Adaptive immune response to *Edwardsiella tarda* infection in ginbuna crucian carp, *Carassius auratus langsdorfii*

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ABSTRACT

Edwardsiella tarda is an intracellular pathogen that causes edwardsiellosis in fish. Although cell-mediated immunity and innate immunity play a major role in protection against intracellular bacterial infection in mammals, their importance in protecting fish against *E. tarda* infection remain unclear. In this study, we examined cell-mediated and humoral immune responses in ginbuna crucian carp (*Carassius auratus langsdorfii*) after *E. tarda* infection. Innate immunity was observed to be the principal immune system for eliminating the majority of *E. tarda*, while a proportion of the bacteria might be resistant to its bactericidal activity. Bacterial clearance in kidney and spleen was also observed following higher cytotoxic activities of cytotoxic T lymphocytes (CTLs) and increased numbers of $CD8\alpha^+$ cells, suggesting that CTLs might contribute to the elimination of *E. tarda*-infected cells with specific cytotoxicity. On the other hand, *E. tarda*-specific antibody titers did not increase until after bacterial clearance, indicating that induction of humoral immunity would be too late to provide protection against infection. Overall, these data suggest that both cell-mediated immunity and innate immunity may play important roles in the protection against intracellular bacterial infection, as they do in mammals. Our study would also contribute towards the understanding of immune responses that provide protection against other intracellular pathogens.

Keywords: *Edwardsiella tarda*; intracellular pathogen; cell-mediated immunity; IFN-γ; CTLs; ginbuna crucian carp

1. Introduction

Edwardsiellosis, caused by the bacterium *Edwardsiella tarda*, is a serious problem in cultured

fish such as the Japanese eel (*Anguilla japonica*), channel catfish (*Ictalurus punctatus*), crimson sea bream (*Evynnis japonica*), Japanese flounder (*Paralichthys olivaceus*) and many other fish species (Hoshima, 1962; Meyer et al., 1973; Kusuda et al., 1977; Kanai et al., 1988; Kusuda et al., 1993). Clinical symptoms of edwardsiellosis may include small cutaneous lesions that can develop into necrotic abscesses, pigment loss, enlarged kidney, distended abdomen and swollen anus, all of which are due to the accumulation of ascitic fluid and abscesses in the internal organs (Plumb, 1999). Because the control of *E. tarda* is difficult using antimicrobial drugs, the development of a vaccine system against edwardsiellosis is an important issue in the aquaculture.

Although vaccine trials with formalin-killed cells (FKC) have been reported, the vaccinations were ineffective in protecting against *E. tarda* infection (Salati et al., 1983; Igarashi and Iida, 2002), which is due to the bacterium's existence as an intracellular pathogen. *E. tarda* is able not only to infect epithelial cells (Ling et al., 2000) but also to be resistant to phagocyte bactericidal activities in the phagosome (Iida et al., 1993; Iida and Wakabayashi, 1993; Rao et al., 2001; Ishibe et al., 2008; Takano et al., 2010). Since antigen-specific antibodies are only effective in an extracellular environment, they are incapable of eliminating intracellular pathogens. On the other hand, cell-mediated immune mechanisms can eliminate pathogen-infected cells by inducing cell death, thereby removing the niche for intracellular pathogen replication (Russell and Ley, 2002). Moreover, cell-mediated immune mechanisms also activate the bactericidal activity of macrophages. In mammals, cell-mediated immunity (CMI) plays a major role in protection against intracellular bacterial infections (Neild and Roy, 2004; Parmer, 2004; Reece and Kaufmann, 2008).

Mammalian IFN-γ produced by Th1, natural killer cells, macrophages, and CTLs promotes CMI activities, including macrophage killing of intracellular pathogens by nitric oxide (NO) and CTL antigen-specific cytotoxicity against intracellular pathogens (Carretelli et al., 2005; Darrah., 2000; Campbell, 1994; Flynn et al., 1993; Saito and Nakano, 1996; Tascon et al., 1998). In teleost fish,

Grayfer and Belosevic (2009) has reported that treatment with recombinant IFN-γ promoted NO production as well as expression of several proinflammatory cytokines, such as IL-1β, TNF-α, and IL-12 in macrophages of goldfish. Moreover, goldfish phagocytes that were primed by recombinant IFN-γ were shown to reduce the survival of intracellular *Mycobacterium marinum* (Grayfer et al., 2011). These findings suggest that CMI might be induced by IFN-γ in fish, in a manner similar to that in mammals. Therefore, CMI could be an effective immune system against intracellular pathogens in fish.

