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Cell-mediated cytotoxicity in rainbow trout, *Oncorhynchus mykiss*, infected with viral haemorrhagic septicaemia virus

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Abstract

Mammalian cytotoxic T cells as part of the adaptive immune system recognize virus-infected target cells by binding of their T-cell receptors (TCR) to classical MHC class I molecules loaded with viral peptides. Our previous studies have shown that the allele of the single dominant polymorphic classical MHC class I locus Onmy-UBA is identical in the rainbow trout clone C25 and in the permanent rainbow trout cell line RTG-2. This enabled us to develop an assay to measure antiviral cytotoxicity in rainbow trout using a system of MHC class I-matched effector and target cells. Peripheral blood leucocytes (PBL) isolated from low dose viral haemorrhagic septicaemia virus (VHSV)-infected rainbow trout killed MHC class I-matched and later also xenogeneic MHC class I-mismatched VHSV-infected cells. When compared to PBL from uninfected control fish PBL from infected fish showed a higher transcriptional level of the CD8α gene which is a typical marker for mammalian cytotoxic T cells. Concurrently, the expression of the natural killer cell enhancement factor (NKEF)-like gene was enhanced as measured by real-time RT–PCR. Taken together, these results suggest that both innate and adaptive cell-mediated immune responses represented by NK and cytotoxic T cells, respectively, are triggered after VHSV infection. PBL that were able to kill VHSV-infected MHC class I-mismatched xenogeneic cells were generated later during infection than PBL capable of lysing VHSV-infected MHC class I-matched targets. This is contradictory to the generally accepted rule that innate immune mechanisms represent the first line of defence after viral infections.

Keywords: Virus infection; Cell-mediated cytotoxicity; Rainbow trout; Teleost

1. Introduction

Cell-mediated cytotoxicity (CMC) represents one of the major protective immune defence mechanisms against viral diseases in mammals [1,2]. Virus-infected cells are recognized by cells either belonging to the innate or to the adaptive immune system. These immune responses are also referred to as non-specific and specific, respectively.

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In mammals, innate CMC reactions are mainly executed by natural killer (NK) cells. NK-like cells that spontaneously kill allogeneic, xenogeneic and virus-infected target cells have been demonstrated in several fish species [3,4].

Alloreactive NK-like cells have been extensively characterized in channel catfish [5]. In this species a number of different PBL-derived alloreactive cytotoxic cell clones were established. Some of these clones were identified as unspecific, NK-like cytotoxic cells, while others showed allospecificity and expressed TCR genes [5,6]. In rainbow trout [7], common carp [8] and channel catfish [9] the so-called NK cell enhancement factor (NKEF) like gene, which is involved in NK-cell regulation in mammals, has been sequenced. Thus, NKEF is the only marker known for rainbow trout that can be used to get information on NK cell activation. A second type of NK-like cells was described in channel catfish. These organ-based non-specific cytotoxic cells (NCCs) express the so-called NCC receptor protein 1 (NCCRP-1) [10] which is absent in other NK-like cells of the same species [11]. There are only a few functional studies on killing of virus-infected target cells by NK-like effector cells in teleosts, e.g. in channel catfish [12] and rainbow trout [13].

Allospecific cytotoxic cells have been found in ginbuna carp [14,15] and rainbow trout [16]. In rainbow trout, alloreactive T cells were negatively enriched by immunomagnetic cell-sorting using population-specific monoclonal antibodies (mAbs) against B cells, monocytes, granulocytes and thrombocytes. Those $sIgM^-$ lymphocytes, in contrast to the other subpopulations, expressed high levels of TCR and CD8α, and specifically killed allogeneic cells [16]. In mammals, the main cellular immune response against virus-infected cells has been shown to be an adaptive response performed by CD8 positive T cells. CD8 positive cells kill cells on which the MHC class I molecules offer virus-derived peptides [17–19]. Recognition of the MHC/peptide complex is mediated by T-cell receptors (TCR) [17,20]. A similar process has also been described for birds [21,22], but for fish no MHC class I-restricted cytotoxicity has been reported yet.

However, many observations indicate that this mechanism of cell killing also exists in fish. The finding of trout and other fish sequence homologues for classical MHC class I [4,23], TCR and CD8 [4] suggests that MHC class I presentation in fish is similar to that in higher vertebrates. Also a number of genes involved in peptide loading of MHC class I molecules such as β2m, low molecular mass protein (LMP) and transporter associated with processing (TAP) [4] have been detected in fish. Moreover, as shown by a monoclonal antibody directed against the recombinant Onmy-UBA*501 protein, rainbow trout classical MHC class I molecules are expressed in similar cell types as mammalian classical MHC class I molecules [24]. All these molecular data strongly suggest a similar function of MHC class I in fish and higher vertebrates.

Besides molecular analysis also functional studies point at similarities regarding the role of MHC class I in fish and mammals. Allogeneity in mammals could largely be attributed to MHC class I function [25]. Allospecific cytotoxic cells were found in ginbunas, rainbow trout and channel catfish [4] and more strikingly, allogeneity between shark siblings appeared to be co-segregating with MHC class I alleles [26]. Further, specific CMC was shown against virus-infected autologous [12] and syngeneic [27] cells, but these studies lacked a concomitant characterization of the MHC class I sequences.

The studies mentioned above indicate an involvement of MHC class I during cell-mediated cytotoxicity in fish. The difficulty with the establishment of a cytotoxicity assay where antigen-specific cytotoxic cells play a role lies in the polymorphism of MHC class I genes. Cytotoxic T lymphocytes (CTLs) kill virus infected target cells only if their TCR matches with the MHC class I of the targets and if the peptide presented by MHC class I originates from the virus by which the CTLs had been originally stimulated [28]. The lack of MHC class I information and/or availability of effector cell donor fish and target cells with identical classical MHC class I alleles have prevented the development of in vitro CMC assays with MHC class I-matched effector and target cells in rainbow trout so far. Recently, though, we discovered that the classical MHC class I sequence *Onmy-UBA**501 (GenBank accession number AF287488) is shared by the clonal rainbow trout strain C25 and the commonly used rainbow trout cell line RTG-2 [24].

Combining these clonal fish and the RTG-2 cell line a cytotoxicity assay with MHC class I-matched effector and target cells has been established. This allowed us to demonstrate that PBL isolated from rainbow trout during early viral haemorrhagic septicaemia virus (VHSV) infection kill only virus infected MHC class I-matched target cells but not virus infected xenogeneic MHC class I-mismatched targets. Due to the lack of anti-CD8 mAbs in any fish species, real time RT–PCRs were conducted in the present work to get information on how this CTL marker is regulated during VHS at the transcriptional level. Accordingly, CD8α expression levels in PBL from infected trout were higher than in PBL from non-infected fish indicating an activation of CTLs during VHS. Although NKEF expression was elevated in PBL from infected fish early in the infection, killing of virus infected MHC class I-mismatched xenogeneic target cells occurred only after a secondary virus injection suggesting a late antiviral NK cell response.

