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Expression profiles of TCRβ and CD8α mRNA correlate with virus-specific cell-mediated cytotoxic activity in ginbuna crucian carp

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Abstract

Our previous studies have demonstrated that virus-specific cell-mediated cytotoxicity of sensitized leukocytes can be induced using clonal ginbuna crucian carp and their syngeneic cell lines. In the present study, we attempt to determine if virus-specific cytotoxic cell populations of fish express CD8α and TCRβ genes. Leukocytes from ginbuna crucian carp were separated into four fractions by immunomagnetic separation and density gradient centrifugation: Fraction A, leukocytes with a density of 1.08 g/ml (primarily lymphocytes); Fraction B, sIg-negative leukocytes with density of 1.08 g/ml; Fraction C, sIg-positive cells (primarily B-lymphocytes); Fraction D, leukocytes with a density of 1.08–1.09 g/ml (primarily neutrophils). Leukocytes in all fractions from uninfected fish do not exhibit cytotoxic activity against virus-infected syngeneic cells and weakly express CD8α and TCRβ mRNAs. In contrast, leukocytes in fractions A and B from virus-infected fish exhibit a high level of cytotoxic activity and strongly express CD8α and TCRβ mRNAs. In addition, mRNA expressions of CD8α and TCRβ in effector cells are upregulated by cocultivation with virus-infected target cells but not uninfected ones. The present study suggests that fish possess virus-specific cytotoxic cells with phenotype and gene expression pattern similar to those of CTLs in mammals.

Keywords: T-cell receptor (TCR); CD8; Cell-mediated cytotoxicity; Viral infection; Ginbuna crucian carp

Introduction

Cell-mediated cytotoxicity (CMC) is an important defense mechanism in the control of virus-infected cells. In mammals, natural killer (NK) cells lyse virus-infected cells in a non-MHC-restricted fashion and are thought to provide a first line of defense by slowing virus replication before antigen-specific responses are generated. Subsequently, following a period of clonal expansion, antigen-specific CD8-positive cytotoxic T-lymphocytes (CTL) attack virus-infected cells in an MHC-class-I-restricted fashion. Therefore, antigen-specific CTLs play an essential role to protect against recurrent infections (Oldstone, 1987; Doherty et al., 1992).

Fish are the most primitive vertebrates that possess an adaptive immune system with lymphocyte subsets (Miller et al., 1985) and can evoke a variety of specific immune functions (Nakanishi et al., 2002). The results of some studies indicate that CTLs are present in fish and that they have functional similarities with those of higher vertebrates (Stuge et al., 2000; Zhou et al., 2001; Nakanishi et al., 2002). We have previously demonstrated that specific CMC against virus-infected cells could be induced by in vivo immunization using clonal ginbuna crucian carp and syngeneic cell lines, which demonstrates that the virus-specific cytotoxic cells may play an important role in protecting fish against viral infection (Somamoto et al., 2000; Somamoto et al., 2002). However, as yet, there is no evidence that specific CMC of fish against viral-infected cells is executed by CTLs.

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T-cell-related genes, such as TCRα, β, γ, δ (Partula et al., 1995, 1996; Hordvik et al., 1996; Wilson et al., 1998; Litman et al., 1999; Wermenstam and Pilstrom, 2001; Nam et al., 2003; Criscitiello et al., 2004), CD8α, β (Hansen and Strassburger, 2000; Moore et al., 2005; Somamoto et al., 2005), CD4 (Suetake et al., 2004; Dijkstra et al., 2006) and CD3 (Park et al., 2001, 2005), have already been identified in several fish species. Since monoclonal antibodies to fish CD8 or TCR have so far not been developed, these sequences are useful tool for monitoring the CTL. Some studies have demonstrated that expression profiles of fish TCR and/or CD8 genes are correlated with antigen-specific cytotoxic activities (Stuge et al., 2000; Fischer et al., 2003). Fischer et al. (2003) reported that effector population, which exhibits alloantigen-specific CMC, strongly expresses TCRα and CD8α mRNA in rainbow trout. Stuge et al. (2000) also showed that TCRαβ genes were expressed in alloantigen-specific cytotoxic cell clones from channel catfish. These findings indicate that alloantigen-specific effector cells of fish are similar to those of CTL in higher vertebrates. However, no information is currently available concerning the functional aspects of TCR and CD8 expressing cells in viral-antigen-specific CMC due to lack of suitable assay for virus-specific CMC in fish.

