Research article

Percentage Dynamics of CD4-1⁺ and CD8 α ⁺ T Cells in Different Tissues at Various Developmental Stages in Ginbuna Crucian Carp *Carassius auratus langsdorffii*

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ABSTRACT—Determination of numbers of T cell subsets in tissues is important to assess the functional maturity of the immune system. The distribution of T cells in lymphoid tissues during ontogenic development has been examined in several fish species using pan-T cell markers. However, information on the age-related changes in the number of T cell subsets in tissues is limited due to the lack of antibodies against CD4 and CD8 in fish. In the present study we examined the percentage dynamics of CD4-1⁺ and CD8 α ⁺ cells in various tissues accompanied with age of ginbuna crucian carp using flow cytometry. We found rapid increases in both CD4-1⁺ and CD8 α ⁺ T cells up until 6 months post-hatch (mph). CD4 and CD8 double-positive cells (DP cells) are present only in the thymus and DP cells increased, while double negative cells decreased, in fish up to 3–6 mph. The percentages of CD4-1⁺ T cells were always higher than those of CD8 α ⁺ T cells in the various tissues examined, except for the thymus and intestine. In contrast, significantly higher percentages of CD8 α ⁺ T cells than CD4-1⁺ T cells were found in the intestine during the period between 6 mph and 2 years post-hatch.

Key words: CD4, CD8, T lymphocytes, ontogeny, *Carassius auratus langsdorffii*, ginbuna crucian carp, thymus, flow cytometry

The thymus is an essential organ for development of T lymphocytes from early thymocyte progenitors to functionally competent T cells. It has been well documented that early steps in T cell (thymocyte) development and thymic organogenesis in fish are similar to those in higher vertebrates, as documented by histology and gene expression patterns in zebrafish (reviewed in Langenau and Zon, 2005) and other fish species (reviewed in Tatner, 1997; Rombout *et al.*, 2005; Nakanishi *et al.*, 2015). However, little is known about the late stages of T cell development within the fish thymus.

Although there have been no reports showing the presence of Peyer's patches, M cells, IgA or J-chain in the intestine of teleost fish, the presence of T cells, including intraepithelial lymphocytes (IELs), has been demonstrated in intestinal tissue of several fish species using monoclonal antibodies that recognize T cell subpopulations, and by expression analysis of T cell marker genes (reviewed in Rombout *et al.*, 2010; Salinas, 2015).

In fish, however, age-related changes in intestinal T cell subsets have yet to be investigated.

The gill is another mucosal tissue exposed to the environment. The presence of considerable numbers of T cells in the gill has been reported in several fish species, e.g. carp (Rombout *et al.*, 1998), Atlantic salmon (Koppang *et al.*, 2010), rainbow trout (Takizawa *et al.*, 2011), and European sea bass (Nuñez Ortiz *et al.*, 2014). In Atlantic salmon Haugarvoll *et al.* (2008) reported intraepithelial lymphoid cell accumulations at the base of the gill filaments, where mRNA expression of MHC class II and TCR α was detected. These data highlight the importance of identifying CD4⁺ and CD8⁺ T cells in the gill to better understand their function.

Monoclonal antibodies (mAbs) against CD4-1 and CD8 α are available for clonal ginbuna crucian carp. With the aid of mAbs against these T cell subsets, CD8⁺ T cells have been identified as cytotoxic T lymphocytes (CTLs) and the helper function of CD4⁺ T cells has been demonstrated (Toda *et al.*, 2009, 2011). Alloantigen- and virus-specific cytotoxicity have also been demonstrated in ginbuna, along with the importance of cell-mediated immunity relative to humoral immunity in the

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protection against intracellular bacterial infection (reviewed in Nakanishi *et al.*, 2015). These findings suggest that CD4⁺ and CD8 α ⁺ T cells in ginbuna are equivalent to helper and cytotoxic T lymphocytes (CTL) in mammals, respectively.