2. Materials and methods

2.1. Animals

Homozygous isogeneic rainbow trout clone C25 was derived from Nagano Prefectural Experimental Station of Fisheries, Akashina, Nagano, Japan. The clone was produced by gynogenesis over two generations by suppression of mitosis and meiosis in the first and second generations, respectively. Clonality was confirmed by DNA fingerprinting (unpublished data). Fish were kept at 15 °C in aerated water tanks and fed commercial dry pellets. Fish at a body weight of about 250 g were used for the experiments.

2.2. Enzyme linked immunosorbent assay (ELISA)

For evaluation of the humoral immune response an antibody ELISA was applied. VHSV, purified by saccharose gradient ultra centrifugation, at a final concentration of 3 μg/ml in bicarbonate buffer (pH 8.6), was used to coat micro titre plates (Polysorb Nunc F) with 100 μl/well at 4 °C overnight. Plates were washed with 200 μl/well PBS containing 0.05% Tween 20 (PBS-T) and blocked with 200 μl/well Roti-Block (Roth, Germany) reagent (10%) for 1 h at room temperature. After washing, serial dilutions of fish sera were added to the wells starting with 1:50 dilutions. Plates were incubated for 1 h, washed and incubated for another hour at room temperature with 100 μl of an anti-rainbow trout IgM mAb (hybridoma cell culture supernatant of mAb 4C10, 1:200 in PBS-T, [29]). Plates were then washed and incubated for 1 h at room temperature with 100 μl of an anti-mouse-Ig-POD conjugate (Sigma, Germany). Plates were washed again and 100 μl of the substrate solution (0.4 mg/ml *o*-phenylenediamine from Sigma; 1 μg/ml 30% H_2O_2, in phosphate citrate buffer, pH 5.0) was added to the wells. Enzyme activity was stopped after 10–20 min with 4 N H_2SO_4 and the absorbance was recorded at 492 nm using an ELISA reader.

2.3. Plaque neutralization test (PNT)

A representative panel of sera was selected for testing in a virus neutralization assay. The 50% plaque neutralization test (PNT) [30] was used as described earlier [31]. In short, a fixed dilution of VHSV (1×10^7 PFU/ml) was mixed with different dilutions of fish sera (heat-treated for 30 min at 45 °C, to inactivate internal complement activity) and incubated for 30 min at 15 °C, before addition of complement in the form of normal trout serum. Following incubation at 15 °C, the mixtures were inoculated onto 96-well EPC cell culture plates. After incubation for 4 days at 15 °C in semi-fluid medium, the cell cultures were fixed in 10% formalin and stained with crystal-violet. The titre of a specific serum was determined as the reciprocal of the serum dilution giving an approx 50% reduction in the number of plaques compared to a normal trout serum control.

2.4. Cells

RTG-2 (rainbow trout gonad [32]; Bank for Cell Lines in Veterinary Medicine, Insel Riems, Germany) and EPC (epithelioma papulosum cyprini [33]; Bank for Cell Lines in Veterinary Medicine, Insel Riems, Germany) cells were grown in mixed medium (MM): Iscove's DMEM/Ham's F12 (Gibco) at a ratio of 1:1, supplemented with 10% foetal bovine serum (FBS) at 20 °C.

2.5. Virus

Viral haemorrhagic septicaemia virus (VHSV) strain FI 13 [34] was propagated in EPC cells at 15 °C. Cells were grown in Eagle's MEM containing 10% fetal bovine serum (FBS). Virus titres based on cytopathic effect (CPE) were determined separately for RTG-2 and EPC cells. The corresponding titres given as tissue culture infective dose ($TCID_{50}$), however, did not necessarily correspond to the number of primarily infected cells before first CPE occurs. Thus, for CMC assays the amount of virus resulting in at least 90% infection of target cells had to be determined by an immunofluorescence test using VHSV-specific mAbs (see below).

2.6. Immunofluorescence test with RTG-2 and EPC cells for the detection of VHSV and MHC class I protein

For the detection of VHSV protein RTG-2 and EPC cells (2×10^4 cells per well) were grown on 96-well cell culture plates (Greiner, Germany) for 10 h and incubated at 15 °C for another 16 h with different amounts of VHSV. Infected cells were then fixed with pre-cooled acetone/methanol (1:1) for 5 min at 4 °C and washed twice with PBS. Fixed cells were incubated with a mAb directed against the G protein of VHSV (Bio 282, BioX, Belgium) for 20 min at 4 °C, washed twice and labelled with an Alexa™ 488 goat-anti-mouse-Ig conjugate (Molecular Probes) for another 20 min at 4 °C. After washing, 50 μl of an anti-bleaching buffer containing DABCO (Sigma) and propidium iodide (2 μg/ml) as a nucleic acid contrast stain were added to each well. Negative control cell cultures were mock infected with cell culture medium and otherwise treated similarly.

For the detection of MHC class I-protein uninfected RTG-2 and EPC cells were mixed and seeded onto cell culture plates. An immunofluorescence test was performed as described above using the anti-Onmy-UBA*501 mAb H9 [24] as a primary antibody.

2.7. Quantitative real time reverse transcription–polymerase chain reaction (RT–PCR)

RNA was isolated from trout PBL using the NucleoSpin RNA II total RNA extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions with few modifications. For later normalization of RNA extraction and RT–PCR a recombinant RNA encoding the enhanced green fluorescent protein (EGFP) was added to the samples after the lysis and denaturation step in a quantity proportional to PBL numbers. Total RNA (cellular RNA and RNA encoding EGFP) was then eluted into a volume of DEPC-treated water (at least 60 μl) that was proportional to the cell numbers used. Ideally, the RNA samples should contain equal concentrations of mRNA encoding EGFP and the following real time RT–PCR should thus yield equal amounts of DNA encoding EGFP.

For quantitative real time RT–PCR analysis of CD8α mRNA expression the following primers/probe combination was used. Forward (CD8-s): 5′-CATCCTGTGAGTTGATTGTTTGGG-3′; reverse (CD8-as): 5′- CCTATAGCCAA CAACAGACT-3′; probe (CD8-s-probe): FAM-5′-ACTGCTGGCTGTGGCTTCCTCTTCC-3′-TAMRA. For real time RT–PCR detection of NKEF mRNA the primers/probe forward (NKEF-s): 5′-AGTAGAGGCAAAGTGAA GATGGC-3′, reverse (NKEF-as): 5′-AGGTGAAGTCCAGCGGGTAG-3′ and probe (NKEF-s-probe) FAM-5′-AAAGCACGCATCGGGCATCTGGC-3′-TAMRA were applied.