We have previously established virus-specific CMC assays employing clonal ginbuna crucian carp and their syngeneic cell lines (Somamoto et al., 2000; Somamoto et al., 2002). Furthermore, TCRβ and CD8α genes have been identified in ginbuna crucian carp (Somamoto et al., 2005), demonstrating that expressions of these genes were enhanced after sensitization with allogeneic grafting. These genes may be useful for addressing the involvement of CTL during the cell-meditated immune responses in fish. The present study was aimed at examining the relationship between CD8α, TCRβ gene expression and CMC against virus-infected cells in ginbuna crucian carp.

Results

Gene expression pattern of each effector cell population and their cytotoxic activities

The compositions of each fraction were described as follows: A; leukocytes with a density of 1.08 g/ml consisted of 72–83% lymphocytes, 6–10% macrophages and 9–12% other cells (Fig. 1A), B; sIg-negative leukocytes consisted of 71–86% lymphocytes (Fig. 1B), C; sIg-positive cells consisted of 90–96% lymphocytes (Fig. 1C), D; Leukocytes with the density of 1.08–1.09 g/ml consisted of 88–93% neutrophils (Fig. 1D).

The expression of TCRβ, CD8α and IgM genes in each cell fractions is shown in Fig. 2A. The leukocytes with a density of 1.08 g/ml (Fraction A) and sIg-negative leukocytes (Fraction B) from CHNV-infected fish strongly expressed TCRβ and CD8α, while a low level of their expressions was detected in the sIg-

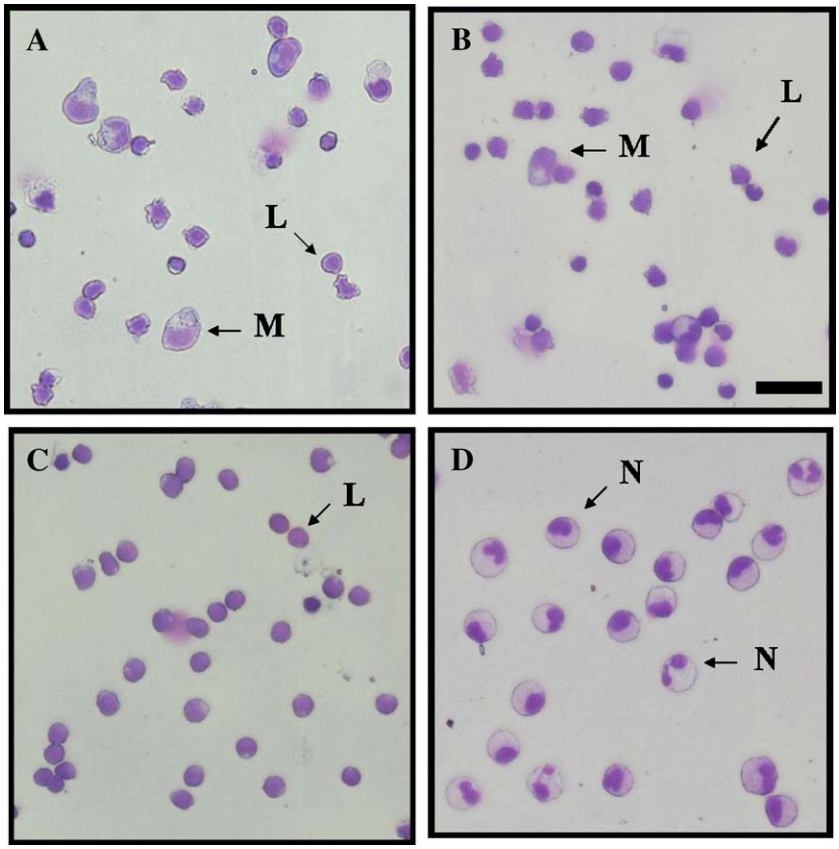

Fig. 1. Leukocytes fraction of head kidney cells from ginbuna. A, leukocytes with a density of 1.08 g/ml; B, sIg-negative leukocytes with density of 1.08 g/ml; C, sIg-positive cells; D, leukocytes with a density of 1.08–1.09 g/ml. L, lymphocytes; M, macrophages; N, neutrophils. Scale bar indicates 50 μm.