Most previous reports on this topic have been focused on early steps in T cell development and thymic organogenesis. However, the appearance of lymphoid organs as well as lymphocytes within them does not necessarily correlate with functional maturity of the immune system (Tatner, 1997). Zapata *et al.* (2006) reported that full maturation of immunological competence develops quite late, despite the early appearance of both lymphoid organs and T- and B-lymphocytes. Therefore, analysis of immunological competence focusing on the quantity of T cells present is important. The distribution of T cells in lymphoid tissues during ontogenetic development has been reported in sea bass (dos Santos *et al.*, 2000; Picchietti *et al.*, 2008, 2009), carp (Romano *et al.*, 1999) and zebrafish (Danilova *et al.*, 2004; Langenau and Zon, 2005). In fish, however, information on the age-related changes in the number of CD4⁺ and CD8⁺ T cells in tissues is limited.

In the present study, we examined the percentages of CD4⁺ and CD8⁺ T cells along with the total number of leukocytes in various tissues according to age of fish. This is the first report to show the distribution of CD4⁺ and CD8⁺ T cells during development, although several similar studies have been conducted using gene expression and/or histological analysis (Huttenhuis *et al.*, 2005; Picchietti *et al.*, 2008, 2009).

Materials and Methods

Fish

Triploid female ginbuna carp (*Carassius auratus langsdorffii*) from Okushiri Island in Hokkaido (OB1 clone) weighing 0.05–60 g (1-month-old–4-years-old) were used for the experiment. Relationship between age and body weight reared at 25°C is shown in Table 1. Juveniles weighing less than 5 g were fed five times daily with a particulate compound solid feed for fish larvae (Scientific Feed Laboratory) using an automatic feeder and were held in a 60-L glass tank. Fish weighing more than 5 g (6 months post-hatch: mph) were fed twice daily with commercial pellets and maintained in 500–1,000-L tanks. Both juvenile and adult fish were held in circulating water at 25 ± 1°C.

Preparation of leukocytes

To obtain peripheral blood leukocytes, fish older than 6 mph were bled from the caudal blood vessels with a heparinized syringe, while fish at 30–90 days post-hatch (dph) were bled from the severed caudal peduncle. The thymus, spleen, head-kidney, trunk-kidney, liver, gill and intestine were then dissected from

Table 1. Relationship between age and body weight in ginbuna crucian carp

Age	Body weight (g)	Note
30–40 days	0.05–0.1	
50–60 days	0.2–0.4	
70–90 days	1–2	
3–4 months	2–3	
4–6 months	3–5	
6–12 months	5–10	
1–2 years	10–20	
2–3 years	20–30	Adult*
3–4 years	>60	Adult*

* Fish more than 15 g are considered as adults since their ovaries contain mature eggs.

sacrificed fish. Tissues from five to ten fish weighing 0.05–5 g were pooled for analysis. All subsequent manipulations of cells were done at 4°C.

For the thymus, spleen, head-kidney and trunk-kidney, the organs were placed on a stainless-steel mesh filter (100 μ m, Q-ho Metal Works), and pressed through with 5 mL of HBSS (Nissui Pharmaceutical) to create single-cell suspensions. For liver, gill and intestine the tissues were incubated with PBS containing 1 mM DTT (Wako Chemicals) and 1 mM EDTA for 15 min after mincing with scissors. After incubation, the organs were washed and dissociated by incubating with calcium- and magnesium-free Hank's Balanced Salt Solution (CMF-HBSS) containing 0.1 mg/mL collagenase (Wako Chemicals), 0.1 mg/mL DNase (Sigma-Aldrich) and 5% FBS for 90 min with shaking at room temperature. Dissociated organs were disaggregated by pressing through the stainless-steel mesh filter into HBSS. The buffy coat from peripheral blood and leukocytes from tissues were collected by centrifugation at 400 × g for 5 min at 4°C.

For FACS analysis cells suspended in OPTI-MEM (Gibco-BRL) supplemented with 0.5% heat-inactivated FBS (OPTI-MEM-0.5) were applied to a Percoll (Pharmacia Fine Chemicals) density gradient of 1.080 g/mL and centrifuged at 450 × g for 30 min at 4°C. The lymphocyte-rich fraction at the interface was collected and washed twice in HBSS. To obtain counts of the total number of leukocytes in lymphoid tissues, cell suspensions including erythrocytes were used. Cell concentration and viability were determined by trypan blue dye exclusion with a haemocytometer. Viability of cells was approximately 90%.