For recalculation of CD8α- and NKEF-mRNA extraction efficiencies, a real time RT–PCR with EGFP-specific primers/probe was performed as internal reference for all PBL samples as previously described [35,36]. The one tube RT–PCR reaction mixture contained the forward primer EGFP1-F: 5′-GACCACTACCAGCAGAACAC-3′, the reverse primer EGFP2-R: 5′-GAACTCCAGCAGGACCATG-3′ and the probe EGFP1-HEX: HEX-5′-AGCACC CAGTCCGCCCTGAGCA-3′-BHQ1. The calculated results finally reflect the relative CD8α- and NKEF-mRNA expression levels in a relative number of PBL. In short, 12.5 μl RT–PCR reaction mixtures were prepared according to the 'One-step RT–PCR kit' (Qiagen, Germany) manual using equal amounts of extracted total RNA (usually 3 μl). To avoid evaporation during the PCR steps, the reaction mixtures were overlaid with 15 μl of mineral oil (Sigma). Protocols for all RT–PCR assays were: 60 °C for 30 min, 95 °C for 15 min, and then 42 cycles of (94 °C for 15 s, 57 °C for 15 s, 62 °C for 30 s). Real-time RT–PCR was performed and analysed using an Mx3000P cycler and software (Stratagene, USA). Threshold cycles (Cts) for CD8α and NKEF expression were normalized to EGFP expression as described before [35] and control RNAs from uninfected fish were regarded as 100% expression references.

2.8. Infection of rainbow trout with VHSV

Forty-four fish were anaesthetized in benzocain (10 mg/L water) and intraperitoneally (i.p.) infected with 10^4 $TCID_{50}$ in 100 μl MEM. An anal application of 10^3 $TCID_{50}$ was given in parallel since anal delivery of antigens was found to trigger cell-mediated immune responses in fish [37]. At 3, 4, 10, 11 and 16 days post infection (d p.i.), four fish were anaesthetized to collect blood and tissue samples. The remaining 20 fish were reinfected 22 d p.i. and samples were taken at the same time points and from the same number of fish as after the primary infection. A third infection was applied to the remaining four fish 98 days after the second infection. Untreated fish served as controls.

2.9. Preparation of effector cells

Fish were anaesthetized and blood was collected by puncture of the caudal vein into a syringe previously rinsed with heparin (Sigma) at 1000 U/ml in PBS. Blood was immediately diluted into a five-fold volume of cold MM supplemented with insulin, transferrin, sodium selenite media supplement (Sigma, 1 vial/5 L medium) and 10% FBS. Prediluted blood was layered onto Percoll (Biochrom, Germany) adjusted to a density of 1.075 g/cm^3 with serum-free MM. After centrifugation at 650 × *g* at 4 °C for 40 min, cells at the interphase were collected and washed twice with MM (200 × *g*, 4 °C, 10 min) then stained with trypan blue (Sigma) and counted using a Thoma haemocytometer. PBL were discriminated by phase contrast microscopy into live lymphocytes, monocytes, granulocytes, thrombocytes, erythrocytes and dead cells. For cytotoxicity assays only the number of viable lymphocyte like cells were taken into account when calculating effector to target cell (E:T) ratios.

2.10. Flow cytometry

Unfixed PBL were incubated with mAbs specific for rainbow trout thrombocytes (mAb 42-1 [38]), B cells (anti-IgM mAb 4C10; [29]) and granulocytes/monocytes (mAb Q4E [39]), and labelled with FITC-conjugated anti-mouse antibodies. Positive and negative cells were counted using a FACSCalibur (Becton Dickinson) flow cytometer and percentages of positive cells were calculated from dot plots for forward scatter and green fluorescence.

2.11. Preparation of target cells

RTG-2 or EPC cells (2 × 10^4/well) were seeded into 96-well flat bottom cell culture plates (Greiner, Germany). This cell number was found to be necessary to yield an LDH release corresponding to an extinction of at least 1.2 in the ELISA reader after total cell lysis and substrate reaction (see below). Cells were propagated for 11 h at 20 °C and VHSV strain Fi13 was added to the target cells at an MOI (multiplicity of infection) that had been previously determined by immunofluorescence to yield an initial infection of at least 90% of the target cells. Control wells were filled with the same amount of virus-free medium. Following inoculation with virus/medium, the target cells were cultured for another 12 h at 15 °C before effector cells were added.

2.12. Lactate dehydrogenase (LDH)-based cytotoxicity assay

The LDH release from killed target cells was compared to the maximum amount of LDH obtained from the same number of artificially lysed target cells. A 96-well plate comprised wells with different control reactions that assayed (1) target cell spontaneous release (TSR; infected or uninfected target cells + MM), (2) target cell maximum release (TMR; infected or uninfected target cells + MM + Triton X-100, Sigma), (3) effector cell spontaneous release (ESR; PBL + MM), (4) medium background (Me; MM only) and (5) a volume correction control (VCC; MM + Triton X-100). The experimental (EXP) wells contained a constant number of infected or uninfected target cells and a varying number of effector cells at different E:T ratios. Highest possible E:T ratios used depended on the number of effector cells sampled per fish and ranged between 40 and 120. In each experiment, triplicate wells with a final volume of 200 μl each were analysed. After adding MM, target and effector cells, plates were centrifuged for 5 min (200 × *g*, 4 °C) and incubated for 4 h at 20 °C. Triton X-100 was added to the TMR and VCC wells 45 min before harvesting 50 μl of supernatant from each well and transferring it into wells of a 96 well flat bottom microtitre plate (Greiner, Germany).

The amount of LDH released from cells was determined using a commercially available Cytotoxicity Detection Kit LDH (Roche, Germany). The test was carried out according to the manufacturer's instructions and plates containing the supernatants from the test wells were read in an ELISA reader at 490 nm. To prevent virus-induced release of LDH from infected target cells incubation with virus should not exceed 16 h including the CMC reaction.

Experiments consisting of combinations of different effector cells (PBL from infected or untreated rainbow trout) and target cells (VHSV-infected and uninfected RTG-2 or EPC cells) were performed in triplicate.

The percentage of specific cytotoxicity was calculated using the following formula.

$$\%\ \text{SPECIFIC CYTOTOXICITY} = \frac{(\text{EXP} - \text{ESR}) - \text{TSR}}{(\text{TMR} - \text{VCC}) - \text{TSR}} \times 100\%$$

2.13. Statistics

Results of all tests were statistically compared by an f-test followed by a two-tailed Student's *t*-test using MS Excel (Microsoft Corp., USA) and were considered to be significantly different at $p \leq 0.05$. Correlation coefficients were calculated by MS Excel 5.0.

3. Results

3.1. Clinical signs after VHSV injection

After primary infection with VHSV fish exhibited varying degrees of pathological signs typical for VHS: petechial bleedings in the skin, peritoneum and skeletal muscles; spleen swelling and pale liver. From the 20 fish that were reinfected 22 days after primary infection only three fish showed clinical signs of VHS. No mortality was recorded during the experiments.

