FCM analysis using anti-PCNA antibodies (Fig. 2). There is no significant difference of the total number of collected cells among each group. The region including CD8α$^+$ cells was gated and regarded as the lymphocyte fraction. Double positive (DP) cells, which express both CD8α and PCNA, were detected in the kidney and intestine (Fig. 2A). In the posterior part of the hindgut from fish sampled at 2 dpi (Intestine 2), the percentage of DP cells in the intestinally immunized fish was significantly higher than that in either the PBS-administrated fish or the intraperitoneally immunized fish. Conversely, there were no significant differences in DP cell composition in the kidney or the anterior part of the hindgut among the treated groups (Intestine 1). Although the normal PCNA protein expression level was higher in the kidney than in the intestine, no significant differences in DP cell percentages among the treated groups were observed for either organ. In all groups that were sampled at 4 dpi, the percentages of DP cells did not increase as a result of FI-CHNV-sensitization.

The CD8$^+$ cell percentages in the intestines of FI-CHNV-immunized fish did not increase or were significantly lower than those of PBS-administered fish at some sampling times.

3.3. Cell-mediated cytotoxic activity of leukocytes from FI-CHNV-immunized fish against

297 *CHNV-syngeneic cells*

298 To determine whether CMI can be induced as a result of immunization with FI-CHNV,
299 the cytotoxicity of PBL against CHNV-infected cells was analyzed using the syngeneic
300 target cell line (Fig. 3). At 8 dpi, the unsorted effector cells from the fish immunized with
301 FI-CHNV efficiently killed CHNV-infected syngeneic cells, but not uninfected cells ($p < 0.05$).
302 Meanwhile, the effector cells from PBS-administered fish did not show significant
303 activity against CHNV-infected cells (Fig. 3A). The effector cells that were collected at 4
304 dpi did not significantly kill the infected cells. The cytotoxicity of $CD8^+$ cells against
305 CHNV-infected cells was significantly higher than that against uninfected cells;
306 conversely, $CD8^-$ cells showed the same level of activity (Fig. 3B). The activities at lower
307 effector/target ratios were reduced in all assays (data not shown). These results indicate
308 that both intestinal and systemic immunization with FI-CHNV can induce CTL activity
309 against virus-infected cells.

310

311 *3.4. Protective effect of FI-CHNV vaccination*

312 The vaccination effects of intestinal and i.p. FI-CHNV administration were determined
313 by evaluating the viral load of the spleen and kidney from the fish that were infected with
314 CHNV after the vaccination (Fig. 4). In both the kidney and spleen, the viral titer of the
315 fish that were intestinally immunized with FI-CHNV was significantly lower than that of
316 the fish that were intestinally administered PBS; however, no significant reduction was
317 observed in the kidney of the i.p. FI-CHNV-immunized fish. Furthermore, there was no
318 significant difference in the viral load between both routes of FI-CHNV immunization in
319 the spleen and kidney of the fish. These results suggest that the administration of the
320 inactivated virus can efficiently prevent viral replication *in vivo*.

4. Discussion

CMC by CD8α$^+$ CTLs is thought to be an effective mechanism for controlling viral infection in teleost fish and in mammals (Somamoto et al., 2014, Zinkernagel and Doherty 1979). MHC class I and antigen-presentation and -processing related molecules have been identified in many fish species (Grimholt 2016, Wilson 2017), and functional and structural analyses suggest that the antigen-presentation mechanism in teleosts is similar to that in mammals (Sever *et al* 2014, Chen *et al*., 2010, 2017). Thus, the induction of viral-antigen-specific CD8$^+$ CTLs in fish is believed to require stimulation with endogenous antigens in MHC class I antigen processing machinery. The cross-presentation is essential for the presentation of exogenous antigens on MHC class I molecules and the initiation of CD8$^+$ T-cell responses in mammals (Joffre et al., 2012). Recent studies suggest that teleost DC-like cells possess cross-presentation ability (Granja *et al*., 2015, Soleto *et al*., 2018). The present study has shown that immunization with exogenous antigens can induce a Th1 response and CMC by CD8$^+$ cells, implying that ginbuna crucian carp can efficiently elicit cross-presentation. Although the mechanism remains unclear, the findings obtained from the present study indicate that the intestine is an important site for viral-antigen specific CD8$^+$ T-cell generation by exogenous antigens, and suggest that the generated CTLs migrate to systemic immune organs or the blood.