In mammals, antigen-specific cell-mediated cytotoxicity (CMC) of CTLs is the principal mechanism for eliminating transformed, virus-infected, and intracellular bacteria-infected cells (Oldstone, 1987; Zinkernagel and Doherty, 1979; Kaufmann and Flesch, 1988; Kaufmann, 1993; Wizel et al., 2008). Mammalian CTLs express the CD8 surface antigen ($CD8^+$ T lymphocytes) and recognize antigens that are processed and presented by antigen-presenting cells only in the context of MHC Class I. In fish, alloantigen- and virus-specific CMC have been demonstrated, suggesting that fish possess CMI similar to that in mammals (Somamoto et al., 2000; Nakanishi et al., 2002; Fischer et al., 2003; Utke et al., 2007; Toda et al., 2009). However, it remains unknown whether CMC plays an important role in targeting intracellular bacterial-infected cells in teleost fish.

The triploid ginbuna crucian carp (*Carassius auratus langsdorfii*) is a useful fish model for investigating CMI in teleost fish (Nakanishi et al., 2011). Ginbuna crucian carp mAbs against cell surface markers of CTLs and helper T cells (CD8α and CD4, respectively) have also been developed recently (Toda et al., 2009; Toda et al., 2011).

To elucidate whether CMI and/or humoral immunity may contribute to protection against *E. tarda* infection in fish, we examined the Th1/Th2- and CMI-related gene expression, antigen-specific antibody titers, bacterial clearance, antigen-specific CMC of CTLs, and lymphocyte populations in *E. tarda*-infected ginbuna crucian carp. We believe that this study will facilitate vaccine development

for protecting fish against intracellular pathogens.

2. Materials and methods

2.1. Fish

Clonal triploid ginbuna crucian carp (*Carassius auratus langsdorfii*), from the island of Okushiri (OB1 clone), were obtained from the National Research Institute of Aquaculture, Japan. The fish, weighing between 20–30 g, were maintained in running water at a temperature of 25 ± 1°C. The fish were fed commercial pellets on a daily basis.

2.2. Bacteria

The *E. tarda* FPC498 strain was isolated from the ascites of a naturally infected Japanese flounder. The bacteria were incubated for 48 h at 25°C in tryptic soy agar (Nissui, Japan) before using it for including experimental infections.

For the cytotoxic assay, pTurboGFP-B Vector (Evrogen, Russia) was transformed into the *E. tarda* FPC498 strain with an electroporation method to generate a strain that express GFP (GFP-*E. tarda*). GFP-*E. tarda* bacteria were incubated for 48 h at 25°C in triptic soy agar with 100 μg/ml ampicillin before being co-cultured with phagocytes.

2.3. Detection of live bacteria and preparation of plasma and kidney leukocytes from *E. tarda*-injected fish

Experimental group of 21 fish were challenged with 1×10^5 CFU/100 g BW (0.2 LD_{50}) of *E. tarda* by i.p. injection. Control group of 21 fish were injected with 1 ml/100 g BW of PBS. Tissues (kidney, liver, and spleen) and plasma were collected from each group at 0, 2, 4, 8, 12, 16, and 30

days post infection (dpi). To obtain the plasma, peripheral blood was isolated with heparinized syringe, and centrifuged at 500 × *g* for 10 min at 4°C. Plasma was collected from supernatant of peripheral blood. Tissues were weighed and then homogenized with PBS. Bacterial numbers were determined using the Miles and Misra method (Miles et al., 1938; Kato et al., 2011). Kidney leukocytes were collected as described previously with slight modification (Toda et al., 2011). Breafly, the kidney cell suspension was prepared by gently pressing the tissue through a 100-μm nylon mesh in RPMI-1640 medium supplemented with 1% FBS (RPMI-1). The cells were subsequently applied to a Percoll™ density gradient 1.040 g/ml of 1.080 g/ml and then centrifuged at 500 × *g* for 30 min at 4°C. The kidney leukocytes were collected 1.040-1.080 g/ml interface, washed 3 times with RPMI-1 by centrifugation (500 × *g*, 30 min, 4°C). Finally, purified kidney leukocytes were suspended in RPMI-1640 supplemented with 5% FBS (RPMI-5).