3.2. Antibody response

Rainbow trout sera were tested for the presence of VHSV-specific antibodies using ELISA. Rising VHSV-specific antibody titres were observed in sera from fish that were sampled after day 11 p.i. After a secondary infection 22 d p.i. antibody titres increased until day 11 post secondary infection (d p.2.i) but decreased at day 16 p.2.i. After a tertiary infection (98 days after secondary infection) antibody titres to VHSV increased again (Table 1).

Testing of a representative panel of sera in PNT revealed the first occurrence of antibody-positive fish around day 11 p.i., while all fish tested from 16 p.i. and onwards were positive (Table 2). Particularly after the re-infection, all fish had high titres of virus-neutralizing activity in their serum.

3.3. Expression of CD8α and NKEF after VHSV infection

At day 10 p.i. with VHSV the level of CD8α mRNA in PBL increased more than 7-fold compared to PBL from fish that had received medium instead of virus (Fig. 1). A similar increase in NKEF mRNA levels was found already at day 3 p.i. increasing to 25 times higher levels than in PBL of uninfected control fish at day 10 (Fig. 2).

CD8α mRNA expression levels still increased after reinfection with highest levels on day 4 and gradually decreased until day 16 p.2.i., although levels of CD8α were still significantly higher (more than 8 times) than in the control (Fig. 1). NKEF mRNA levels were lower after secondary VHSV infection when compared to the levels after primary infection but still significantly higher than control levels on days 4 and 11 p.2.i. The NKEF mRNA expression reached almost control levels on day 16 p.2.i. (Fig. 2).

3.4. Total leucocyte capacity

For the cytotoxicity assays as much blood as possible was drawn from each experimental fish to maximize the E:T ratios. The total number of PBL from each fish served as a measure for the circulating leucocyte capacity. After primary infection with VHSV a leucopenia was observed. This leucopenia was characterized by a significant ($p < 0{,}001$) drop

Table 1
Antibody response after VHSV infection

Antibody titres	3 d p.i.	4 d p.i.	10 d p.i.	11 d p.i.	16 d p.i.	3 d p2.i.	4 d p2.i.	10 d p2.i.	11 d p2.i.	16 d p2.i.	36 d p3.i.
3200									**2**		
1600						**1**	**1**	**2**	**2**		
800						**1**		**2**			**3**
400				**1**	**2**	**2**	**3**			**1**	**1**
200	**1**	**2**	**1**		**1**					**2**	**2**
<200	3	2	3	3	1					1	

Reciprocal ELISA titres were calculated as mean values from at least four sera per sampling day. Numbers in **bold** depict fish with positive antibody titres. Values less than 200 were regarded as negative.

Table 2
Antibody response after VHSV infection

Days p.i.	Titres	No. of PNT-positive sera
4 d p.i.	≤40	0 of 4
11 d p.i.	<160	0 of 4
16 d p.i.	≥1280	4 of 4
4 d p2.i.	≥2560	4 of 4
11 d p2.i.	>20480	4 of 4
16 d p2.i.	>20480	4 of 4
36 d p2.i.	>20480	4 of 4
Pos. control	10240	Reference pool of 10 sera
Neg. control	<160	1 reference serum

PNT titres are given as reciprocals of the serum dilution yielding an approx 50% reduction in the number of plaques compared to a normal trout serum control. Values less than 160 were regarded as negative.

of the total PBL capacity on days 10 and 11 p.i. to less than 50% of the total PBL capacity determined for uninfected control fish. When fish were infected a second time with VHSV 22 days after the primary infection their PBL pool had been replenished to almost normal levels and remained at those levels until the end of sampling 16 d p.2.i. (Fig. 3).

3.5. Ratio of PBL subpopulations after VHSV infection

PBL applied in CMC assays were characterized in terms of subtype proportions by flow cytometry using leucocyte subpopulation-specific mAbs against rainbow trout thrombocytes, B cells and granulocytes/monocytes (Fig. 4). After primary infection with VHSV the percentages of thrombocytes did not significantly change, but were increased after secondary and tertiary infection. The percentages of B cells and neutrophils/monocytes were significantly decreased 16 days after primary infection. Considering the absolute numbers of PBL isolated per fish (Fig. 3) the absolute numbers for most subpopulations decreased after primary infection but more dramatically for neutrophils/monocytes (absolute and relative decrease). On the other hand there was a relative increase of cells that could not be labelled by any of the mAbs. The relative numbers of these cells representing the Ig negative lymphocytes increased from 8.6% (100%−42.6% thrombocytes−13.8% B cells−25% monocytes/granulocytes) to 33.4% (100%−49.5% thrombocytes−8.7% B cells−8.4% monocytes/granulocytes) on day 16 after primary infection. This fourfold increase of Ig negative lymphocytes together with a 50% decrease of the absolute PBL capacity means an approx. twofold increase of the Ig negative lymphocyte capacity in infected fish.

3.6. Testing of target cells on the expression of Onmy-UBA*501 protein

To confirm the presence or absence of the MHC class I protein Onmy-UBA*501 in RTG-2 and EPC target cells, respectively, an immunofluorescence test using mAb H9 was applied (Fig. 5). This mAb was produced against the

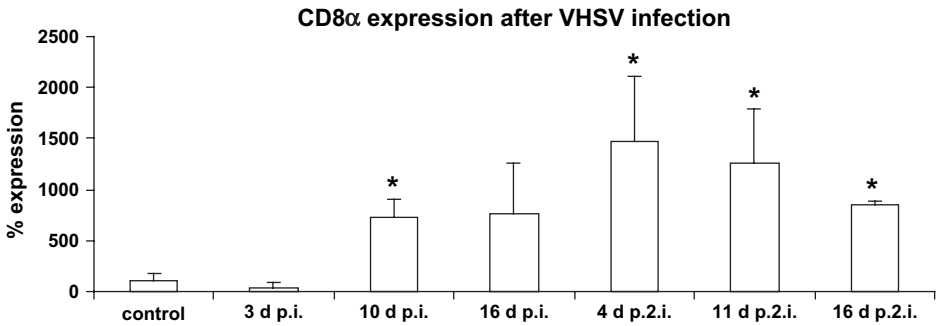

Fig. 1. Expression of mRNA encoding CD8α in PBL after VHSV infection. Some fish received a second VHSV infection 22 d after primary infection (d p.i. = days post primary infection; d p.2.i. = days post secondary infection). Expression values were calculated after quantitative real time RT−PCR ($n = 4$). Asterisks depict significant differences ($p \leq 0.05$) from control PBL of fish injected with cell culture medium (mock infection).

