Gene expression analysis of common carp (*Cyprinus carpio* L.) lines during Cyprinid herpesvirus 3 infection yields insights into differential immune responses

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ABSTRACT

Cyprinid herpesvirus 3 (CyHV-3), also known as koi herpesvirus (KHV), is the etiological agent of a virulent and lethal disease in common and koi carp. This study aimed to determine the genetic basis underlying the common carp immune response to the CyHV-3 virus. Two common carp lines (R3 and K) were infected with CyHV-3 by immersion. The R3 line presented a 20% higher survival rate compared to the K line and significantly lower viral loads as measured at day 3 post infection (p.i.). Microarray analysis using a common carp slides containing a number of 10,822 60-mer probes, revealed that 581 genes in line K (330 up-regulated, 251 down-regulated) and 107 genes in line R3 (77 up-regulated, 30 down-regulated), showed at least a 2-fold difference in expression at day 3 p.i. compared to day 0. Genes which showed at least a 4-fold difference in expression in both lines were selected as potential markers of a CyHV-3 infection in common carp. Additionally, 76 genes showed at least 2-fold differentially expression between K and R3 lines at day 3 p.i. Significantly higher expression of several immune-related genes including number of those which are involve in pathogen recognition, complement activation, MHC class I-restricted antigen presentation and development of adaptive mucosal immunity was noted in more resistant R3 line. Further real-time PCR based analysis provided evidence for higher activation of CD8^{+} T cells in R3 line. This study uncovered wide array of immune-related genes involved into antiviral response of common carp toward CyHV-3. It is also demonstrated that the outcome of this severe disease in large extent could be controlled by genetic factors of the host.

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1. Introduction

Cyprinid herpesvirus 3 (CyHV-3), also known as koi herpesvirus (KHV), is the etiological agent of a highly contagious and extremely virulent disease of common carp (*Cyprinus carpio* L.) and its ornamental koi varieties (Bretzinger et al., 1999; Hedrick et al., 2000; Michel et al., 2010; Pokorova et al., 2005). The mortality rate in affected ponds is as high as 70–95%, causing very large economic losses in fish farms in many countries worldwide. CyHV-3 is a member of the *Alloherpesviridae* family comprising of piscine and amphibian herpesviruses (Davison et al., 2009; Waltzek et al., 2009). The genome of CyHV-3 is a 295-kb, linear, double-stranded DNA molecule, encoding for 156 potential protein-coding open reading frames (ORFs). The CyHV-3 genome is packaged within an icosahedral capsid which is surrounded by a proteinaceous tegument and finally by a host-derived lipid envelope containing virus glycoproteins. The sequence analysis of the whole genome of three CyHV-3 strains isolated in Israel (CyHV-3 I), Japan (CyHV-3 J) and United States (CyHV-3 U) revealed high sequence identity between the strains (Aoki et al., 2007).

Knowledge about immune mechanisms and immunological traits that can correlate with disease resistance in fish, is essential for the development of prophylactic strategies (such as vaccination) as well as for the development of more resistant strains by the use of molecular marker assisted selection. Genetic differences in resistance to CyHV-3 of different carp crosses has been reported (Dixon et al., 2009; Ødegård et al., 2010; Rakus et al., 2009; Shapira

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et al., 2005). However, to the best of our knowledge the number of studies on immune responses of carp against CyHV-3 is very limited. Perelberg et al. (2008) described the different kinetics of anti-CyHV-3 antibody formation in the serum of Koi kept at different temperatures and exposed to CyHV-3. It was shown that protection against CyHV-3 is proportional to the titer of specific antibodies during the primary infection. Nevertheless, no studies provided further insights in the immune response of carp against CyHV-3 using DNA microarray and real-time quantitative PCR.

In general, *in vivo* studies in human and other mammals have shown that the innate immune response against herpesviruses mostly include an activation of natural killer (NK) cells, the production of type I interferons (IFN) and interleukin-15 (IL-15) (Mossman and Ashkar, 2005) whereas the adaptive immune response includes an activation of both CD8+ and CD4+ T cells as well as the production of specific anti-viral antibodies (Chan et al., 2011; Wills et al., 2007). However, the genomes of many herpesviruses contain multiple genes that code for various proteins which counteract different aspects of the host immune response and involved in immune evasion processes. In the CyHV-3 genome, such candidate genes are ORF12, ORF16, and ORF134 which respectively code for a TNF receptor homolog, a potential G protein-coupled receptor and an IL-10 homolog (Michel et al., 2010).

In recent years, many genes encoding different immuno-regulatory peptides of the innate and the adaptive immune system as well as markers of immune cells have been discovered in teleost fish, including common carp. The teleost thymus, head kidney and spleen are considered as the major lymphoid organs (Rombout et al., 2005) and represent suitable sources for immune cells which respond to pathogen challenge. A genome-wide analysis of gene expression in cells derived from lymphoid organs would therefore allow a measurement of the expression pattern of a large number of genes with immune-related functions and of genes with currently unknown functions. Following the expression pattern at different time points during an infection of carp with CyHV-3 should therefore lead to a better understanding of the mechanisms of immune responses of carp to this virus, and will be very useful for development of future vaccines.