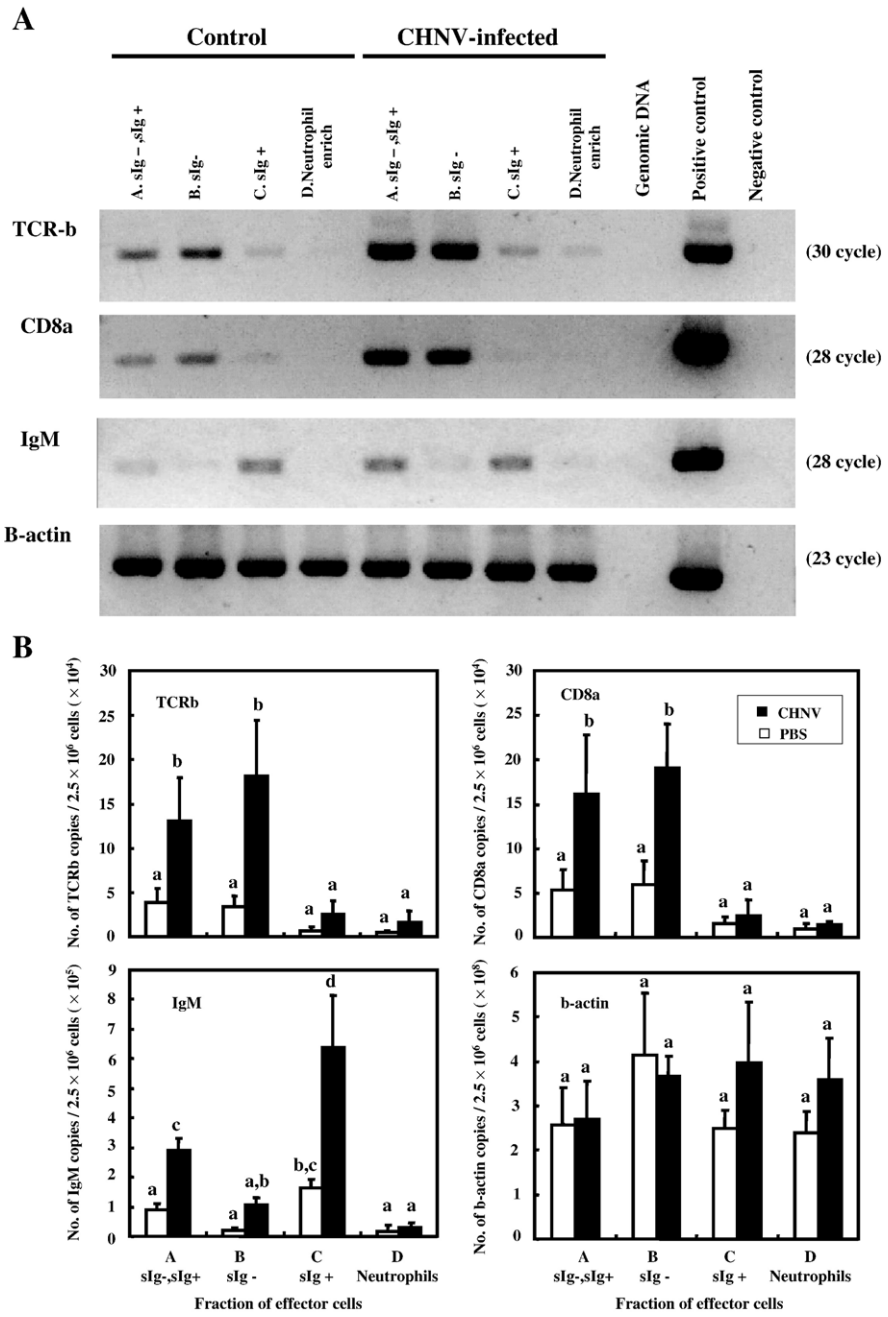

Fig. 2. (A) Expression of TCRβ, CD8α, IgM and β-actin genes in each cell fractions from virus-infected and uninfected fish. Leukocytes are sampled 8 days after secondary injection with CHNV or PBS. Data shown are representative of four fish analyzed. (B) The copy number of TCRβ, CD8α, IgM and β-actin mRNA in each cell fraction from CHNV-infected and uninfected fish. Results, which were obtained from four fish, are presented as mean (±standard deviation) copies of target gene per well after cocultivation with effector and target cells. Each sample was run in duplicate, together with known dilutions of respective plasmid cDNA ranging from 10^6 to 10^2 copies and appropriate non-template controls. In all quantitative real-time PCRs, melting curve analyses were performed and single specific melting peaks were observed, indicating amplification specificity (data not shown). Means with different letters are significantly different ($P < 0.05$).