T-bet is a transcription factor that regulates Th1 cell differentiation and controls the expression of IFN-γ in mammals (Szabo *et al*., 2000, Sullivan *et al*., 2003), and its expression is skewed by Th-1. Several studies have indicated that the expression level of the T-bet gene is correlated to Th-1 responses in teleost fish (Munang'andu *et al*., 2013,

345 Yamasaki *et al*., 2014, 2015, Yang *et al*., 2017). An interesting observation in the present
346 study is that the enhancement of T-bet expression in FI-CHNV-immunized fish was
347 detected in the intestine, but not in the kidney. The IFN-γ mRNA expression profile agrees
348 with this observation, although higher IL-10 mRNA expression levels show conflicting
349 results. The expression of GATA-3, which is essential for the differentiation of mature
350 naive $CD4^+$ T-cells to Th-2 cells, was not elevated in the kidney or intestine by the
351 stimulation. Comprehensively, the expression analysis of T-cell related genes shows that
352 the Th1 response is induced by intestinal and systemic administration of the inactivated
353 virus. Furthermore, we have demonstrated that the posterior part of the intestine is the site
354 of $CD8^+$ cell generation by immunization with the inactivated virus. In IPNV-sensitized
355 rainbow trout, T cells mobilized to the mid/hindgut region and pyloric caeca, but not the
356 foregut region (Ballesteros *et al*., 2014). Although it cannot simply correspond to the
357 regions in trout and crucian carp, which have physiologically different digestive tracts,
358 this finding and our results suggest that the posterior region of the digestive tract is an
359 important T cell mobilization site in teleosts. There are various phenotypes of $CD8^+$ IELs,
360 including $TCR\alpha\beta^+$ T cells, $TCR\gamma\delta^+$ T cells, and natural killer T (NKT) cells in mammals
361 (Bannai et al., 2003, Konjar et al., 2017), suggesting that teleost IELs are also classified
362 into various phenotypes. Thus, further studies should identify the population of $CD8^+$
363 IELs present in ginbuan crucian carp.
364 CMC against CHNV-infected syngeneic cells was induced by immunization with FI-
365 CHNV. This result is consistent with the report that CMC activity was induced by oral
366 immunization with FI-CHNV (Sato and Okamoto 2010). Furthermore, the present study
367 has determined that $CD8\alpha^+$ cells are effectors against virus-infected cells in FI-CHNV-
368 immunized fish. Previous studies have shown that $CD8\alpha^+$ (2C3 mAb^+) lymphocytes

strongly express TCRβ, lck, CD3ε, and ZAP70 mRNA (Toda et al., 2011, Somamoto et al., 2013, Miyazawa et al., 2018) as well as ZAP70 and CD3ε proteins (Miyazawa et al., 2018). In addition, a previous study reported that CHNV-sensitized effector cells, which express TCRβ and CD8α mRNA, possess CMC against CHNV-infected syngeneic cells but not against infected allogeneic cells, implying that they recognize virus-infected targets in an MHC class I restriction manner (Somamoto et al., 2002, 2009). These findings suggest that $CD8\alpha^+$ CTLs are the dominant effector cells in the present study, although further identification of the effector cells would be required. Although $CD8\alpha^-$ cells also significantly killed CHNV-infected cells in this study, the effector cells except for CTLs contributed to CMC against CHNV-infected cells, as shown in the previous study (Somamoto *et al*., 2013). We suggest that monocytes and/or NK-like cells were activated by Th-1 cytokines such as IFNγ and thus play an important role in the clearance of viral infections. In fact, a large number of $CD8^-$ cells were proliferated by the immunization with FI-CHNV, suggesting that leukocytes other than CTLs also contribute to controlling CHNV-infection. Intestinal immunization with FI-CHNV provided significant prevention against CHNV-replication in the kidney and spleen, suggesting that CMC by CTLs and other effecter cells contributed to this protection. Evidence at the cellular and biological levels indicates that vaccination with extracellular antigens can induce efficient CMC, thus demonstrating its availability as a useful viral vaccine for fish.