The present study describes for the first time a transcriptional analysis of immune-related genes of common carp during CyHV-3 infection. We investigated gene expression differences between two lines of common carp that showed differences in both survival rates and viral loads during CyHV-3 infection. Our results demonstrate that differences in resistance of various genetic groups of fish could be associated with variations in the expression of selected immune-related genes. We also describe a group of genes which can serve as potential markers of an infection of common carp with CyHV-3.

2. Materials and methods

2.1. Fish

European common carp (*Cyprinus carpio carpio* L.) used in this study included four groups of carp: 2 lines (K and R3) and 2 crosses between them (KxR3 and R3xK) (Irnazarow, 1995). Fish were obtained from the Institute of Ichthyobiology and Aquaculture in Gołysz (Poland). Each group was grown in aquaria with UV treated recirculating water and bio-filters. The total capacity of the system was 3 m³ and the water temperature was maintained at 21 ± 1 °C. Daily feeding with commercial carp pellets (Aller Aqua, Poland) was applied at 3–5% body weight per day. Before the challenge experiments, fish (mean weight ± SD = 120 g ± 38 g) from the four genetic groups were randomly sampled, marked by fin clipping and transported to the Laboratory of Fish Diseases, National Veter-

inary Research Institute in Puławy (Poland). Once there, fish were divided into two experimental groups: (i) mortality recording group ($n = 30$ fish/line and cross), and (ii) gene expression study group ($n = 40$ fish/line K and $n = 28$ fish/line R3), and kept in two separate 800 l tanks. In addition, a control group (non-infected) was kept in a third 800 l tank ($n = 10$ fish/line and cross). Daily feeding with carp pellets at 3% body weight per day was applied. The water temperature was 23 ± 1 °C in the mortality recording group and 21 ± 1 °C in the gene expression study group. Fish were allowed to acclimatize to the new recirculating system for a period of 4 weeks. No mortalities were observed during the acclimatization period.

2.2. Cells and virus

C. carpio brain (CCB) cells (Neukirch et al., 1999) were cultured in minimum essential medium (Gibco, Germany) containing 4.5 g/liter glucose (D-glucose monohydrate), 10% fetal calf serum (FCS), Penicillin (200 i.u./ml), Streptomycin (0.2 mg/ml), and 1% MEM non-essential amino acid solution (SIGMA, Germany). The cells were cultured at 22 °C in a humid atmosphere containing 5% CO_2. CCB cultures were inoculated with CyHV-3 virus isolated in the Laboratory of Fish Disease, National Veterinary Research Institute in Puławy (Poland) from infected common carp in 2005 (passage no. 4). Infected CCB cultures were incubated at 22 °C in a humid atmosphere containing 5% CO_2 for 10 days and examined for cytopathic effect (CPE). Medium from infected cells including the virus was harvested and used for infection of the fish. The virus titer of the original virus suspension, given as tissue culture infective dose ($TCID_{50}$/ml), was estimated by the method of Reed and Muench (1938). This titer was 8×10^4 $TCID_{50}$/ml.

2.3. Fish challenge and sampling protocol

Fish were infected with the CyHV-3 virus by bath immersion challenge for 1 h at 22 °C, in small plastic containers containing tank water with virus suspension (3.2×10^2 $TCID_{50}$/ml in the final volume of water), and subsequently returned to their original tanks. The same treatment was applied to the control fish, but the water contained medium harvested from uninfected CCB cultures. For the mortality recording group, fish were observed three times per day over a period of 32 days. Moribund fish were removed from the tanks and the kidney and fin clips were collected and stored in 96% EtOH until DNA extraction. Fish from the gene expression study group ($n = 5$/line/sampling point) were sampled on days 0, 1, 3, 5 and 14 post infection (p.i.). An additional $n = 5$ fish/line were sampled at day 3 p.i. Organs (head kidney, spleen, kidney, liver, skin, gills, posterior gut (beginning part), and anterior gut (ending part)) were collected and divided into two parts. One part was placed into empty, sterile tubes, immediately frozen in liquid nitrogen and stored at −80 °C. The second part was placed into tubes containing RNAlater (Qiagen, Germany) and stored at 4 °C until RNA extraction. In addition, blood was collected and serum was stored at −80 °C until complement activation assay.

2.4. Detection and quantitation of CyHV-3

Virus detection was performed by PCR amplification of a CyHV-3 genome fragment localized between ORF55 and ORF56, using the M-KHV1-F and M-KHV1-R primers (Table 1). In short, DNA was isolated from 25 mg of kidney samples from infected and control fish using the Genomic Mini Kit (A&A Biotechnology, Poland) according to the manufacturer's manual. Amplification of the target PCR product was carried out in 20 μl of reaction mix using 200–300 ng of total DNA, 200 nM of each primer, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 1x Flexi Buffer, and 0.5 units of *GoTaq* Flexi

Table 1
Primers and probes sequences.