positive cells (Fraction C) and neutrophil-rich fractions (Fraction D). The level of TCRβ and CD8α expression of leukocytes from uninfected fish was lower than that from CHNV-infected fish. The IgM gene was strongly expressed in the sIg-positive leukocytes (Fraction C) and unsorted leukocytes with a density of 1.08 g/ml (Fraction A). The amount of TCRβ, CD8α, IgM and β-actin mRNA from the effector and the target cells assessed by real-time PCR is shown in Fig. 2B. The copy numbers of TCRβ and CD8α mRNA in the fractions containing sIg-negative lymphocytes from CHNV-infected fish (Fractions A and B) were significantly higher than those in sIg-positive lymphocytes and neutrophil-rich fraction from both CHNV-infected and uninfected fish (Fractions C and D). In Fractions A and B, the copy numbers of TCRβ and CD8α mRNA in CHNV-infected fish were significantly higher than those in uninfected fish. In the both CHNV-infected and

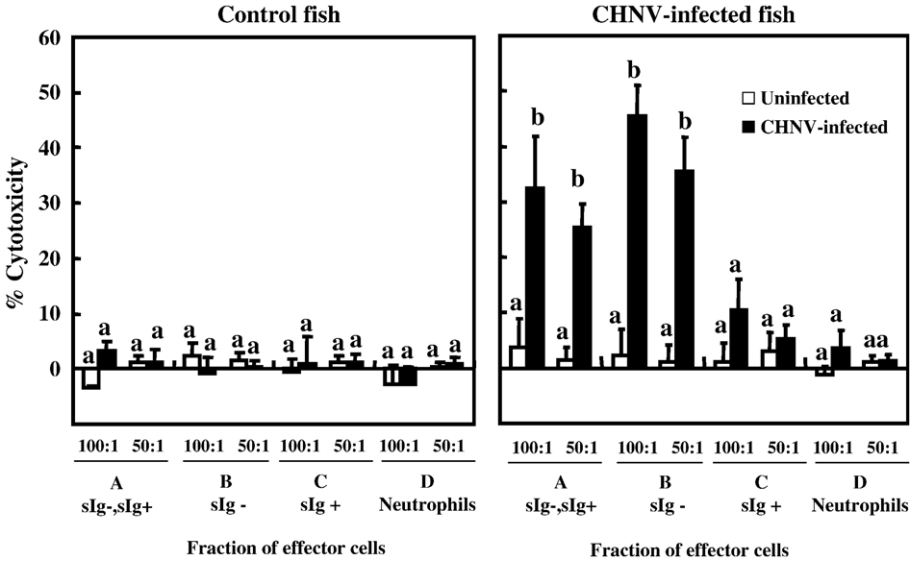

Fig. 3. Cell-mediated cytotoxicities of each cell fractions from uninfected or CHNV-infected fish against uninfected and CHNV-infected syngeneic cells. Effector cells are sampled 8 days after secondary injection with CHNV or PBS. The results are presented as the mean of 4 individuals and brackets show standard deviation. Cytotoxicities were measured at 100:1 of effector-to-target cell ratio. Means with different letters are significantly different ($P < 0.01$).

uninfected fish, the copy numbers of IgM mRNA in Fractions A and C were significantly higher than those in Fractions B and D.

The cytotoxic activities of each fraction against CHNV-infected and uninfected syngeneic cells are shown in Fig. 3. The high levels of cytotoxicity (32, 45%) were detected in the sIg-negative leukocytes and leukocytes with a density of 1.08 g/ml. The sIg-positive cell fraction (Fraction C) and neutrophil-rich fraction (Fraction D) did not exhibit significant cytotoxic activity. The cytotoxic activities of all cell fractions from uninfected fish did not show any cytotoxicities. The cytotoxicities at effector-to-target ratio (E:T ratio) of 50:1 were lower than that at E:T ratio of 100:1.