2.4. Measurement of *E. tarda*-specific antibody titers

Bacterial cells were lysed in the BugBuster™ Protein Extraction Reagent (Merck, Germany) at a final concentration of 200 mg/ml at room temperature for 20 min. The suspension was centrifuged at 16,000 × *g* for 15 min at 4°C, and the collected supernatant was used as the antigen for ELISA. We added 10 mg of bacterial antigen to each well of a 96-well plate (Thermo Fisher Scientific, USA) and incubated for 2 h at room temperature. Antigen solution was discarded, and each well was blocked with 250 μl of Immunoblock™ (Dainippon Sumitomo Seiyaku, Japan) at 4°C overnight. The plate was washed 3 times with PBS containing 0.05-% Tween 20 (PBS-T), and the plasma prepared from sampled fish was diluted (1:5) in PBS, was added to each well and incubated for 2 h at 25°C, followed by 3 washes with PBS-T. Mouse anti-ginbuna IgM mAb was produced by injecting mice with purified ginbuna IgM according to the standard protocol (Köhler et al., 1975) and has been used to separate $sIgM^+$ and $sIgM^-$ cells (Somamoto et al., 2006; Toda et al., 2009; Toda

et al., 2011). Mouse anti-ginbuna IgM mAb (50 µl) (1:5,000 in PBS) was then added to the plate and incubated for 2 h at 25°C. After 3 washes with PBS-T, the third antibody solution, 50 µl of peroxidase-conjugated goat anti-mouse IgG and IgM (1:5,000 in PBS; Life Technologies, USA) was added. The plate was incubated for 1 h at 37°C and washed 3 times with PBS-T. Substrate buffer (100 µl; 0.7 mg/ml 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) in distilled water) with 0.1% H_2O_2 was added to each well and incubated for 15 min in the dark. The absorbance was read at 405 nm using a microplate reader (Thermo Fisher Scientific).

2.5. Real-time PCR

Total RNA was extracted from kidney leukocytes (5×10^6) using the RNeasy Plus Mini Kit (Qiagen, Germany), according to the manufacturer's instructions. First-strand cDNA was synthesized from purified total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Real-time PCR were carried out using specific primer sets (Table 1) for the following genes: IFN-γ1 (*ifng1*), IFN-γ2 (*ifng2*), IFN-γrel (*ifngrel*), T-bet (*tbx21*), GATA-3 (*gata3*), and for housekeeping gene, EF1α (*eef1a*). Real-time PCR, using a Mini OpticonTM System (BIORAD, USA), were performed with 5 µl of 5-fold diluted cDNA, 10 µl of Powere SYBR Green PCR Master Mix (Applied Biosystems, USA) and 200 nM of each primer set for IFNγ1, IFNγ2, IFN-γrel, T-bet, GATA-3, and EF1α in 20 µl mixtures. The amplification was carried out as follows: 3 min at 95°C, 50 cycles of 10 s at 95°C, 30 s at 60°C. A standard curve was generated by plotting the threshold cycle (ct) versus known copy number for each plasmid template in dilutions. A normalized amount of target gene was calculated by dividing the amount of target gene by the amount of EF1α as an endogenous control.

2.6. Percentage of CD8α$^+$ and sIgM$^+$ lymphocytes in kidney leukocytes

Rat anti-ginbuna CD8α mAb was prepared, as described by Toda *et al*. (2009). Kidney leukocytes (KLs) collected from *E. tarda*-infected fish (1×10^7 cells/ml) were incubated with either rat anti-ginbuna CD8α mAb or mouse anti-ginbuna IgM mAb (1:5,000) for 60 min at 4°C. After 3 washes with RPMI-1, the cells (1×10^8 cells/ml) were incubated with either FITC-conjugated anti-rat IgG or anti-mouse IgG (1:100) for 30 min at 4°C, followed by 3 washes. Propidium iodide (PI, 20 μg/ml) used for dead cell stain was added to cell suspensions before the flow cytometric analysis. Flow cytometry was performed using Cytomics FC 500 (Beckman Coulter, USA).