Primer/probe name	Primer/probe sequence (5′→3′)	Accession No.
Primers and probes for detection and quantification of CyHV-3		
M-KHV1-F	TACCGAAACACGAAACACTTGCTC	AY660950
M-KHV1-R	CTCTTTACCCAGGAGGACCAGAC	
KHV-86F	GACGCCGGAGACCTTGTG	AF411803
KHV-163R	CGGGTTCTTATTTTTGTCCTTGTT	
KHV-109P	(FAM) CTTCCTCTGCTCGGCGAGCACG (BHQ1)	AF411803
EGFP1-F	GACCACTACCAGCAGAACAC	–
EGFP10-R	CTTGTACAGCTCGTCCATGC	
EGFP1-HEX	(HEX) AGCACCCAGTCCGCCCTGAGCA (BHQ1)	–
Primers for RT-qPCR gene expression study		
40S-F	CCGTGGGTGACATCGTTACA	AB012087
40S-R	TCAGGACATTGAACCTCACTGTCT	
TCRα1-F	TTCACACAACATAATGCTGTAAATGA	EU025122
TCRα1-R	TTGACTGGATGATCCTGACATGC	
TCRα2-F	CAGTGCGATATGCAGAACGAAGT	EU025123
TCRα2-R	TTGACTGGATGATCCTTGCTGA	
CD8α1/2-F	TGCGCAGCCATGAACAACAATA	EU025118
CD8α1-R	GCCATTGTTGTTGTCGATGGTA	
CD8α2-R	CGTAATCACTGGTGTTGCAGTT	EU025119
CD8β1-F	AATCAACGGCTCGGAAACTATC	EU025120
CD8β1-R	CGCTCTCTGCCAGCGCTGTTACA	
CD8β2-F	AATCAATGGCTCGGAAGTTCTGT	EO025121
CD8β2-R	CCAGCACTGTTGACATACACTAA	
IL-1β1-F	AAGGAGGCCAGTGGCTCTGT	AJ245635
IL-1β1-R	CCTGAAGAAGAGGAGGCTGTCA	
IL-10-F	CGCCAGCATAAAGAACTCGT	AB110780
IL-10-R	TGCCAAATACTGCTCGATGT	
IL-12p35-F	TGCTTCTCTGTCTCTGTGATGGA	AJ580354
IL-12p35-R	CACAGCTGCAGTCGTTCTTGA	
IL-6M17-F	CACATTGCTGTGAGGGTGAA	AY102633
IL-6M17-R	GCATCCATAGGCTTTCTGCT	
IFNαβ-F	GGCAGATATGGGACGGTGAG	EC393381
IFNαβ-R	GTCCTCCACCTCAGCTTTGTC	

polymerase (Promega, USA). The PCR profile included an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 62 °C for 30 s, 72 °C for 45 s, and a final extension step at 72 °C for 7 min. PCRs were performed using a MJ Research PTC-200 thermocycler (Biorad, USA). PCR products were detected on a 1% agarose gel with ethidium bromide staining and visualized using a TFX-35 M UV transilluminator (Vilber Lourmat, France).

Virus quantitation was performed by real-time TaqMan PCR as described by Gilad et al. (2004) with modifications. The kidneys from fish at day 0 and 3 p.i. were collected, snap frozen in liquid nitrogen and stored at −80 °C until further use. The DNA was isolated from 25 mg of tissue after mechanical lyses in QIAgen Tissuelyser II (Qiagen, Germany) with the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's manual. After isolation the quantity of DNA was evaluated in a Nanodrop ND-1000 spectrophotometer and the samples were diluted to 50 ng/μl and stored at −80 °C. Real-time TaqMan PCR was used to amplify a fragment of the CyHV-3 genome (Accession No. AF411803) by the use of KHV-86F and KHV-163R primers and the KHV-109P probe (see Table 1). In each sample, TaqMan PCR amplification control was also performed by the use of an INTYPE IC-DNA control (Labor Diagnostik Leipzig, Germany), EGFP1-F and EGFP10-R primers and the EGFP1-HEX probe (see Table 1). The real-time TaqMan PCR reaction mix contained 1x master mix (QuantiTect Multiplex PCR Kit, Qiagen, Germany), 800 nM of each primer, 100 nM of each fluorescent probe, 5×10^4 copies of INTYPE IC-DNA, and 250 ng of DNA template. The real-time TaqMan PCR was performed on a Stratagene Mx 3005P thermocycler (Agilent, USA). Plasmid based virus copy quantification was performed using the product amplified with the primers KHV-86F and KHV-163R (Table 1), ligated

into the pGEM-T Easy vector (Promega, USA) and propagated in JM109 competent *Escherichia coli* bacteria (Promega, USA). The plasmids were isolated by the alkalies-lyses method and purified with the Clean-Up Kit (A&A Biotechnology, Poland) according to the manufacturer's protocol. A standard curve from 10^2 to 10^7 of gene copies was prepared and used for quantification of the viral load from each sample. The results are presented as the total number of virus copies per 250 ng of DNA.

2.5. RNA extraction and cDNA synthesis

For microarray analysis, the total RNA was isolated from head kidney samples using the RNeasy Mini Kit (Qiagen, Japan) according to the manufacturer's instructions. For real-time quantitative PCR analysis, the total RNA was isolated from spleen samples of the same individuals using the RNAiso Plus kit (Takara, Japan) including a second DNase treatment of isolated RNA (RNase-Free DNase, Promega, Japan). The concentration of RNA was measured by means of a spectrophotometer at OD_{260nm}. The purity of extracted RNA was determined as the OD_{260nm}/OD_{280nm} ratio with expected values between 1.8 and 2.0. Total RNA was stored at −80 °C until use.