Expression analysis of effector cells after cocultivation with target cells

To further examine if the CD8 and TCR molecules are involved with the recognition of effector cells, the expression profiles of TCRβ, CD8α, IgM and β-actin genes after cocultivation with effector and target cells were investigated by real-time PCR. The copy numbers of TCRβ and CD8α mRNA in the effector cells were significantly increased after cocultivation with virus-infected target cells, but not with uninfected targets. In contrast, the expressions of IgM and β-actin genes were not enhanced after the incubation. Since TCRβ, CD8α and IgM mRNA were not detected in virus-infected and uninfected target cells (data not shown), it was concluded that TCRβ, CD8α and IgM mRNA were derived from the effector cells.

Discussion

We have previously established an assay system for induction of specific cell-mediated cytotoxicity (CMC) against virus-infected syngeneic targets, employing clonal ginbuna and their target cell lines (Somamoto et al., 2000, 2002). The cytotoxic activity was boosted during the secondary immunization, and the effector cells exhibited viral antigen specificity and killed virus-infected syngeneic targets but not uninfected syngeneic targets nor infected or uninfected allogeneic targets. Although the above activity was similar to mammalian virus-specific CTL activity, it remains unclear whether the effector cells are equivalent to CD8- and TCR-positive CTLs. The present study demonstrates that sIg-negative leukocytes with density of 1.08 g/ml from virus-infected fish, which consist of approximately 80% lymphocytes, exhibit the cytotoxicity against virus-infected syngeneic cells and strongly expressed TCRβ and CD8α mRNA. Thus, the present study suggests that fish possess virus-specific cytotoxic cells with phenotype and gene expression pattern similar to those of CTLs in mammals. In mammal, CTL recognize virus-infected targets through the interaction of their TCR and CD8 with MHC/epitope complexes. The present study showed that the expression of TCRβ and CD8α genes in effector cells from virus-infected fish was enhanced after cocultivation with virus-infected targets, suggesting that virus-specific cytotoxic cells may recognize the virus-infected targets through these molecules in fish. In order to identify the recognition mechanisms of fish CTL, future studies will require examining whether CD8 and TCR contact with MHC class I molecule on the virus-infected target cells.

It has been reported that alloantigen-specific cytotoxic cells of fish are TCR and CD8-positive (Stuge et al., 2000; Fischer et al., 2003). Fischer et al. (2003) demonstrated that an sIg-negative lymphocytes fraction exhibited alloantigen-specific CMC and strongly expressed TCRα and CD8α in rainbow trout. In channel catfish, the cloned cytotoxic cells, which exhibit alloantigen-specific cytotoxicity, express TCRαβ

(Stuge et al., 2000), through a cytotoxic mechanism involving perforin/granzyme (Zhou et al., 2001). These studies indicate that fish possess alloantigen-specific CTLs corresponding to mammalian ones. On the other hand, the study concerning virus-specific CD8 and TCR-positive CTL has not been reported, probably due to the lack of an assay system for virus-specific cell-mediated immune responses. Boudinot et al. (2001) reported that primary and secondary infection with viral hemorrhagic septicemia virus (VHSV) dramatically skewed the repertoire of trout TCRβ, and the profiles were reminiscent of the public and private virus-specific T-cell responses observed in mammals. This indicates that clonal expansion of T cells of fish is induced by stimulation with viral antigen. Our results obtained in the present study showed that effector cells from virus-infected fish expressed TCRβ mRNA stronger than that from uninfected fish and the number of TCRβ gene products in effector cells increased at 2–6 h after cocultivation with virus-infected targets. These results demonstrate that the regulation pattern of expressions of TCR and CD8 mRNA correspond well to the specific cytotoxic activities obtained in the previous and present studies (Somamoto et al., 2002).

Although viral infections are the most serious disease in many fish species (Wolf, 1988), the importance of T-cell response in fish during viral infections is still largely unknown because suitable assay systems for virus-specific CMC have been limited (Nakanishi et al., 2002). Researches on fish immune response to viruses have been focused on humoral or non-specific immune systems (Lorenzen and LaPatra, 1999; Lorenzen et al., 1999; Kim et al., 2000), whereas many studies suggest that fish utilize cell-mediated immunity in protecting from infectious disease. Our previous results suggest that virus-specific CMC provides an important role in antiviral defense system (Somamoto et al., 2002). In addition, the present study shows that gene expression pattern of virus-specific cytotoxic cells is similar to those of CTLs in mammals. Therefore, the analysis of virus-specific CTLs using ginbuna and their CTL markers will help to better understand the host defense to virus in fish.