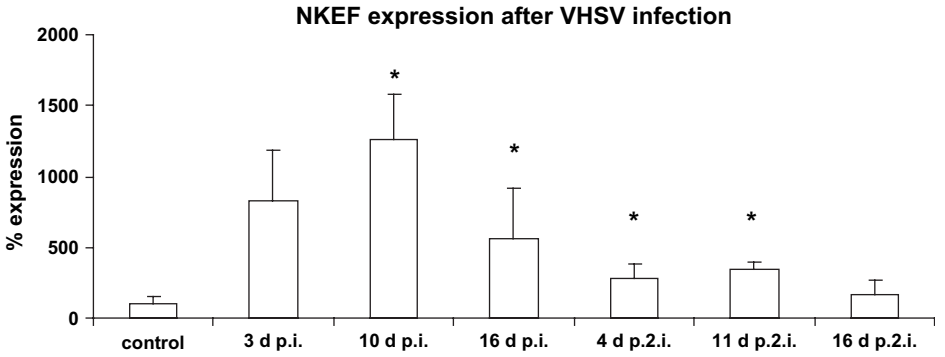

Fig. 2. Expression of mRNA encoding NKEF in PBL after VHSV infection. Some fish received a second VHSV infection 22 d after primary infection (d p.i. = days post primary infection; d p.2.i. = days post secondary infection). Expression values were calculated after quantitative real time RT–PCR ($n = 4$). Asterisks depict significant differences ($p \leq 0.05$) from control PBL of fish injected with cell culture medium (mock infection).

recombinant Onmy-UBA*501 protein, and the locus *Onmy-UBA**501 is shared by RTG-2 cells and homozygous C25 rainbow trout [24].

3.7. Cell-mediated cytotoxicity

Before setting up CMC assays, each virus batch was tested by immunofluorescence. Virus concentrations required to infect at least 90% of the target cells in the primary infection cycle corresponded to an MOI ranging from 5 to 10. Fig. 6 shows monolayers of RTG-2 and EPC cells with an infection rate of almost 100%.

3.8. CMC after single VHSV infection

CMC was measured in vitro by LDH release from target cells. Target cells used were either VHSV-infected or uninfected RTG-2 or EPC cells, and effector cells were PBL isolated from mock infected (PBS injection) or VHSV-infected C25 rainbow trout. It has been previously shown that the homozygous rainbow trout clone C25 and the RTG-2 cells share the MHC class I allele *Onmy-UBA**501 [24].

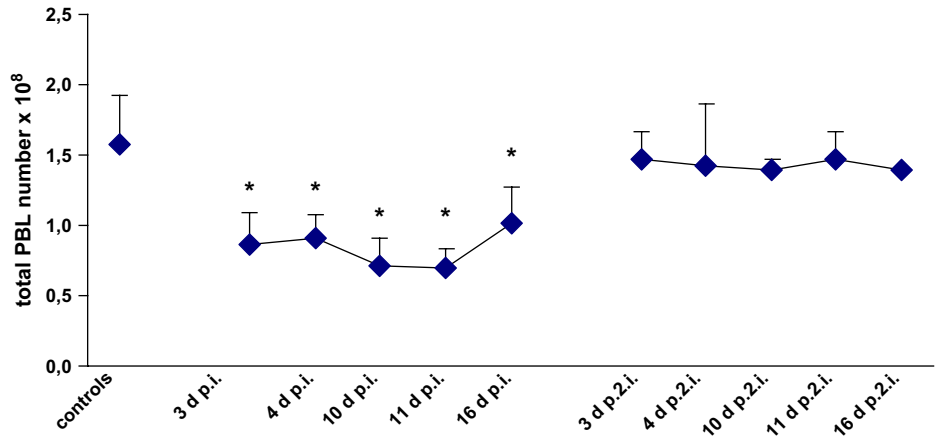

Fig. 3. Total number of PBL isolated from each experimental fish. Total amounts of separated PBL per fish are means of 4 fish sampled at 3, 4, 10, 11 or 16 days after primary/secondary infection, respectively. Asterisks depict significant differences ($p \leq 0.05$) from control PBL of fish injected with cell culture medium (mock infection).

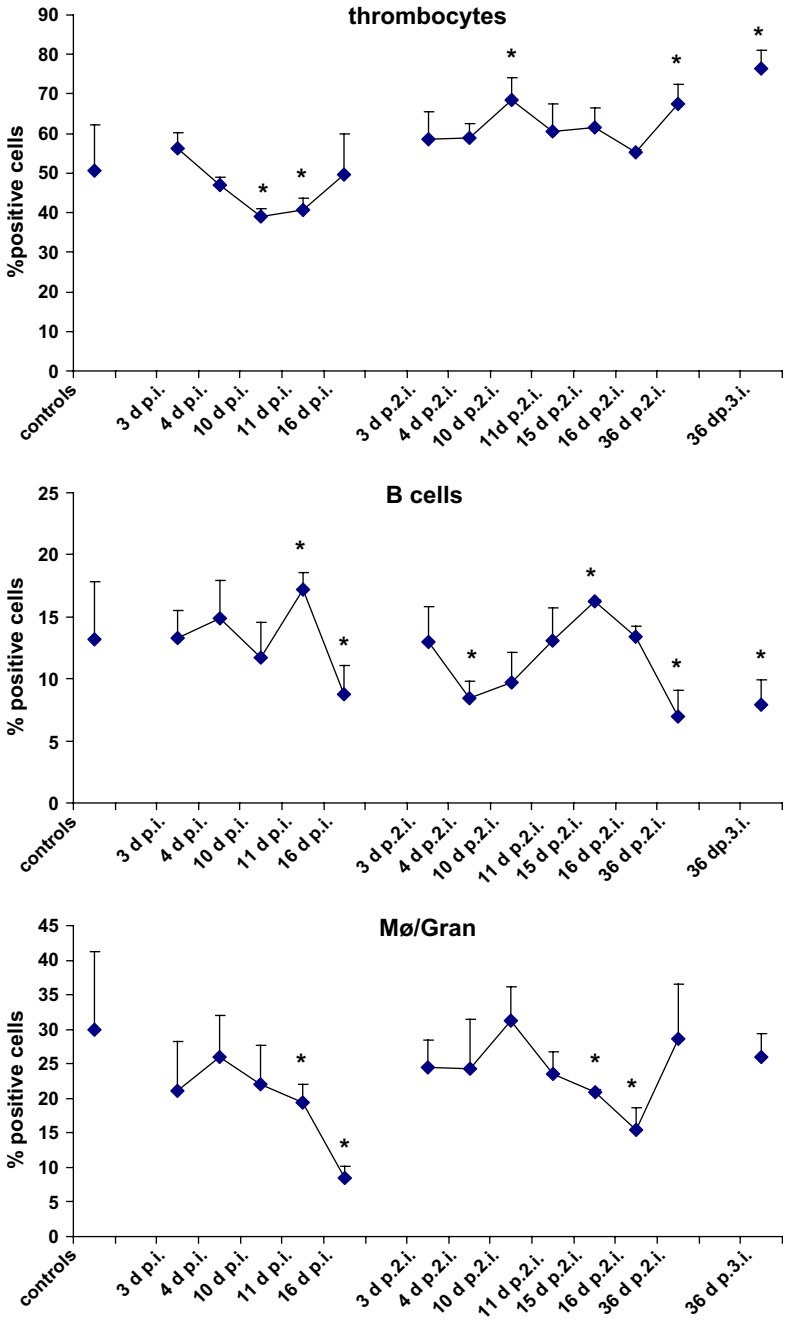

Fig. 4. Flow cytometric analysis of PBL after VHSV infection. Mock infected controls: $n = 30$; infected fish: $n \geq 4$ from 3 d p.i. to 11 d p.2.i. and on day 36 d p.3.i.; $n = 2$ for days 15 and 16 d p.i.). Asterisks depict significant differences ($p \leq 0.05$) from control PBL of fish injected with cell culture medium (mock infection) Mø/Gran = monocytes/granulocytes.