For microarray analysis, 200 ng of total RNA was used for synthesis and labeling of cDNA by using the Low Input Quick Amp Labeling Kit (Agilent Technologies, USA) following the manufacturer's instructions. The cDNA was labeled by cyanine 3-CTP (Cy3). For real-time quantitative PCR analysis, 1 μg of RNA was used for the synthesis of cDNA by using the M-MLV cDNA synthesis System (Invitrogen, Japan) according to the manufacturer's instructions. cDNA samples were further diluted 40 times in nuclease-free water before use as the template in real-time quantitative PCR experiments.

2.6. One-color microarray-based gene expression measurement and statistical analysis

A microarray analysis was performed on head kidney samples of fish from lines K and R3 ($n = 4$ fish/line/sampling point) sampled at day 0 and day 3 after CyHV-3 infection. A custom microarray was designed for 8×15 K glass slides (Agilent Technologies, USA). Common carp transcript clusters were derived from Genbank mRNA transcripts and EST sequences of *C. carpio*. Based on these sequences a number of 10,822 60-mer probes were designed. In addition, a number of 152 60-mer probes were designed to target each of CyHV-3 annotated ORFs. Probe design was performed by Agilent. The arrays were hybridized with Cy3-labeled first strand cDNA in hybridization buffer. Hybridization was carried out for 17 h at 65 °C. After 17 h, the glass slides were washed with GE Wash Buffer for 1 min at RT and subsequently with GE Wash Buffer 2 for 1 min at 37 °C using the Low Input Quick Amp Labeling Kit (Agilent Technologies, USA). The two glass slides were dried and scanned immediately using a Gene Pix 4000B (Axon Instruments, USA).

Images obtained from scanning the slides were extracted to numerical data (.txt format) by GenePix Pro ver. 4.0 (Axon Instruments, USA) and analyzed in R using the 'limma' package from the Bioconductor project (Gentleman et al., 2004; R Development Core Team, 2011; Smyth, 2004). The single-channel arrays were first background corrected with the normexp method and an offset of 50 (Ritchie et al., 2007) and then normalized with a quantile normalization (Bolstad et al., 2003; Yang and Thorne, 2003). The experiment included four biological replicates with no dye swaps for each strain (K and R3) and condition (day 0 and day 3). A linear model was fit to the data using a least squares method (Smyth, 2004). In order to identify the genes differentially expressed in each of the model's contrasts, an empirical Bayes moderated

t-statistics was used (Smyth, 2004). A moderated *F*-statistic (*F*) combining the *t*-statistics for all the contrasts was also used to infer genes bearing differential expression in at least one of the contrasts (Smyth, 2004). The resulting *p*-values were corrected for multiple testing with Benjamini–Hochberg's procedure to control for a false discovery rate (Benjamini and Hochberg, 1995). Genes with 2-fold changes or greater were considered differentially regulated and with adjusted *p*-values below 0.05 were considered significantly differentially regulated.

The raw and normalized data resulting from this experiment have been deposited in the NCBI Gene Expression Omnibus (GEO) database with Accession Nos. GSM754677–GSM754692, and the microarray design has been deposited to GEO with accession number GPL13819, following MIAME guidelines (Brazma et al., 2001).

2.7. Real-time quantitative PCR (RT-qPCR) and statistical analysis

RT-qPCR using SYBR Green I technology was performed using an Applied Biosystems 7300 instrument (Applied Biosystems, USA) with the Fast Start Universal SYBR Green Master (ROX) (Roche Diagnostics GmbH, Germany) as detection chemistry. The primers used for RT-qPCR were previously described by Forlenza (2009) and are listed in Table 1. The master-mix for each RT-qPCR run was prepared as follows: 1x Fast Start Universal SYBR Green Master (ROX), 200 nM of each primer, 5.0 µl of 40x diluted cDNA and sterile water to a final volume of 20 µl. The amplification program included an initial denaturation at 95 °C for 10 min, followed by 40 cycles with denaturation at 95 °C for 15 s and annealing and elongation in one step at 60 °C for 1 min. At the end, the dissociation stage was performed as follows: 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. Fluorescence data from RT-qPCR experiments were analyzed using Applied Biosystems 7300 software and exported to Microsoft Excel. The threshold cycle (Ct) was determined using the Auto method for all runs. The expression of analyzed genes was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The 40S ribosomal protein S11 was used as a reference gene.

Significant differences ($p \leqslant 0.05$) in RT-qPCR gene expression between CyHV-3 infected and uninfected samples, as well as between fish from lines K and R3 at each sampling time point were assessed using one-way ANOVA in cases where the data were normally distributed, or with the non-parametric Kruskal–Wallis test when not. All the statistical analysis was performed using MedCalc version 9.3.0.0 software.

2.8. Complement hemolytic activity in the classical pathway (CH50) analysis

2.8.1. Carp anti-SRBC serum
One individual of *C. carpio* (500 g) was injected (i.p.) with 10^9 SRBC in PBS. Four weeks later a booster injection (10^9) was given. Ten days later, fish were bled for hemolysin and carp anti-SRBC antibody was determined by haemagglutination test. Complement was inactivated by incubation at 45 °C for 15 min. Hemolysin was stored at −80 °C. Optimal dilution of hemolysin for SRBC sensitization 1:800 (v/v) was determined according to Yano (1992).