Material and methods

Fish, cell line and virus

Clonal triploid ginbuna crucian carp (*Carassius auratus langsdorfii*), an isogeneic strain from Lake Suwa in Nagano prefecture (S3n), weighing 46–62g, were maintained in flowing 25 °C water at the National Research Institute of Aquaculture, Tamaki Branch. The fish were fed daily with commercial pellets.

A CFS cell line derived from the S3n strains of ginbuna crucian carp was established and maintained as described by Hasegawa et al. (1997). The origin of these cell lines was confirmed with DNA fingerprinting (Hasegawa et al., 1998).

Following as described in previous reports (Somamoto et al., 2002), crucian carp hematopoietic necrosis virus (CHNV) belonging to Rhabdoviridae was inoculated into the CFS cells growing in Eagle's minimal essential medium (MEM, Nissui) with 2% heat-inactivated fetal bovine serum (FBS, Commonwealth), as described by Somamoto et al. (2002).

Preparation of antigen-specific effector cells

The immunization protocol was as described in Somamoto et al. (2002). Briefly, S3n ginbuna crucian carp were infected with 10^6 $TCID_{50}$/ml of CHNV or PBS by i.p. injection. The second immunization was carried out 3 weeks later. The kidney was excised 8 days after the last inoculation. Kidney effectors were disaggregated by passing through a 150-gauge mesh stainless steel sieve in OPTI-MEM I (Gibco). The cells were washed with OPTI-MEM I, applied to a Percoll density gradient of 1.08 g/ml and 1.09 g/ml and centrifuged at 350 × g for 30 min at 4 °C. The cells at each of the two interfaces were collected and washed three times with OPTI-MEM I. The cell number from each fraction was adjusted to 1×10^7 cells/ml with OPTI-MEM supplemented with 10% heat-inactivated FBS (OPTI-MEM–10).

Cell suspensions with a density of 1.08 g/ml (1 ml) were incubated with 100 μl of mouse anti-ginbuna IgM monoclonal antibody (hybridoma supernatants) for 45 min on ice. The cells were washed twice with OPTI-MEM–10 and incubated on ice for 20 min with 1 ml of a 1:5 dilution of magnetic-beads-conjugated goat anti-mouse Ig antibody (Miltenyi Biotec GmbH, Bergisch Glabach, Germany) and then re-washed further three times. Surface Ig (sIg)-positive and negative cells were separated with a magnetic separation system (Mini Macs, Miltenyi Biotec) by applying the cell suspension to a plastic column equipped with an external magnet. The sIg-positive cells were retained in the column, while the sIg-negative cells were not. Both cell fractions were collected and viability confirmed to greater than 95% by the trypan blue dye exclusion method. The sIg-positive and negative cells were then resuspended in OPTI-MEM–10, and a sub-sample of the separated cells used for Giemsa-stained smear preparations to determine (from a total of 1000 cells) the composition of each leukocyte population. Four cell fractions used in the present study were as follows: (A) leukocytes with a density of 1.08 g/ml, (B) sIg-negative leukocytes with a density of 1.08 g/ml, (C) sIg-positive cells with a density of 1.08 g/ml, (D) leukocytes with 1.08–1.09 g/ml.

Cytotoxic assays

Cytotoxicity was assayed by the method of Somamoto et al. (2000, 2002). Target cells (CFS cells) were seeded in 96-well, flat-bottom microtiter plates (Corning) at 10^4 cells/well and allowed to settle down in the wells for 6 h. Then, 20 μl of MEM-10 containing 2 μCi of $Na_2^{51}CrO_4$ (NEN) was added to each well, and the plates were incubated overnight at 25 °C. After washing three times with OPTI-MEM I, the cells were infected with a virus at 25 °C for 5–6 h (M.O.I. = 10) following by washing three times with OPTI-MEM. Effector cells (5×10^5 and 10^6 cells/well) were added to each well in a final volume of