2.7. Cytotoxicity assay using flow cytometry

$CD8\alpha^+$ cells isolated from KLs of *E. tarda*-injected fish were used as effector cells. KLs were collected from *E. tarda*-injected fish at 0, 4, 8, 12, and 16 dpi. KLs (1×10^7 cells/ml) were incubated with rat anti-ginbuna CD8α mAb (1:5,000) for 60 min at 4°C. After 3 washes with RPMI-1, the cells (1×10^8 cells/ml) were incubated with magnetic bead-conjugated goat anti-rat IgG antibody (1:5; Miltenyi Biotec GmbH) for 30 min at 4°C. After 3 times washes, $CD8\alpha^+$ cells were isolated by magnetic cell sorting and resuspended in RPMI-10 containing 10 U/ml penicillin, 10 μg/ml streptomycin, and 10 μg/ml gentamicin. Cells were cultured for 12 h at 25°C to enhance the activity of effector cells (Suzumura et al., 1994).

Phagocytes isolated from KLs of naïve fish were used as target cells. KLs were applied to a Percoll™ density gradient of 1.080 g/ml and 1.090 g/ml and centrifuged at 500 × *g* for 30 min at 4°C. Phagocytes in the 1.090 g/ml layer were collected and washed 3 times with RPMI-1. Phagocytes (5×10^6 cells/ml) were cultured with GFP-*E. tarda* (multiplicity of infection of 10) for 2 h at 25°C. After 3 washes with the medium, and to sterilize extracellular bacteria, cells (5×10^6 cells/ml), were subsequently incubated with RPMI-5 containing 200 U/ml penicillin, 200 μg/ml streptomycin, and 200 μg/ml gentamicin for 1 h at 25°C. The GFP-*E. tarda*-phagocytizing target cells were confirmed

187 by flow cytometry and then placed in 96-well plate (1×10^4 cells/well).

188 Effector cells (5×10^4 cells) were added to each well in a final volume of 200 µl and incubated
189 for 6 h at 25°C. Cytotoxicity was assayed following the protocol, as described by Jedema *et al.*
190 (2004) with slight modification. In brief, cytotoxicity was calculated based on the absolute number
191 of viable target cells, which was determined by flow cytometry as follows. 10,000 microbeads (6 µm
192 in diameter; Becton Dickinson) and PI (2.5 ng/ml) were added to cell suspensions, mixed thoroughly,
193 and immediately analyzed by flow cytometry. Counts in gated "Live target" in GFP-PI dot plots
194 were recorded when the number of acquired microbeads reached 2,000. The percentage of survival
195 was calculated as follows:
196 % survival = (number of target cells with effector cells) × 100/(target cells without effector cells)
197 The percentage of cytotoxicity was calculated as follows: % cytotoxicity = 100 - % survival
198
199 **2.8. Statistics**
200 The results were shown as mean ± standard deviation (SD). Differences in *E. tarda*-specific
201 antibody titers were determined by performing *t* test. Statistical analysis of the quantitative
202 expression, kinetics of lymphocytes, and cytotoxic assays were performed using analysis of variance
203 (ANOVA). A P value of <0.05 was considered statistically significant.
204
205 **3. Results**
206
207 **3.1. Bacterial clearance in tissues after infection**
208 All fish that were injected with *E. tarda* (10^5 CFU/100 g BW) showed no clinical signs during
209 the challenge test. Bacterial counts of *E. tarda* in kidney, spleen, and liver decreased within 8 dpi.
210 Bacterial levels were undetectable in the kidney and spleen at 12 dpi and undetectable in all tested

211 tissues at 16 dpi (Fig. 1).

212

213 **3.2. Antibody responses against *E. tarda***

214 *E. tarda*-specific antibody titers in infected fish plasma increased significantly at 16 dpi, and

215 higher level was observed 30 dpi (Fig. 2).

216

217 **3.3. Quantitative expression analysis of IFN-γ, T-bet and GATA-3 genes in kidney leukocytes**

218 The *ifng1* gene was significantly up-regulated at 2 and 12 dpi (Fig. 3A). The *ifng2* gene was

219 significantly up-regulated at 2 dpi (Fig. 3B). The *ifngrel* gene showed no changes during the

220 challenge test (Fig. 3C). The expression level of *tbx21* gene was significantly up-regulated from 8 to

221 16 dpi (Fig. 3D). The *gata3* gene was significantly up-regulated from 12 dpi, and higher level was

222 observed 30 dpi (Fig. 3E).