2.8.2. Hemolytic assay
The gelatin veronal buffer (GVB^{2+}) containing 10 mM Mg^{2+}, 1 mM Ca^{2+}, pH 7.6 was used for the assay. Test serum samples were diluted 1:20 (v/v) in the GVB^{2+} buffer and the volumes of 15, 45, 75 and 97.5 µl were placed in triplicate in the U-bottom 96 well plates. Samples were combined with GVB^{2+} buffer to the final volume of 97.5 µl. Next, 15 µl of sensitized SRBC in GVB^{2+} was added. A positive control (100% hemolysis) reference contained water and sensitized SRBS, the negative control contained sensitized SRBC and GVB^{2+} buffer. Finally the control sensitization of SRBC with non-immune pooled carp serum and a standard complement sample (serum pool) for correction of plate differences were included.

The plate were incubated for 60 min at 25 °C and centrifuged (Labofuge II, Heraeus, Germany) for 5 min at 500*g* to spin down SRBC. The supernatant in each well was carefully aspirated and placed in a new flat bottom microtitre plate (F.L. Medical S.r.l., Italy). The optical density of the supernatant was measured at 540 nm. A lysis curve was obtained by plotting the percentage of hemolysis against the volume of serum; the volume which yielded 50% hemolysis was determined and used for calculating the complement activity of the samples (CH50 unit/ml) according to Yano (Yano, 1992).

3. Results

3.1. CyHV-3 challenge and virus quantification

After infection of common carp with CyHV-3, the first mortalities were recorded on day 6 p.i. in line K ($n = 5$) and cross R3xK ($n = 1$) carp. The last mortalities were recorded on day 18 p.i. The highest cumulative mortality (76%) was observed in line K and the lowest (56%) in line R3. The mortality of crosses KxR3 and R3xK were 62% and 66%, respectively.

The presence of CyHV-3 was confirmed by PCR in all infected fish (dead and the survivors). All control fish were negative for CyHV-3. The quantitative analysis of viral load by real-time TaqMan PCR was performed on a group of fish from line K and R3 dedicated for microarray analysis. At day 0, no virus DNA was revealed in fish from both lines ($n = 5$ fish/line). At day 3 p.i. the number of virus DNA copies was significantly higher in the K line (higher mortality; higher susceptibility) than in the R3 line (lower mortality; moderate susceptibility) ($n = 10$ fish/line) (Fig. 1).

3.2. Differentially expressed genes between day 0 and day 3 p.i. in line K (susceptible) and R3 (moderately susceptible) as revealed by microarray analysis

Changes in gene expression between day 0 and day 3 after CyHV-3 infection was analyzed in each line separately. At day 3 p.i., 581 genes in line K (330 up-regulated, 251 down-regulated) and 107 genes in line R3 (77 up-regulated, 30 down-regulated), were at least 2-fold differentially expressed compared to day 0. Among them, genes which were at least 4-fold differentially expressed in both lines were selected as potential markers of an infection of common carp by CyHV-3. This group includes 17 up-regulated and only 1 down-regulated genes (Table 2). Among up-regulated genes, there were 8 immune-related genes (47%): two interferon-inducible protein Gig-1 like genes, signal transducer and activator of transcription 1 (STAT1), novel protein belonging to the chemokine CC superfamily, ubiquitin-like protein, ubiquitin-specific protease-18, galectin-3-binding protein A, and suppressor of cytokine signaling 1. Only one gene, a novel protein containing a lectin C-type domain, was significantly down-regulated in both lines.

3.3. Differentially expressed genes between line K (susceptible) and R3 (moderately susceptible) at day 3 p.i. as revealed by microarray analysis

Microarray analysis revealed no significant differences in gene expression between line K and R3 at day 0. Three days after CyHV-3 infection, 76 genes showed at least 2-fold difference in

expression between two lines. In line R3, 55 genes were expressed significantly higher compared to line K. Twenty-two of them were immune-related genes (40%). Comparatively, 21 genes were expressed significantly higher in line K compared to line R3, including six immune-related genes (29%) (Table 3).

A more detailed analysis of the nature of expression ratios for these 55 genes (R3 > K variant), between day 3 and day 0 in both lines revealed that in most of the cases the difference was a result of down-regulation of gene expression in line K, rather than an up-regulation of gene expression in line R3, during the course of infection. In the other 21 genes (R3 < K variant), most of the changes resulted from an up-regulation of gene expression in line K during the course of infection (data not shown).

3.4. Differential complement activation in the classical pathway (CH50) between lines K (susceptible) and R3 (moderately susceptible) during CyHV-3 infection

Complement hemolytic activity in the classical pathway (CH50) measured in line K maintained the same level during course of observation with the exception of day 1 p.i. when it was significantly lower as compared to day 0. In contrary, carp from line R3 showed significantly elevated activity of complement at day 5 p.i. Line R3 presented significantly higher level of complement activation compared to line K at day 1 and 5 p.i. (Fig. 2). Due to limited number of fish in the R3 carp we could not measure CH50 values of the complement at day 14 p.i.