The cytotoxic activity of PBL isolated from uninfected control fish and VHSV-infected rainbow trout toward uninfected and VHSV-infected target cells was determined 3, 10, 11 and 16 days after infection (Fig. 7). From the 10th day after infection onwards a CMC reaction against infected RTG-2 cells bearing the same MHC class I as the donors of effector cells was detected; and the level of cytotoxicity was dose-dependent on the E:T ratio (data not shown). Highest possible E:T ratios for uninfected control fish were 100:1. Due to a lower yield of PBL from single-infected fish, E:T ratios of 50:1 were used to compare control levels with cytotoxicities measured on the different days after

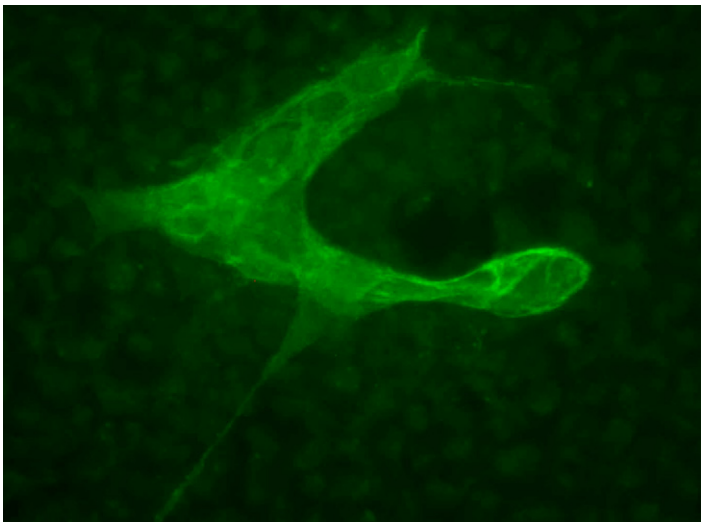

Fig. 5. Immunofluorescence staining of uninfected target cells for detection of the rainbow trout MHC class I protein Onmy-UBA*501. RTG-2 (light green) and EPC (dark green background) cells were mixed in equal numbers, incubated overnight and stained with an anti-Onmy-UBA*501 mAb and Alexa™ 488 (green) goat-anti-mouse-Ig conjugate. Only the fibroblastoid RTG-2 cells expressed the Onmy-UBA*501 protein.

infection. Cytotoxicity levels increased gradually until day 16 after infection while high individual differences with respect to the level of the CMC reaction were observed. Due to high individual variations, the response measured on day 16 p.i. was not significantly different from the control. After single VHSV infection a CMC reaction was only detected against VHSV-infected target cells, whereas infected MHC class I-mismatched EPC cells remained intact. Neither PBL from infected rainbow trout nor PBL from uninfected control fish killed uninfected RTG-2 cells or uninfected EPC cells (data not shown).

3.9. CMC after secondary VHSV infection

To ascertain if a CMC reaction can be boosted by a second viral administration, some fish were reinfected 22 days after the first infection. A CMC reaction of PBL from infected fish against infected RTG-2 cells was detected throughout the experiment until day 16 p.2.i. In contrast to the observations made after primary infection, the response was significantly different from the control at all measuring points. Furthermore, statistically relevant killing of VHSV-infected MHC class I-mismatched EPC target cells was measured after the secondary infection reaching CMC levels of about 15% on day 11 p.2.i. (Fig. 7).

The data on CMC against VHSV-infected cells are summarized in Table 3.

4. Discussion

Virus infections induce both CTL and NK cell responses in mammals [2,40] and birds [41]. In fish a number of studies confirmed these observations. In channel catfish two populations of naïve PBL-derived NK-like cytotoxic cells can be distinguished, one capable of spontaneously lysing allogeneic cells and the other capable of spontaneously killing virus-infected autologous but also allogeneic target cells [12]. In the same species another organ-derived NK-like cell population termed NCC is able to spontaneously kill traditional xenogeneic NK cell targets used in mammalian immunology [42]. In contrast, naïve leucocytes isolated from ginbuna crucian carp [14] and rainbow trout [16] are not able to spontaneously kill foreign (allogeneic) cells. Our experiments confirm these previous observations since neither infected nor uninfected foreign (xenogeneic EPC) cells were spontaneously lysed by C25 clonal rainbow trout PBL. Only sensitized PBL of VHSV-infected C25 trout but not naïve PBL from non-infected fish killed VHSV infected (MHC class I-matched) RTG-2 but also VHSV infected (MHC class I-mismatched) EPC cells. The latter observation suggests that killing of virus-infected target cells in our experiments was executed both by cells of the