223

224 **3.4. Kinetics of CD8α$^+$ and sIgM$^+$ lymphocytes in the kidney from *E. tarda*-infected fish**

225 The percentage of CD8α$^+$ lymphocytes in kidney increased significantly from 4 to 8 dpi,

226 followed by a decrease at 12 dpi, and the levels remained the same up to 30 dpi (Fig. 4A). On the

227 other hand, the percentage of sIgM$^+$ lymphocytes increased significantly at 12 dpi and reached a

228 much higher level (>2-fold) at 30 dpi (Fig. 4B).

229

230 **3.5. Cytotoxic activity of CD8α$^+$ cells**

231 The cytotoxic activity of CD8α$^+$ cells against *E. tarda*-phagocytizing isogenic target cells

232 increased significantly at 4 dpi and peaked to the highest level at 8 dpi, followed by a decrease at 12

233 dpi (Fig. 5).

234

4. Discussion

The importance of CMI in the host defense against intracellular bacterial infection has been well established in mammals (Neild and Roy, 2004; Parmer, 2004; Reece and Kaufmann, 2008). In fish, however, the principal component of the immune system that protects them from infection by intracellular bacteria such as *E. tarda* remains unclear. Therefore, this study designed to investigate the cell-mediated and humoral immune responses in *E. tarda*-infected fish. Our results show that both cell-mediated and innate immunity play an important role in the protection against intracellular bacterial infection, as they do in mammals. These findings also provide insights into the immune responses that protect against other intracellular pathogens, such as *Mycobacterium* sp., *Nocardia seriolae*, and *Photobacterium damselae* subsp. *piscicida* in fish.

IFN-γ is an essential cytokine that promotes CMI against various infectious agents in mammals. Similar function was also observed in fish IFN-γ (Grayfer and Belosevic, 2009). In this study, the IFN-γ1 and 2 genes expression was up-regulated at day 2 after *E. tarda* infection (Fig. 3A), suggesting that CMI was induced in *E. tarda*-infected fish. In contrast, the IFN-γrel expression level was not modulated by infection (Fig. 3C). Fish possess 2 types of IFN-γ: IFN-γ and IFN-γrel (Iwaga et al., 2006). Although ginbuna crucian carp IFN-γrel lacks the carboxyl-terminal cationic residues that are thought to be required for the IFN-γ activity, Yabu *et al*. (2011) has shown that recombinant IFN-γrel conferred virus-resistant activity to host cells in ginbuna crucian carp. A number of cytokine isoforms such as IL-1β and TNF-α are generally present in cyprinid fish. The expressions of these isoforms lead to different responses against various antigens, suggesting that they might share multiple functions in certain situations (Engelsma et al., 2003; Grayfer et al., 2008). Nevertheless, our results suggest that IFN-γrel, although may exert other functions in ginbuna crucian carp, are not involved in the clearance of *E. tarda* from the fish kidney.

In mammals, the differentiation of naïve helper T cells to Th1 and Th2 is controlled by two

opposing transcription factors, T-bet and GATA-3, respectively. In fish, T-bet and GATA-3 genes have also been identified and well characterized. High expression level of T-bet and GATA3 genes was observed in sIgM$^-$ cells and lymphocytes in ginbuna crucian carp, suggesting that fish T-bet and GATA-3 are expressed in T cells (Takizawa et al., 2008a; Takizawa et al., 2008b). It has been reported that mice with T-bet deletions exhibit increased susceptibility to intracellular bacteria and viruses (Szabo et al., 2002; Ravindran et al., 2005; Matsui et al., 2005), reveal that T-bet is essential for CMI in mammals for enhancing the differentiation and activation of Th1. In this study, we observed that T-bet gene was up-regulated from 8 to 16 dpi (Fig. 3D), suggesting that Naïve helper T cells was differentiation to Th1 during this periods. Moreover, expression of IFN-γ1 was observed at 12 dpi, suggesting that Th1 might produce IFN-γ1 and induce CMI. On the other hand, GATA-3 is recognized as the master regulator of Th2 cells (Zheng and Flavell, 1997; Zhang et al., 1997). GATA-3 conditional gene-knockout studies showed that Th2 differentiation completely depends on GATA-3 expression (Zhuet al., 2004; Pai et al., 2004). Th2 cells were also reported to produce IL-4, IL-5, and IL-13, which is important for immune responses against extracellular parasites (Zhu, 2010). In this study, GATA-3 gene was up-regulated from 12 to 30 dpi (Fig. 3E), suggesting that Th2 was differentiation from Naïve helper T cells during this period. These findings suggesting that the adaptive immunity with helper T cells was induced from 8 dpi. In addition, T-bet and GATA-3 genes showed the opposed expressions, suggesting helper T cells of fish might have the same differentiation mechanism of Naïve helper T cells to Th1 and Th2 as in mammals.