3.5. Differential kinetic of the expression of T cell markers between lines K (susceptible) and R3 (moderately susceptible) during CyHV-3 infection as revealed by RT-qPCR

The kinetics of gene expression of T cell markers TCRα1, TCRα2, CD8α1, CD8α2, CD8β1 and CD8β2 were determined in spleen samples derived from fish from the lines K and R3 (n = 4 fish/line/sampling point). Samples were collected over a period of 5 (line R3) to 14 (line K) days after CyHV-3 infection and analyzed by RT-qPCR. At day 14 p.i. gene expression in line R3 was not investigated due to the limited number of fish (Fig. 3).

Challenge with CyHV-3 significantly up-regulated the expression of TCRα1 and TCRα2 genes in fish from line R3 at day 1 p.i. By day 5 p.i., however, the expression of these genes was significantly down-regulated. In fish from line K we did not observe any differences in expression of TCRα1 during infection but the expression of the TCRα2 gene was significantly down-regulated at day 3 p.i. and up-regulated by day 14 p.i. In addition, both genes – TCRα1 and TCRα2, were expressed on significantly higher level in line R3 compare to line K at day 1 and 3 p.i.
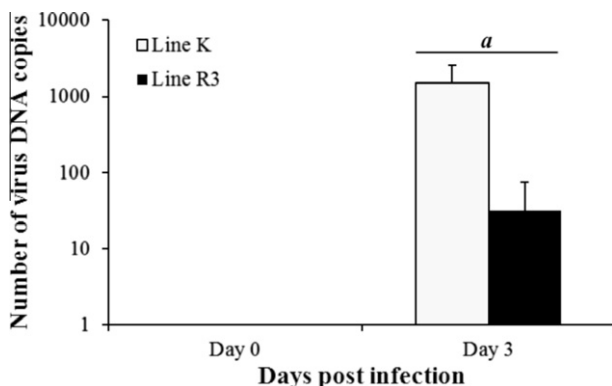
Fig. 1. CyHV-3 load in the kidney of carp from line K and R3 as measured by TaqMan-qPCR at day 0 and 3 p.i. Data are shown as mean + SD of n = 5 fish (day 0) and n = 10 fish (day 3 p.i.). Symbol (_a_) indicates statistical difference (p < 0.05) in the viral loads between fish from 2 lines (K vs. R3).

Expression of CD8β1 was significantly up-regulated in line R3 at day 1, 3 and 5 p.i. and in line K at day 5 p.i. In addition, at day 1 p.i. expression of this gene was significantly higher in line R3 than in line K. The challenge with CyHV-3 did not significantly change the expression level of the CD8β2 gene in carp from line R3, while the expression of this gene was significantly down-regulated in carp from line K at 3 days p.i. No significant differences were detected in the expression of CD8α1 and CD8α2 genes in carp from both lines K and R3 during the challenge with CyHV-3.

3.6. Differential kinetic of the expression of cytokine genes between line K (susceptible) and R3 (moderately susceptible) during CyHV-3 infection as revealed by RT-qPCR

The kinetics of gene expression of the cytokines IL-1β1, IL-10, IL-12p35, IL-6M17 and IFNαβ were determined in spleen samples derived from fish from the lines K and R3 (n = 4 fish/line/sampling point). Samples were collected over a period of 5 (line R3) to 14 (line K) days after CyHV-3 infection and analyzed by RT-qPCR. At day 14 p.i. gene expression in line R3 was not investigated due to the limited number of fish (Fig. 4).

Challenge with CyHV-3 significantly up-regulated the IL-1β1 gene expression in individuals from line R3 at day 5 p.i and from line K on days 3 and 5 p.i. Significant differences in the expression of IL-1β1 between fish from lines K and R3 were however observed at day 5 p.i. Indeed, expression level of IL-1β1 in carp from line R3 was about five times higher than in carp from line K at that time point. At day 14 p.i. the expression of IL-1β1 in line K carp was on the same level as in the fish sampled at day 0. Challenge with CyHV-3 also induced significant up-regulation of IL-10 in line R3 at day 5 p.i. and in line K at day 14 p.i. At day 3 p.i. the expression of this gene was significantly higher in fish from line R3 than in line K, although not significantly different when compared to individuals sampled at day 0 due to high individual variations in line R3.

Expression of IL-12p35 was down-regulated during the CyHV-3 infection in line K on days 3 and 5 p.i. In those sampling points the expression of IL-12p35 was significantly higher in line R3 than in line K. We also observed an up-regulation of IL-12p35 expression in line R3 at day 3 p.i., although not statistically significant due to high individual variations. Challenge with CyHV-3 induced an up-regulation of IL-6M17 in line R3 on days 1, 3 and 5 p.i., although these differences were significant only at day 5 p.i. because of high individual variations at earlier sampling points. At day 5 p.i. the expression of this gene was also significantly higher in line R3 as compared to line K. No significant differences in expression of the IFNαβ gene was detected in lines K and R3 during the infection with CyHV-3.