Monoclonal antibodies

Flow cytometric analysis was performed using the previously reported rat mAbs 6D1 (IgG2a) and 6C10 (IgG1) that recognize ginbuna crucian carp CD4-1 and CD8 α , respectively (Toda *et al.*, 2009, 2011; Shibasaki *et al.*, 2010).

Immunofluorescence staining and Flow cytometry

For two-color immunofluorescence analysis of cell surface CD4-1 and CD8 α with 6D1 and 6C10 mAbs, 1×10^6 cells/mL of leukocytes from the various tissues in HBSS were first incubated with 1 μ g/mL of mAb 6D1 for 45 min on ice, followed by staining with a 1:500 dilution of FITC-conjugated anti-rat IgG + IgM + IgA (Rockland) for 30 min. Then the cells were resuspended and incubated with 1 μ g/mL of mAb 6C10 for 45 min on ice followed by staining with PE conjugated anti-rat IgG1 goat IgG (Beckman Coulter) diluted 1:500 for 20 min. Cells were washed three times with HBSS after incubation with antibodies at each step. Finally, cells were suspended in 1 mL of HBSS with 2.5 μ g/mL propidium iodide (Invitrogen) and dead cells were excluded for the analysis. Lymphocytes were gated on FS & SS dot plot and then analyzed for double staining with the mAbs using a FACSCanto flow cytometer (Becton Dickinson).

Statistics

Results of FCM analysis were statistically compared using one-way or two-way ANOVAs, followed by Tukey's multiple comparisons tests to detect significant difference between means in the percentage of positive cells. A p-value of < 0.05 was considered statistically significant.

Results

Tissue distribution of CD4⁺ and CD8⁺ T cells in adult fish

Since we previously reported the distribution of mAbs 6D1 (anti-CD4-1)- and 6C10 (anti-CD8 α)-positive lymphocytes in lymphoid tissues and PBL of adult fish by FACS analysis (Toda *et al.*, 2011), here we summarize the percentages of mAb-positive cells among lymphocytes in tissues including lymphoid tissues and PBL of adult fish (Table 2). In brief, significantly higher percentages of CD4-1⁺ cells than CD8 α ⁺ cells were present with the exception of the thymus, intestine and gill. In

the present study we also examined the distribution of mAbs 6D1- and 6C10-positive lymphocytes in non-lymphoid tissues by FACS analysis. MAb 6D1 reacted with 11.9%, 7.4% and 6.0% of liver, intestine and gill lymphocytes of adult fish, respectively and mAb 6C10 reacted with 4.0%, 10.0% and 4.7% of liver, intestine and gill lymphocytes, respectively (Fig. 1 and Table 2).

Changes in percentages of CD4⁺ and CD8⁺ T cells with age

In the thymus, percentages of CD4-1 and CD8 α double-positive lymphocytes (DP cells) greatly increased from 6% at 30–40 days to 35% at 6–12 mph (Fig. 2). In contrast, percentages of double-negative lymphocytes (DN cells) decreased from 66% at 30–40 dph to 28% at 6–12 mph. The percentage of CD8 α single-positive cells was significantly lower in 30–40 dph (4%) than in fish older than 50 dph (approx. 20%), while the percentages of CD4-1 single-positive cells were constant throughout life (approx. 20%), except in 50–60 dph (14%). The percentages of CD4-1⁺ T cells increased in juveniles between 30 dph and 4 mph in the head- and trunk-kidney, spleen, liver and gill, but not intestine (Fig. 3). The percentages of CD4-1⁺ T cells were always higher than those of CD8 α ⁺ T cells in the tissues examined with the exception of the intestine and thymus. The percentage of CD8 α ⁺ T cells was lower in younger fish at 30–60 dph and increased from 6 mph onwards adult in most of tissues, although the change was not significant in liver. The percentage of CD8 α ⁺ T cells remarkably increased in the intestine from 6 mph to 2 years post-hatch (yph) and that was significantly higher than that of CD4-1⁺ T cells, although CD4-1⁺ T cells also increased through development and adulthood. There was a dramatic decrease in the number of CD4-1⁺ T cells in the spleen and liver in fish at 6 mph, while in the gills an increase of CD4-1⁺ T cells was evident in fish at 3 mph.