Although *E. tarda* is known to be resistant to phagocyte bactericidal activity in the phagosome (Iida et al., 1993; Iida and Wakabayashi, 1993; Rao et al., 2001; Ishibe et al., 2008; Takano et al., 2010), our results showed that the bacterial counts decreased from day 2 to 4 after *E. tarda* infection (Fig. 1). During the period, adaptive immune response had not been induced, indicating that bacterial reduction resulted from innate immune response. In addition, the highest expression level of IFN-γ1

283 and 2 genes were also observed at day 2 after *E. tarda* infection. Grayfer *et al*. (2011) previously
284 reported that goldfish macrophages primed by recombinant IFN-γ reduced the survival of
285 intracellular *Mycobacterium marinum*. Therefore, ginbuna crucian carp macrophages may also be
286 activated by IFN-γ and contribute to bacterial reduction from day 2 to 4. However, bacterial counts
287 were unchanged from 4 to 8 dpi, suggesting that a proportion of *E. tarda* might be able to avoid
288 bactericidal activity of the innate immune system.

289 Mammalian CTLs play an important role for immunity against intracellular pathogens
290 (Kaufmann and Flesch, 1988; Kaufmann, 1993; Wizel et al., 2008). In this study, we reported that
291 $CD8\alpha^+$ lymphocytes increased from 4 to 8 dpi (Fig. 4A). Although the increasing rate of $CD8\alpha^+$ cells
292 showed lower than those of $sIgM^+$ cells, the highest expression level of CD8α molecule on the cell
293 surface was observed at the same period by flow cytometry (data not shown), indicating that $CD8\alpha^+$
294 cells was activated from 4 to 8 dpi. In addition, during the same period, $CD8\alpha^+$ cells showed higher
295 cytotoxic activities against *E. tarda*-infected isogenic cells (Fig. 5). These results indicate that
296 cell-mediated immunity was induced from 4 to 8 dpi. Moreover, bacterial clearance in kidney and
297 spleen of infected fish was observed following higher $CD8\alpha^+$ cytotoxic activities (Fig. 1),
298 implicating that CTLs might contribute to the bacterial clearance by developing specific cytotoxicity
299 against *E. tarda*-infected cells in the tissues.

300 Antigen-specific antibodies produced by B cells are effective in protection from extracellular
301 pathogens. In fish, humoral immunity plays a primary role in protection against bacterial infections
302 that are not resistant to bactericidal mechanisms in phagosomes, such as *Streptococcus agalactiae*
303 and *Streptcoccus iniae* (Pasnik et al., 2006; LaFrentz et al., 2011). However, the role of humoral
304 immunity in protecting fish against intracellular bacterial infections is unclear. In this study, we
305 showed that the percentage of $sIgM^+$ lymphocytes in kidney leukocytes increased significantly only
306 from day 12 to 30 after *E. tarda* infection (Fig. 4B) and that *E. tarda*-specific antibody titers