4. Discussion

Recently, several studies described that variation in disease resistance between different populations of fish can be correlated with differences in expression profiles of immune-related genes (Baerwald et al., 2008; Bilodeau-Bourgeois et al., 2008; Yasuike et al., 2010). In this work we studied the immune response of two different lines of common carp (K and R3) infected with CyHV-3 by bath immersion. The two carp lines presented a 20% difference in survival rate and significant difference in viral loads at day 3 p.i.

4.1. CyHV-3 infection induces the up-regulation of immune-related genes and activation of type I IFNs-dependent pathways in both carp lines

Microarray analysis revealed that infection of common carp with CyHV-3 resulted in changes of expression of many genes at

Table 2
Differently expressed genes from the carp head kidney during CyHV-3 infection. Listed below are genes which showed at least a 4-fold difference in expression between day 0 and day 3 p.i. in lines K and R3 ($p < 0.05$). Immuno-related genes are printed in bold.

Putative gene product [Species of best BLAST hit]	Accession	Fold change	
		K line	R3 line
Up-regulated genes			
KIAA0650-like protein [*Danio rerio*]	AAW34265	16.0	14.2
Novel protein belongs to chemokine CC superfamily [*Danio rerio*]	CAM14364	29.7	12.9
Ubiquitin-like protein [*Carassius auratus*]	AAF17609	10.5	11.2
Zgc:112492 protein [*Danio rerio*]	AAI64862	25.9	10.1
Galectin-3-binding protein A	Q24JV9	11.0	8.6
Signal transducer and activator of transcription 1 (**STAT1**) [*Carassius auratus*]	AAO88245	11.4	7.8
Novel protein similar to vertebrate phosopholipid scramblase family [*Danio rerio*]	CAN88616	10.8	6.3
Novel protein [*Danio rerio*]	CAM14206	4.6	5.8
Interferon-inducible protein Gig1-like [*Danio rerio*]	XP_002660606	4.2	5.4
Ubiquitin specific protease 18 [*Carassius auratus*]	ABC86864	4.9	5.2
Poly (ADP-ribose) polymerase family, member 14-like [*Danio rerio*]	XP_691115	10.1	4.9
Novel protein similar to vertebrate hect domain and RLD 3 (HERC3) [*Danio rerio*]	CAX12367	4.1	4.4
Suppressor of cytokine signaling 1 [*Ctenopharyngodon idella*]	ADA60714	6.0	4.3
Interferon-inducible protein Gig1-like [*Danio rerio*]	XP_001336615	5.8	4.1
Hypothetical protein LOC557223 [*Danio rerio*]	XP_685328.3	5.6	6.3
Hypothetical protein LOC335618 [*Danio rerio*]	XP_001920014	4.5	4.9
Hypothetical protein [*Danio rerio*]	XP_699055	5.7	4.5
Down-regulated genes			
Novel protein containing a Lectin C-type domain [*Danio rerio*]	CAX14642	−11.3	−5.9

day 3 p.i. We were able to identify group of genes which showed at least a 4-fold difference in expression in both lines. These genes serve number of potential CyHV-3 infection markers of common carp. Such markers of infection can be useful, for example in the design and evaluation of new vaccines, as well as for studying immune responses and host-pathogen interactions of common carp. Our results clearly showed the importance of type I IFNs activated pathways in the response of carp against CyHV-3. Several genes involved in the type I IFN response, including the gene encoding for signal transducer and activator of transcription 1 (STAT1) protein, as well as type I IFN-inducible genes (Gig1-like protein, ubiquitin-like protein (ISG15) and ubiquitin specific protease 18) were highly up-regulated in both carp lines.

The type I IFN system is involved in the first line of defense against viral infections. The antiviral effect is exerted through the binding of type I IFNs to specific cell-surface receptors and triggering signal transduction through the JAK-STAT signal transduction pathway, which in-turn induces the expression of a large number of IFN-stimulated genes, some of which encode antiviral proteins (Katze et al., 2002; Robertsen, 2006). STAT1 is one of the cytoplasmic transcription factors that mediate intracellular signaling in IFN-induced pathways. STAT1 expression was highly up-regulated in the kidney of salmon at day 6 and day 4 after injection with ISAV and IPNV, respectively (Collet et al., 2008). Gig1 is an IFN-inducible protein activated by JAK-STAT signal transduction pathway and seems to play an essential role in the host-defense response (Zhang and Gui, 2004).

Ubiquitin-like protein and ubiquitin specific protease 18 (USP18) were also highly up-regulated in both carp lines.

Ubiquitin-like proteins (Ubls) are small proteins related to ubiquitin, that all possess the same three-dimensional structure, and conjugated to diverse cellular proteins which results in degradation or modulation of the target protein's stability, function, and localization (Kerscher et al., 2006). The conjugation can be reversed by USP18, which is an IFN-inducible cysteine protease. In our study the highly up-regulated gene which showed the best BLAST hit with the goldfish ubiquitin-like protein sequence – IFN-stimulated gene 15 (ISG15) (Liu et al., 2002) – most probably represent the ISG15 homolog in common carp. In general, ISG15 is induce to a very high level by type I IFNs and can inhibit the replication of different viruses in human and mice. ISG15 play a role in ISGylation process, which unlike ubiquitinylation is an IFN-stimulated and regulated process and does not promote the degradation of the target protein but modulate the protein's function and activity (Harty et al., 2009). In addition, the secreted form of ISG15 exhibits immunomodulatory functions, inducing production of IFNγ in T cells and stimulating NK cell proliferation and activity (D'Cunha et al., 1996). Several studies described the up-regulation of ISG15-like genes in fish in response to virus infection (reviewed in Verrier et al., 2011).