Our hypothesis was that mucosal vaccination can induce a more efficient cell-mediated immune response than systemic vaccination because several studies have suggested that Th1 responses were induced by bath or oral vaccinations with inactivated virus (Sato and Okamoto 2010, Ou-yang *et al*., 2012, Kai *et al*., 2014, Munang'andu *et al*., 2015). Systemic immunization with FI-CHNV can also induce CMC against CHNV-infected

393 cells and protection against CHNV-infection, whereas the effect was slightly lower than
394 that by intestinal immunization. Interestingly, both systemic and intestinal immunization
395 generated viral antigen reactive $CD8^+$ cells and enhanced Th1-related genes in the
396 intestine, but not in the kidney. This fact implies that the posterior part of the intestine
397 possesses an important site for cross-presentation, which can present exogenous antigens
398 on MHC class I, by dendritic cells (DCs). Teleost DC-like cells have recently been
399 identified in rainbow trout and are inferred to possess cross-presentation ability (Granja
400 *et al*., 2015, Soleto *et al*., 2018). The trout DC-like cells were found in the gills and skin
401 and showed different phenotypic and functional characteristics, suggesting the existence
402 of distinct DC subsets in the mucosal tissues. Therefore, mucosal tissues may be
403 important sites for developing DCs in teleost fish, which indicates that the mucosal DCs
404 play a major role in generating antigen-specific $CD8^+$ cells. According to the findings
405 from the present study, we hypothesize that the intestinal resident DCs ingested FI-CHNV,
406 which was directly exposed to or circulated into the intestine, by endocytosis, processed
407 it via proteasomes, and presented it on MHC class I molecules to $CD8^+$ cells. The cross
408 presentation frequently occurs in mucosal tissues such as the intestine. Further studies on
409 intestinal DCs would provide a better understanding of the mechanisms of how
410 inactivated vaccines induce virus-specific CTLs.
411

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416

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Figure legends

Figure 1. Quantitative expression profiles of T-cell-related genes (TCRβ, T-bet, and GATA-3) (A) and cytokine genes (IFNγ1, IFNγ2, IFNγ-rel2, and IL-10) (B) in kidney (left graph) and intestine (right graph) cells after CHNV-sensitization. Data from the three individual fish are shown as the mean fold change in mRNA expression relative to the PBS-administered fish (white bars). The fish were immunized with FI-CHNV (black bars) or live CHNV (hatched bars) via the intestine and with FI-CHNV via i.p. injection (gray bars). The error bars indicate the standard deviation (SD). The asterisks indicate significant differences between the PBS-administered and CHNV-sensitized fish (*$P < 0.05$).

Figure 2. Detection of $PCNA^+$ and $CD8\alpha^+$ cells by FCM in the kidney and intestines of FI-CHNV-immunized fish. (A) Two color histograms of cells expressing CD8α (BL1) and PCNA (BL3). Cells that were sensitized by intestinal (CHNV-Int) and intraperitoneal (CHNV-Ip) immunization with FI-CHNV or intestinal administration with PBS were analyzed by FCM. The cells were collected from the kidney, the anterior portion of the hindgut (intestine 1), and the posterior portion of the hindgut (intestine 2). Four individual samples were independently analyzed, and representative data are shown. The numbers indicate the percentages of positive cells within each region. The control samples were stained with isotype control antibodies (BL3) or with only secondary antibodies (no primary antibody) (BL1). (B) Percentages of CD8α-positive and double positive (DP) cells. The results are presented as the means of four individual fish, and the error bars indicate SD. The asterisks indicate significant differences between each group (*$P < 0.05$).

Figure 3. Cell-mediated cytotoxic activity of unsorted leukocytes (A) or $CD8\alpha^+$/$CD8\alpha^-$

555 cells (B) against CHNV-infected syngeneic cells. Unsorted leukocytes that were
556 sensitized by intestinal (Int) and intraperitoneal (Ip) immunization with FI-CHNV or
557 intestinal administration with PBS were used as effector cells. $CD8\alpha^+$ and $CD8\alpha^-$ cells
558 that were sensitized by intestinal immunization with FI-CHNV were used as effector cells.
559 The unsorted cells or the $CD8\alpha^+/CD8\alpha^-$ cells were incubated with target cells at an
560 effector:target ratio of 80:1 or 20:1, respectively. The unsorted cells were collected from
561 fish sampled at 4 (a) and 8 (b) dpi. $CD8\alpha^+/CD8\alpha^-$ cells were isolated from fish sampled
562 at 8 dpi. The results are presented as the means of four individual fish, and the error bars
563 indicate the SD. The black and white bars indicate the activities against the infected and
564 uninfected cells, respectively. The asterisks indicate significant differences from the
565 activity against uninfected targets (*$P < 0.05$).
566
567 **Figure 4.** Viral loads of kidneys and spleens from vaccinated fish. The vaccinated fish
568 were anally or intraperitoneally immunized with FI-CHNV. The results are presented as
569 the means of four fish, and the error bars indicate the standard deviations (SDs). Means
570 with different letters indicate significant differences (*$P < 0.05$).
571