Table 2. Percentages of CD4 and CD8 positive T cells in tissues of adult fish^{*1}

	CD4 single positive	CD8 single positive	CD4/CD8 double positive
Thymus	19.8 $\pm$ 2.8 ^{*2}	21.9 $\pm$ 3.0	26.4 $\pm$ 5.6
Head-kidney	20.7 $\pm$ 4.8	8.7 $\pm$ 2.6 ^{§3}	0.4 $\pm$ 0.2
Trunk-kidney	20.0 $\pm$ 5.1	10.7 $\pm$ 3.8 ^{§2}	0.6 $\pm$ 0.2
Spleen	7.6 $\pm$ 3.4	4.3 $\pm$ 1.6 ^{§1}	0.2 $\pm$ 0.1
Liver	11.9 $\pm$ 2.8	4.0 $\pm$ 2.6 ^{§3}	0.1 $\pm$ 0.1
Intestine	7.4 $\pm$ 3.9	10.0 $\pm$ 6.2	0.3 $\pm$ 0.2
Gill	6.0 $\pm$ 3.1	4.7 $\pm$ 3.2	0.1 $\pm$ 0.1
PBL	5.5 $\pm$ 2.8	2.0 $\pm$ 0.8 ^{§2}	0.1 $\pm$ 0.1

^{*1} Six fish more than 2 years old (20 g) were used.

^{*2} Mean $\pm$ SD

[§] Significant differences in the percentage between CD4 and CD8 single positive T cells (§1; $p < 0.05$, §2; $p < 0.01$, §3; $p < 0.0001$).

Statistical significance was calculated using a one-way ANOVA, followed by Tukey's multiple comparisons test.

Fig. 1. Staining pattern of anti-CD4-1 and anti-CD8 α mAbs in tissues of adult fish. Leukocytes from liver, intestine and gill from fish at 2–3 yph (20–30 g) were stained with mAb 6D1 (CD4-1) and FITC-conjugated goat IgG to rat IgG2a, and with mAb 6C10 (CD8 α) and PE-conjugated goat IgG to rat IgG1. Lymphocytes/thrombocytes were gated on FS and SS dot plot and analyzed for double staining with the mAbs. Each figure is representative of more than five analyses.

Fig. 2. Changes in percentages of T cell subsets in thymus according to age. Open box, CD4 single-positive cells; black box, CD8 single-positive cells; striped box, double-positive cells; grey box, double-negative cells. Bars represent means $\pm$ standard deviation (SD). $n \geq 3$. Statistical significance was calculated using a two-way ANOVA, followed by Tukey's multiple comparisons test. Columns without characters or those have the same characters are not significantly different. Significant differences among CD8 single-positive cells are denoted by the different uppercase letters (B; $p < 0.05$, C; $p < 0.01$ vs. the percentages in fish at 30–40 dph [A]). Significant differences among double-positive cells are denoted by the different lowercase letters (c; $p < 0.01$, e; $p < 0.0001$ vs. the percentages in fish at 30–40 dph [a]). Significant differences among double-negative cells are denoted by the different Roman numerals (ii; $p < 0.05$, iii; $p < 0.01$, v; $p < 0.0001$ vs. the percentages in fish at 30–40 dph [i]).