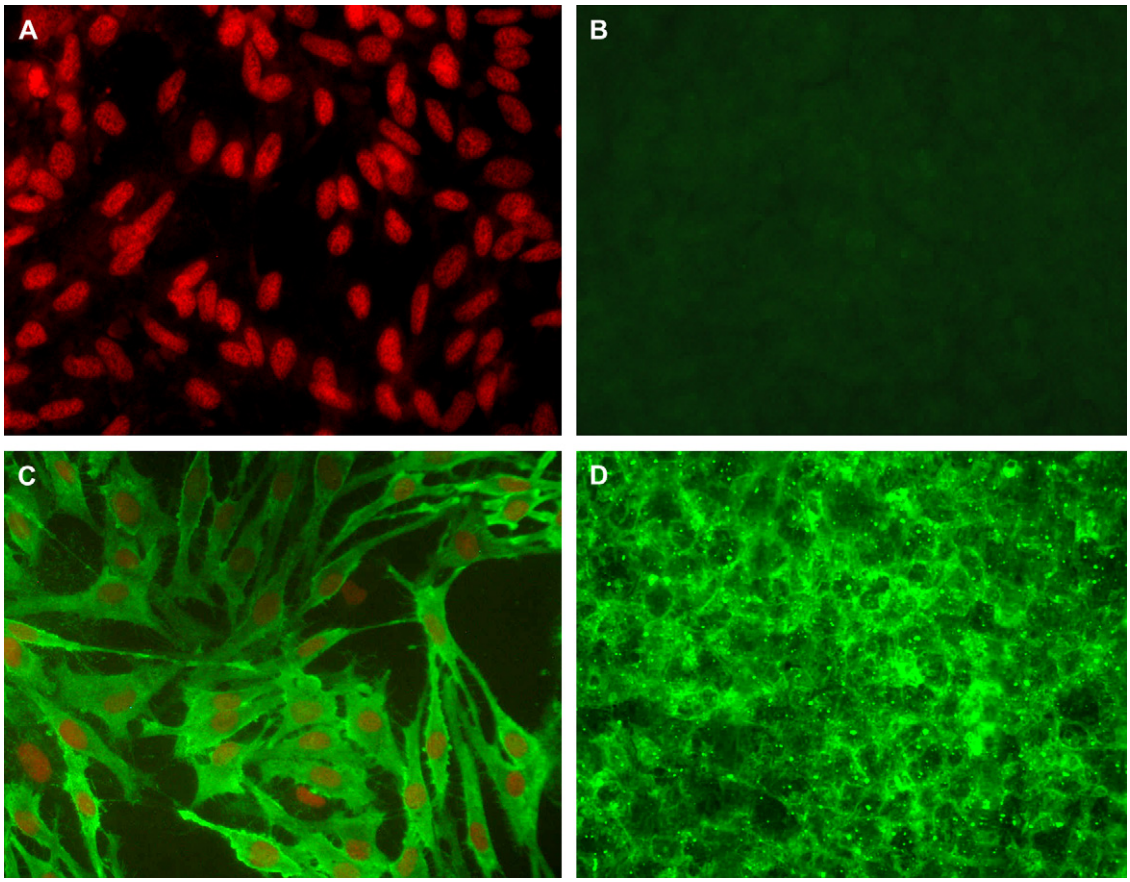

Fig. 6. Immunofluorescence staining of VHSV infected (C, D) and uninfected (A, B) target cells (12 h p.i.). RTG-2 cells (A, C) and EPC cells (B, D) were stained with a mAb directed against the G protein of VHSV and Alexa 488 (green) goat-anti-mouse-Ig conjugate; red counterstaining of nucleic acids with propidium iodide (only images A and C).

adaptive and the innate immune system corresponding to CTLs and NK cells, respectively. This differs from data obtained for virus infection of ginbuna crucian carp [27]. Although the authors also applied a booster infection like we did in our experiments here, leucocytes from ginbunas infected with crucian carp haematopoietic necrosis virus (CHNV) only lysed CHNV-infected syngeneic (MHC class I-matched) cells, while no such cytotoxicity was found against allogeneic (MHC class I-mismatched) CHNV-infected cells. This suggests that only CTLs but not NK cells showed antiviral CMC activity in CHNV infection.

It has been previously demonstrated by PCR analysis and with a monoclonal antibody directed against the recombinant allomorph Onmy-UBA*501 that RTG-2 cells express the same MHC class I allele as the homozygous rainbow trout clone C25 [24]. Thus, this combination of target cells and effector cell donor represents an ideal tool to study antiviral CMC in an MHC class I-matched system. The carp cell line EPC expresses a different MHC class I than rainbow trout. This was confirmed by sequence alignment analysis of MHC class I for rainbow trout and carp (data not shown). In addition, PCR using rainbow trout MHC class I specific primers (data not shown) and an immunofluorescence test using a mAb directed against rainbow trout MHC class I were negative for EPC cells. The EPC cells are not the optimal target cells, since they may differ from the RTG cells in many other aspects than MHC class I. However, rainbow trout cell lines mismatched in MHC class I with clone C25 that are able to replicate VHSV in sufficient amounts and, at the same time, to release sufficient amounts of LDH upon lysis were not available. Thus, MHC class I-restriction of antiviral cell-mediated cytotoxicity still remains to be shown in fish.

In the present study we also detected an absolute decrease in total PBL numbers (leucopenia). Similar observations were made previously [43] where a decrease of blood leucocyte counts after VHSV infection was detected too. In addition, we found a significant decrease in the percentages of several leucocyte subpopulations after VHSV infection

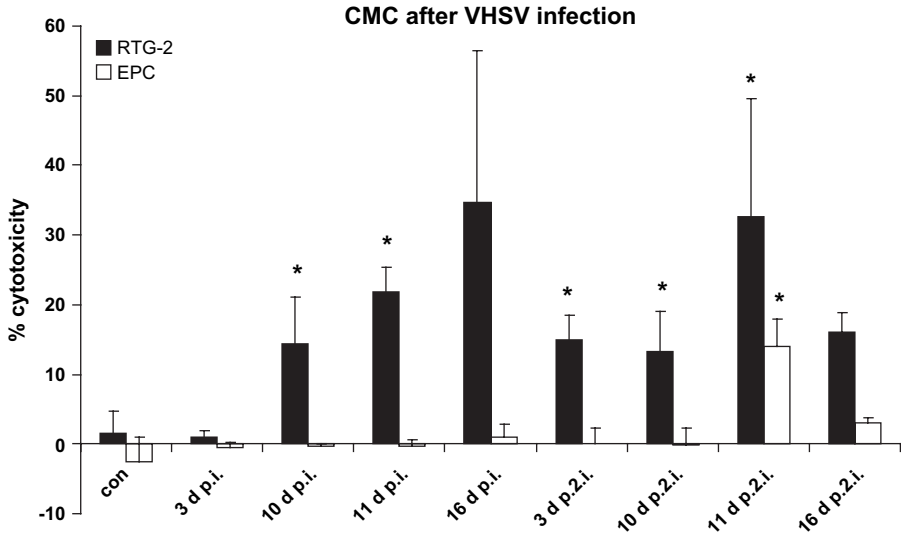

Fig. 7. Cytotoxic activity in VHSV infection. Fish received two VHSV injections. d p.i. represent sampling days after first injection and d p.2.i. represent days after a second injection that was given 22 d p.i. PBL isolated from infected rainbow trout were tested against VHSV-infected RTG-2 (black columns) and EPC (white columns) target cells at E:T ratios of 50:1 (controls: $n = 38$; infected fish: $n = 4$, except for day 11 p.2.i.: $n = 3$ and day 16 p.2.i.: $n = 2$). Asterisks depict significant differences ($p \leq 0.05$) from cytotoxicity levels determined for uninfected control fish.

as shown by flow cytometry. In particular, numbers of the monocyte/granulocyte subpopulation considerably dropped after VHSV infection by about sixfold when taking into account both the decrease in percentages and absolute cell numbers. A corresponding observation was reported [44] where a decrease of granulated cells in the head kidney occurred after VHSV infection.