307 increased significantly only after the bacteria clearance (Fig. 2). In addition, the induction of Th2
308 was observed at the same period. These results indicate that induction of humoral immunity may be
309 too late to provide protection against *E. tarda* infection. Moreover, Igarashi and Iida (2002) reported
310 that it has no relationship between antibody titer and survival ratio in the challenge test with *E. tarda*.
311 Taken together, these observations indicate that protection against *E. tarda* can occur without
312 humoral immune response. Somamoto *et al*. (2002) previously reported that humoral immunity is
313 not effective in protecting against intracellular pathogens such as viruses and that CMI contributes to
314 controlling of viral infections in fish. Our results demonstrate that CMI is also an important immune
315 mechanism for protecting fish from intracellular bacterial infection.
316 The vaccinations with formalin-killed cells (FKC) have been ineffective in protecting fish
317 against intracellular pathogens, such as *E. tarda*, *Mycobacterium* sp., and *N. seriolae* (Salati et al.,
318 1983; Igarashi and Iida, 2002; Kato et al., 2010; Kato et al., 2012). Kato *et al*. (2012) reported that
319 the vaccination with FKC was ineffective in protection against *N. seriolae* in Japanese flounder and
320 that the IFN-γ expression was not up-regulated by the vaccination with either. Our previous works
321 also indicated that the expression of CMI-related genes, such as IFN-γ, CD8α, and TCR-β, was
322 induced more weakly by sensitization with *E. tarda*-FKC than by that with *E. tarda*-live cells in
323 ginbuna crucian carp (unpublished data). These findings suggest that IFN-γ production provides an
324 early and effective protection against intracellular bacterial infection. Jung *et al*. (2012) recently
325 reported that recombinant IFN-γ induced the expression of immune-related genes such as IL-1β and
326 IFN-γ and inhibited *E. tarda* infection in olive flounder, indicating that IFN-γ is necessary for the
327 elimination of *E. tarda*. Therefore, CMI induced by IFN-γ might play important role in protection
328 against *E. tarda* infection.
329 In summary, our data indicate that although a proportion of the bacteria might be resistant to
330 bactericidal activity of the innate immune system, innate immunity is still one principal immune

system for eliminating the majority of *E. tarda*. Moreover, the bacterial clearance in kidney and spleen was observed following higher cytotoxic activities of CTLs, suggesting that CTLs play a role in the elimination of *E. tarda*-infected cells with specific cytotoxicity. On the other hand, induction of humoral immunity may be too late to provide protection against infection. These data indicate that CMI as well as innate immunity play a crucial role in protection against *E. tarda* infection. These findings also provide novel insights into the development of a CMI-induced vaccine against intracellular pathogens of fish.

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565

566 **Figure legends**

567

568 **Fig. 1.** Reduction of bacterial counts in fish kidney, spleen, and liver after *E. tarda* infection.
569 Bacterial counts are shown as mean ± SD of 3 fish. N.D. indicates that no colony was detected in the
570 media.

Fig. 2. Antibody responses in plasma *E. tarda* infection. Fish were injected with PBS (●) or *E. tarda* (□). Antibody titers are shown as the mean ± SD of 3 fish. Statistical analysis was performed using Student's unpaired *t*-test. Asterisks indicate significant differences between the *E. tarda*- and PBS-injected groups during the same period (*$P < 0.05$, **$P < 0.01$).

Fig. 3. Quantitative expression analysis of IFN-γ (A, *ifng1*; B, *ifng2*; C, *ifngrel*), T-bet (D, *tbx21*), and GATA-3 (E, *gata3*) in kidney leukocytes after *E. tarda* infection. Fish were injected with PBS (open bar) or *E. tarda* (closed bar). The expression levels were calculated as a ratio to the EF1α (*eef1a*) level, and are shown as fold change in expression based on naïve fish sample (day 0). Data are shown as mean ± SD of 3 fish. Statistical analysis was performed using analysis of variance (ANOVA). The labels with different letters above each bar denote statistical significance ($P < 0.05$); labels with the same letter on each bar of a graph indicate no statistical difference between the groups.

Fig. 4. Kinetics of $CD8\alpha^+$ (A) and $sIgM^+$ (B) lymphocytes in the kidney after *E. tarda* infection. Fish were injected with PBS (open bar) or *E. tarda* (closed bar). The percentages of cell populations in kidney leukocytes (KLs) are shown as mean ± SD of 3 fish. Statistical analysis was performed using analysis of variance (ANOVA). The labels with different letters above each bar denote significant difference ($P < 0.05$); labels with the same letter on each bar of a graph indicate no statistical difference between the groups.

Fig. 5. Cytotoxic activity of $CD8\alpha^+$ cells after *E. tarda* infection. Data are shown as mean ± SD of 3 fish. Statistical analysis was performed using analysis of variance (ANOVA). The labels with

595 different letters above each bar denote significant difference ($P < 0.05$); labels with the same letter
596 on each bar a graph indicate no statistical difference between the groups.