Fig. 3. Changes in percentages of T cell subsets in tissues according to age. The open and black boxes indicate the percentages of 6D1 (CD4-1) and 6C10 (CD8 α) positive cells, respectively. Bars represent means $\pm$ standard deviation (SD). $n \geq 3$. Statistical significance was calculated using a two-way ANOVA, followed by Tukey's multiple comparisons test. Significant differences in the percentage between CD4-1⁺ and CD8 α ⁺ T cells are denoted with asterisks above columns (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). Significant differences among CD4-1 single-positive cells are denoted by the different uppercase letters (B; $p < 0.05$; C; $p < 0.01$; D; $p < 0.001$ vs. the percentage in fish at 30–40 dph [A]). Significant differences among CD8 α single-positive cells are denoted by the different lowercase letters (b; $p < 0.05$; c; $p < 0.01$; d; $p < 0.001$; e; $p < 0.0001$ vs. the percentage in fish at 30–40 dph [a]).

Changes in numbers of total leukocytes in tissues with age

In the thymus, the total number of leukocytes (consisting of more than 90% thymocytes) greatly increased from 4.2×10^5 cells at 40 dph (0.1 g) to 86.4×10^5 cells at 12 mph and then dropped to 24.0×10^5 cells at 3–4

yph (Fig. 4). Head- and trunk-kidney leukocytes increased with age, while the numbers were higher in the head-kidney than those in trunk-kidney except at 40–80 dph (0.1–1 g). The number of leukocytes in the spleen was very low at 40–80 dph (3.4 – 8.6×10^4 cells) and increased to 7.2 – 19.0×10^5 cells from 6 mph

Fig. 4. Changes in total number of leukocytes in tissues according to age. Open box, head-kidney leukocytes; grey box, trunk-kidney leukocytes; striped box, spleen leukocytes; black box, thymus leukocytes. Bars represent means $\pm$ standard deviation (SD). $n = 5$. Statistical significance was calculated using a two-way ANOVA, followed by Tukey's multiple comparisons test. Columns without characters or those have the same characters are not significantly different. Significant differences among the number of head-kidney leukocytes with age are denoted by the different uppercase letters (B; $p < 0.05$, E; $p < 0.0001$ vs. the number of leukocytes in fish at 40 dph [A]). Significant differences among trunk-kidney leukocytes with age are denoted by the different lowercase letters (d; $p < 0.001$, e; $p < 0.0001$ vs. the number of leukocytes in fish at 40 dph [a]). Significant differences among thymus leukocytes with age are denoted by the different Roman numerals (iv; $p < 0.001$, v; $p < 0.0001$ vs. the number of leukocytes in fish at 30–40 dph [i]).

onward, although the number was lower than that in other lymphoid organs.

Discussion

In the present study, we report a rapid increase of CD4-1⁺ and CD8 α ⁺ T cells through 6 months of age in ginbuna crucian carp. This is in good agreement with previously reported knowledge on the maturation of both cell-mediated and humoral immunity in fish. Namely, the strength of immune reactivity increases through 6 months of age, although the onset of immune responses occurs earlier as described below. CD4 and CD8 double-positive cells (DP cells) are present only in the thymus and DP cells increased, while DN cells decreased, in fish at 3–6 mph, suggesting enhanced T cell differentiation in early stage of life. The percentages of CD4-1⁺ T cells were always higher than those of CD8 α ⁺ T cells in the various tissues examined with the exception of the thymus, intestine and gill. A higher percentage of CD4-1⁺ cells than CD8 α ⁺ cells is considered to be a common feature in ginbuna throughout their life from juvenile to adults. In contrast, significantly higher percentages of CD8 α ⁺ T cells than that of CD4⁺ T cells were found in the intestine of ginbuna during the period between 6 mph and 2 yph, although no significant difference was observed older than 2 yph (more than 20 g) as shown in Fig. 3 and Table 2. Accordingly, the higher

percentages of CD8 α ⁺ T cells suggest an abundance of CD8 α ⁺ T cells in the intestine during the period between 6 mph and 2 yph, although the reason remain unknown. Further studies are needed to clarify the reason including the count of total number of leukocytes in the intestine.