Flow cytometry using mAb Q4E [39] revealed relatively high percentages of granulocytes in naïve fish (approx. 30%). The majority of Q4E positive cells, however, consisted of small (low FSC) less granulated (low SSC) cells when compared to mature granulocytes (data not shown). The percentage of those smaller, less granulated granulocytes was significantly lower (right shift) after VHSV infection, especially on day 16 p.i. A similar observation was made by Amlacher et al. [45], who found a right shift in granulocyte maturation during VHS. Considering both the decrease of absolute PBL numbers estimated for each fish and a concurrent increase of the percentages of IgM negative lymphocytes, the absolute number of IgM negative lymphocytes per fish doubled. The antigen specificity of this population was not investigated in this study, but a clonal expansion of VHSV-specific CTLs is a probable occurrence as supported by the observation of Boudinot et al. [46], who discovered skewed complementarity-determining region 3 size profiles for VβJβ combinations after VHSV infection.

Mammalian IgM negative lymphocytes comprise NK cells and T cells. This, however, cannot be proved for rainbow trout yet, because of the absence of NK and T-cell specific surface markers of the CD nomenclature. The only way to corroborate this presumption was to detect the expression of specific T cell- and NK cell markers at the genomic level. We observed increased transcription levels of the CTL marker CD8α after infection with VHSV which coincided with an increased antiviral CMC activity against MHC class I-matched cells. Similarly, NKEF-like transcription

Table 3
Cell-mediated cytotoxicity of trout PBL against VHSV-infected cells

Target cells	Target cells VHSV-infected	Target cells lysed by PBL from uninfected donors	Target cells lysed by PBL from primary VHSV-infected donors	Target cells lysed by PBL from secondary VHSV-infected donors
EPC	−	No	No	No
EPC	+	No	No	Yes
RTG-2	−	No	No	No
RTG-2	+	No	Yes	Yes

Negative cytotoxicity means that cytotoxicity levels for all fish of the corresponding group were less than 4% or less than 1% on average.

was markedly elevated in PBL of infected fish. Although NKEF is not a direct marker for NK cells, its up-regulation suggests a trigger effect upon NK cells as described for mammals. In humans, NKEF is highly expressed during erythroid differentiation [47]. Since VHSV is a haemorrhagic disease it can be hypothesized that a compensatory erythropoiesis is accompanied with an increased release of NKEF which in turn triggers NK cells. The observed increase of thrombocyte numbers, especially after a second VHSV infection, also supports the notion of compensatory reactions occurring in parallel with haemorrhagic processes.

In mammals, it is generally accepted that shortly after a virus infection innate immune mechanisms such as IFN-γ and NK cells are activated to ensure antiviral protection and followed by adaptive immune mechanisms represented by antibodies and CTLs. The more rapid up-regulation of NKEF transcription compared to that of CD8α observed here supports that a similar scenario takes place in fish. Similarly, in a gene expression profiling study with IHNV infection in rainbow trout [48] a significant up-regulation at day 3 post infection of a number of cytokine genes related to the innate immune response was found. In the experiments described in this paper, significant antiviral antibody titres were only observed from the 16th d p.i. onwards while an antiviral CTL-like response against MHC class I-matched cells was measured already on the10th d p.i. Although the NKEF-like gene was already up-regulated shortly after VHSV injection an NK cell-like response, as measured by killing of infected xenogeneic EPC cells, was only detected after a secondary infection. This indicates a different function of the trout NKEF homologue when compared to mammals. Considering the functional data collected during this study it rather suggests that the adaptive cell-mediated cytotoxic response to VHSV infection is induced more rapidly than the innate NK cell-like response. However, since analysis of antigen specificity in the CMC experiments was not performed and since the EPC cells differ from RTG cells in aspects other than MHC class I, the kinetics in the killing of the two types of infected target cells may not be fully comparable. The late NK cell-like response could also be explained by an initial depletion of virally infected NK cells shortly after infection followed by a late recovery of these cells.

Rainbow trout that were injected with VHSV developed an antiviral antibody response. Since even low levels of neutralizing antibodies have been found to be protective against salmonid rhabdoviruses [49], the response found in the PNT-assay at 16 d p.i. was most probably able to protect the fish against further development of disease. And the high neutralizing titres found at all later sampling times would presumably be fully protective. In ELISA, the kinetics of the antibody response appeared somewhat different, probably reflecting the fact that neutralizing antibodies are directed against conformational epitopes on the viral G protein [31], whereas antibodies to all viral proteins and different types of epitopes may be detected in ELISA where purified virus particles were used as antigen. Especially after a secondary booster infection, the antibody titres as measured by ELISA increased followed by a drop on day 16. A possible explanation for this drop could be a decrease in natural antibodies after the 11th day post secondary infection followed by a late response of more specific antibodies during affinity maturation. A late-affinity maturation has been described in rainbow trout after immunization with TNP-lipopolysaccharide [50].

Antibody-dependent cell-mediated cytotoxicity (ADCC) is a specific killing mechanism where the antigen bearing cells are opsonized by specific antibodies and then recognized via the Fc receptor on NK cells. Although there is evidence for ADCC killing in several fish species [51–53], prima facie this mechanism is unlikely to have caused lysis of the target cells in the present study, as the effector cells were carefully separated from the blood plasma and washed. Likewise, no antibodies were detected during the first days after infection when CMC against virus-infected cells was already recorded. However, ADCC cannot be completely ruled out since highest cytotoxicities against infected EPC cells were observed on day 11 after secondary infection when ELISA antibody titres were highest. Likewise, there was a drop of ELISA antibody titres on day 16 when cytotoxicities against infected EPC cells were low again. It has been shown in channel catfish that NK-like cells armed with antibodies specific to antigens on target cells via a putative FcμR are able to kill the corresponding target cells [53]. It could have happened in our experiments that NK-like cells were already armed with VHSV-specific antibodies in vivo. In vitro, those antibody-armed NK-like effector cells could have attached to VHSV-infected target cells. Since it has been demonstrated that VHSV-infected cells incorporate G protein into their cell membrane [54], this attachment could result in ADCC. Therefore the question of ADCC should be a subject of further investigations.

Summarizing this study, it was demonstrated for the first time that rainbow trout generate antiviral cytotoxic cells during a virus infection, thus leading to a better understanding of the immune response to an economically important rhabdovirus of salmonids. Antiviral CMC was detected both in an MHC class I-matched and in an MHC class I-mismatched system of effector cell donors and target cells. This, together with an increased CD8α and NKEF expression, suggests the involvement of both CTLs and NK cells, respectively. Surprisingly, the NK-like cell-mediated

cytotoxicity observed against virus infected xenogeneic target cells was found later than the CTL-like response against virus infected MHC class I-matched target cells. This does not agree with the generally accepted rule in mammals that NK cell responses occur immediately after viral infections while CTL responses need a certain time to develop. Further studies on CMC in rainbow trout including allogeneic MHC I-mismatched target cells as well as analysis of antigen specificity are needed to clarify this point. Similarly, the relative importance and potential interdependence of humoral and cellular mechanisms for protection of rainbow trout against VHS remains to be determined in detail.

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