Table 1
Primers used to amplify cDNA for CD8α, TCRβ, IgM and β-actin

Name	Sequence (5′ → 3′)
CD8α	
CD8α-F1	CTAGTATTGTAAAAGAACTGAATGTCTGTG
CD8α-F2	GGAGGTCTGACAGAAATTAGAGGAC
CD8α-R1	GATAGGCTTCGGGTCTGGTTTTTTACAGTT
TCRβ	
TCRβ-F1	GTAGAAATCCTGAAGCCCTCTGAAATCGAG
TCRβ-F2	GCCATCCTAGACAATGCCAC
TCRβ-R1	GTGCTGAATTCACATAGTTTTCTGGCTCA
IgM	
IgM-F1	CCAAACTGGATGTTGGCAGCGTC
IgM-R1	TGTCTCGTCACGTGTCCAGTGAGC
IgM-R2	TTCCAGCTCAGGCTCCAGTCTG
β-actin	
β-actin-F1	CACTGTGCCCATCTACGAG
β-actin-F2	GGCCAACAGGGAAAAGATGACACAGATCA
β-actin-R1	AGGAGGAGGAAGCAGCAGTGCCCAT
β-actin-R2	ACTGTGCCCATCTACGAGGGTT

200 μl. After incubation at 25 °C for 6 h, supernatants (200 μl) were harvested with an automated supernatant collection device (Skatron) and radioactivity was measured using COBRA II auto-gamma counting system (Packard Instruments). The effector cells from each individual were divided and tested against uninfected and virus-infected target cells. Radioactivity of the supernatants from target cells without effector cells and from target cells lysed with detergent (10% 7×, ICN Biomedicals) served as the spontaneous and maximum release controls, respectively. Percent cytotoxicity was calculated as follows:

$$\frac{(\text{Test release}) - (\text{Spontaneous release})}{(\text{Maximum release}) - (\text{Spontaneous release})} \times 100$$

Expression analysis of TCRβ and CD8α genes in leukocyte subpopulations

Total RNA was extracted from 2.5 × 10^6 cells of the leukocyte subpopulations using NucleoSpin RNA II (Machery-Nagel), according to the manufacturer's protocol. Total RNA was reverse-transcribed to cDNA using Transcription Reagents with random hexamer primers (Applied Biosystems, USA) according to the manufacturer's protocol. PCRs were carried out with the following specific primer sets: CD8α-F1 and-R1 for CD8α (AB186397), TCRβ-F1 and-R1 for TCRβ (AB186399), IgM-F1 and -R1 for constant region of immunoglobulin light chain (AB201791) (see Table 1). Additional PCRs were run using β-actin (β-actin-F1 and β-actin-R1) designed from crucian carp β-actin (AB020852) to ensure that equal quantities of the transcribed RNA were included in each PCR reaction. PCR was performed with a GeneAmp PCR system 9700 (Applied Biosytems, USA) in 20 μl reactions containing 1× PCR buffer (Takara Shuzou, Japan), 200 μM of dNTPs, 0.5 U *Taq* DNA polymerase (Takara Shuzou, Japan), 0.5 μM each of the primer pair and 2