It is generally agreed that the ability to react to histoincompatible tissue develops early in teleost fish, as determined by allograft rejection. For instance, young fry rejected allografts as early as 16 dph in carp at 22°C (Botham and Manning, 1981) and 14 dph in rainbow trout at 14°C (Tatner and Manning, 1983), although the rejection takes longer compared to that in adults. In our study with rock fish (marine teleost, *Sebastes marmoratus*) we found that fish as young as 1.5 mph showed allograft rejection in the same manner as adult fish as assessed by eye transplantation, and fish of 3–5 mph could reject scale grafts more rapidly than adults at 23°C (Nakanishi, 1986). However, Rijkers and van Muiswinkel (1977) reported that rosy barb (*Barbus conchionius*) was fully immunocompetent with respect to the allograft response by 6 mph at 24°C. In the present study, considerable numbers of CD4⁺ T cells were found to be present even in fish of 30 dph and percentages increased to the highest level, comparable to that of adults, at 3–6 mph. Taken together, it is possible that strength of immune reactivity increases with age by 6 months, although the onset of allo-immune responses

occurs around two weeks after hatch in carp and rainbow trout (Reviewed in Tanner, 1997; Zapata *et al.*, 2006).

Humoral immunity develops later than cell-mediated immunity in fish (reviewed in Tatner, 1997; Zapata *et al.*, 2006). In carp, fry at 4 weeks post-hatch (wph) were unable to mount a plaque forming cell (PFC) response to sheep red blood cells (SRBC) which act as thymus-dependent antigen (van Loon *et al.*, 1981), while older carp fry at 8 wph showed antibody response against human gamma globulin (HGG) in Freund's complete adjuvant (FCA, Manning *et al.*, 1982). Similarly, immune-competence in zebrafish as measured by humoral response to thymus-dependent and -independent antigens, is not reached until 4–6 wph (Lam *et al.*, 2004). We also found that young rock fish at 2 mph are capable of eliciting a humoral immune response, although the titer level is lower and the duration is shorter than in adults (Nakanishi, 1986). Interestingly, carp fry at 4 wph were able to elicit a humoral response with memory against formalin-killed *Aeromonas salmonicida* which acts as a thymus-independent antigen. Accordingly, the present results showing the rapid increase of CD4⁺ T cells through 6 months of age coincides with the maturation of humoral immunity to thymus-dependent antigens.

The thymus contains heterogeneous populations of thymocytes, CD4⁺ CD8[−] (DN, double-negative), CD4⁺ CD8⁺ (DP, double-positive), CD4⁺ CD8[−] (CD4 SP, single-positive), and CD4[−] CD8⁺ (CD8 SP) thymocytes and the developmental sequence can be delineated as: DN→DP→TCR $\alpha\beta$ ⁺CD4/CD8 SP (Ellmeier *et al.*, 1999; Laky *et al.*, 2006). In the present study, percentages of DP cells increased while DN cells decreased up to 3–6 mph suggesting that differentiation from DN cells to DP cells in thymus is occurring during this period. This increased differentiation from DN cells to DP cells parallels the rapid increase of both CD4 SP and CD8 SP cells in other lymphoid tissues (Fig. 3). It should be noted that the total number of both CD4⁺ and CD8⁺ T cells increase with age/weight as growth occurs, although the percentages are constant.

We found significantly higher percentage of CD8 α ⁺ T cells than CD4⁺ T cells in the intestine of ginbuna during the period between 6 mph and 2 yph. The presence of T cells in the intestine has been reported in carp (Rombout *et al.*, 1998), sea bass (Romano *et al.*, 2007), and Atlantic salmon (Koppang *et al.*, 2010). It has been known that $\gamma\delta$ T cells express CD8 α/α homodimers in rodents (Leishman *et al.*, 2001) and the presence of $\gamma\delta$ T cells has been suggested in sea bass (Buonocore *et al.*, 2012). Furthermore, intraepithelial lymphocytes (IELs) are also present in a variety of teleost species (reviewed in Rombout *et al.*, 2010). In the mouse, intestinal IELs contain predominantly CD8⁺CD4[−] T cells (>70% of intestinal IELs) differing from other peripheral T cell

populations (Hayday *et al.*, 2001). Accordingly, the present results may reflect the abundant presence of T cells, most of which are CD8 α ⁺, in the intestine of ginbuna from 6 mph to 2 yph.