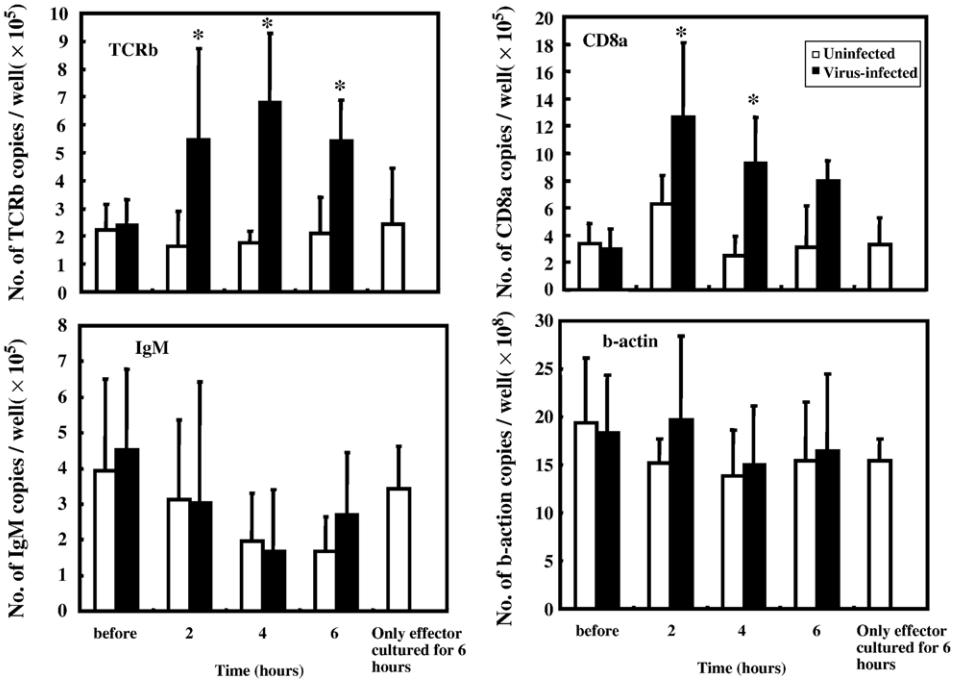

Fig. 4. Quantitative expression profiles of TCRβ, CD8α, IgM and β-actin after cocultivation with effector and target cells generated by real-time PCR. Leukocytes with a density of 1.08 g/ml from CHNV-immunized fish were used as effector cells. Results, which were obtained from four fish, are presented as mean (±standard deviation) copies of target gene per well after cocultivation with effector and target cells. Each sample was run in duplicate, together with known dilutions of respective plasmid cDNA ranging from 10^6 to 10^2 copies and appropriate non-template controls. In all quantitative real-time PCRs, melting curve analyses were performed and single specific melting peaks were observed, indicating amplification specificity (data not shown). Asterisks indicate that the copy number is significantly higher than that of the corresponding cocultivation with uninfected targets (*$P < 0.05$).

μl of cDNA template. The thermal cycling protocols for analysis of tissue distribution were as follows: for CD8α, 94 °C for 2 min, 30 cycles of 94 °C for 25 s, 56 °C for 30 s, 72 °C for 1 min, finally 72 °C for 2 min; for TCRβ, 94 °C for 2 min, 30 cycles of 94 °C for 25 s, 56 °C for 30 s, 72 °C for 1 min, finally 72 °C for 2 min; for β-actin, 94 °C for 2 min, 23 cycles of 94 °C for 25 s, 58 °C for 30 s, 72 °C for 1 min.

To quantify the copy number of each mRNA, real-time PCR was performed with a Quantitect SYBR Green PCR kit (QIAGEN) as previously described (Somamoto et al., 2005). The reverse-transcribed cDNA sample (TCRβ, CD8α, IgM; 2 μl or β-actin; 0.1 μl) was used in 20 μl PCR reactions. The following primers were used for real-time PCR: CD8α: CD8α-F2 and-R1; TCRβ: TCRβ-F2 and-R1; IgM: IgM-F1 and R2; β-actin: β-actin-F2 and β-actin-R2 (see Table 1). Genomic DNA was not amplified since the sequence of either the forward or the reverse primer in each pair spans the putative intron–exon boundary. Amplification was carried out as follows: 15 min at 95 °C, 45 cycles of 15 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C. Thermal cycling and fluorescence detection were conducted using the GeneAmp 5700 sequence detection system. All samples were run in duplicate. A standard curve was generated by plotting the threshold cycle (ct) versus known copy number for each plasmid template in the dilutions. Statistical significance was analyzed by a one-way ANOVA and Tukey.

Expression analysis of effector cells after cocultivation with target cells

We investigated whether TCRβ and CD8α gene expressions of effector cells were enhanced after conjugation with virus-infected cells, by real-time PCR. Effector cells with a density of 1.08 g/ml from CHNV-immunized fish were prepared as described above. Target cells (CFS cells) were seeded in 48-well, flat-bottom microtiter plates (Corning) at 5×10^4 cells/well and allowed to settle down in the wells for 6 h. After washing three times with OPTI-MEM I, the cells were infected with a virus at 25 °C for 5–6 h (M.O.I. = 10). Then, the cells were washed three times with OPTI-MEM. Effector cells (5×10^6 cells/well) were added to each well in a final volume of 500 μl. Total RNA was extracted from each well on the time indicated in Fig. 4 and cDNAs were synthesized following the above method. The real-time PCR was performed as described above. Statistical significance was analyzed by a paired Student's *t* test.

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