Recent studies have highlighted the significance of gills as mucosal immune tissues in fish. Haugarvoll *et al.* (2008) first demonstrated the presence of intraepithelial cell accumulations on the caudal edge of the interbranchial septum at the base of the gill filaments in Atlantic salmon. Koppang *et al.* (2010) confirmed the presence of T cells using sera recognizing a peptide sequence of the CD3 ϵ chain and reported accumulations of T cells in interbranchial lymphoid tissue (ILT). More recently, Hetland *et al.* (2010) confirmed the abundance of CD8 α ⁺ or MHC class II β ⁺ cells in the gill from Atlantic salmon infected with infectious salmon anaemia virus (ISAV). In European sea bass, Nuñez Ortiz *et al.* (2014) reported the presence of considerable numbers of T cells in the gill epithelium where 10%–20% of cells were positive for the T cell-specific mAb DLT15. In the present study, there is a tendency that percentages of CD4-1⁺ T cells were higher than those of CD8 α ⁺ T cells in gills of ginbuna and the difference was significant at 3–6 mph and 1–2 yph (Fig. 3). This finding coincides well with the report by Dalum *et al.* (2015) who found higher expression of CD4-1 genes than CD8 α genes in all gill segments investigated. Interestingly, the higher proportion of CD4-1⁺ T cells in the gill is in large contrast with the intestine where percentages of CD8 α ⁺ T cells tend to be higher than that of CD4-1⁺ T cells.

The presence of several forms of CD4⁺ cells, CD4-1⁺ and CD4-2 (CD4 related or CD4-rel)⁺ cells has been reported in several fish species. Recently, Takizawa *et al.* (2016) reported the presence of three types of CD4⁺ cells in rainbow trout, i.e. CD4-1 single positive, CD4-2 single positive and CD4-1/CD4-2 double positive cells. They reported that CD4-1/CD4-2 double positive cells were predominant population accounting for 83–91% of the total CD4⁺ lymphocytes in all tissues examined, while CD4-2 single positive cells were around 10%, and CD4-1 single positive cells turned out to be monocytes/macrophages. However, it has been reported that CD4-1 and CD4-2 are expressed in different T cell lines in channel catfish (Edholm *et al.*, 2007), common carp (Yamaguchi *et al.*, 2013) and Japanese flounder (Kato *et al.*, 2013). In our study with ginbuna we found that CD4-1 and CD4-2 transcripts were expressed on different cell populations and CD4-1⁺ cells did not contain CD4⁺ monocytes (Somamoto *et al.*, 2014). Therefore, tissue distribution of three types of CD4⁺ cells and their expression of CD4-1 and CD4-2 may be different among fish species, although the presence of CD4-1/CD4-2 double positive cells is possible in ginbuna since we found the expression of both genes in MACS (Somamoto *et al.*, 2014) and FACS sorted (Unpublished data) CD4-1⁺ cells as has been reported in

zebrafish (Yoon *et al.*, 2015; Dee *et al.*, 2016). Accordingly, present results may reflect the real or approximate percentages of CD4⁺ lymphocytes present in tissues provided that CD4-1 and CD4-2 are coexpressed on major CD4⁺ lymphocytes. In any case the exact number of CD4⁺ cells can be determined by further studies with a mAb for CD4-2.

In conclusion, we demonstrated that there are considerable numbers of CD4⁺ T cells present even in 1-month-old fish, and rapid increases of both CD4⁺ and CD8⁺ T cells occur in lymphoid tissues that parallel development of cell-mediated and humoral immunity. Furthermore, we showed progression of T cell differentiation in the thymus through 6 months of age. However, there are several findings without suitable explanations at present. For example, there was a sudden decrease in the percentage of CD4-1⁺ T cells at 6-months-old fish in the spleen and liver, while an increase of CD4-1⁺ T cells was observed at 3-months-old fish in gills. Furthermore, we were not able to analyze the percentages of CD4⁺ and CD8⁺ T cells at stages earlier than 30 days due to the paucity of lymphocytes. This should be examined by histological analysis. A current study on the localization of CD4⁺ and CD8⁺ T cells in tissues by immuno-histochemical analysis of ginbuna young fry is in progress.

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