In addition to genes involved in the type I IFN response, two other immune-related genes (a novel protein belonging to the chemokine CC superfamily and suppressor of the cytokine signaling 1) were also very highly up-regulated in both lines during CyHV-3 infection. The members of the CC chemokine family are known to guide leukocytes to sites of infection and inflammation (Rossi and Zlotnik, 2000). It has been described that the number of leukocytes in carp blood increases 2.5 times in the first 2 days after a CyHV-3 infection and is about two times higher during 14 days of the clinical infection compared to control fish (Negenborn, 2009). The suppressor of the cytokine signaling 1 (SOCS1) belongs to the SOCS family that consists of eight members, designated SOCS1 to SOCS7 and CIS (cytokine-inducible SH2 domain protein), which are known to participate in negative feedback loops in cytokine receptor signaling by inhibiting a variety of signal transduction pathways (Starr et al., 1997). In mammals, the up-regulation of SOCS1 expression is mainly induced by interferons as well as by several other cytokines (Fenner et al., 2006). SOCS1 acts to inhibit the proinflamatory effect of IFNs and IL-6 by suppressing signaling in response to these cytokines (Fenner et al., 2006). Different viruses, however, evolved mechanisms to induce SOCS expression in order to inhibit IFNs signaling and to reduce antiviral response of the host (Bode et al., 2003; Oshansky et al., 2009; Pothlichet et al., 2008; Zhao et al., 2007). Thus, the up-regulation of SOCS1 observed in our study might also indicates the presence of immune evading mechanisms of CyHV-3.

4.2. Differentially expressed genes between carp lines R3 and K at day 3 p.i. might result in differences in resistance to CyHV-3

In our study we did not observe differences in gene expressions between lines K and R3 before an infection. However the microarray analysis revealed 76 genes at least 2-fold differentially expressed between the two lines at day 3 p.i. Seven annotated genes: fucolectin-4 and fucolectin-6, fucose binding lectin, FBP32 precursor, FBP32 II precursor, class I metallothionein, and cysteine proteinase were expressed at a very high level in line R3 compared to line K (>7-fold change). These genes represent immune-related genes among which many play a role in pathogen recognition and activation of innate immune responses. Fucolectins (F-type lectins; lectins that bind fucose) have been described as immune-recognition molecules in both invertebrates (Saito et al., 1997) and vertebrates (Honda et al., 2000) and play a role in the regulation of cellular defense responses and in complement activation (Bianchet et al., 2002). Two other genes that can be involved in complement activation (proteins containing a C-type lectin domain and

Table 3

Differently expressed genes in head kidney between CyHV-3 infected carp from line K and R3 at day 3 p.i. Immuno-related genes are printed in bold.

Putative gene product [Species of best BLAST hit]	Accession	Fold change	p-value
Line R3 > line K (55 genes)			
Fucolectin-4 [*Anguilla japonica*]	BAB03526	10.4	0.0415
Class I metallothionein [*Cyprinus carpio*]	AAB61577	10.2	0.0185
FBP32 precursor [*Morone chrysops*]	ABB29990	10.2	0.0392
Cysteine proteinase [*Cyprinus carpio*]	AAA49207	9.0	0.0165
Fucose binding lectin [*Dicentrarchus labrax*]	ACF94293	8.9	0.0415
Fucolectin-6 [*Anguilla japonica*]	BAB03528	8.9	0.0408
FBP32II precursor [*Morone saxatilis*]	ABB29991	7.9	0.0437
Liver-basic fatty acid binding protein b [*Cyprinus carpio*]	ACA64701	5.7	0.0295
Neurotoxin/C59/Ly-6-like protein-like [*Danio rerio*]	XP_002660983	5.4	0.0165
Alcohol dehydrogenase 8a [*Danio rerio*]	CAX14458	4.7	0.0165
Chemokine CCL-C25ab [*Danio rerio*]	BAF98246	4.3	0.0287
Tyrosine-protein kinase receptor (Ntrk1 protein) [*Danio rerio*]	AAI62577	4.2	0.0391
Invariant chain like protein 1 [*Cyprinus carpio*]	BAC53767	4.0	0.0300
40S ribosomal protein S23 [*Salmo salar*]	ACN10021	3.9	0.0403
Novel protein containing a Lectin C-type domain [*Danio rerio*]	CAX14642	3.5	0.0419
Microfibrillar-associated protein 4-like isoform 1 [*Danio rerio*]	XP_001340028	3.5	0.0267
Cystatin C (zgc:136227) [*Danio rerio*]	CAX14260	3.2	0.0221
Transgelin [*Danio rerio*]	AAI22181	3.1	0.0221
Keratin [*Carassius auratus*]	AAC38008	3.0	0.0165
Extended synaptotagmin-like protein 1a [*Danio rerio*]	XP_695611	2.9	0.0391
Keratin, type I cytoskeletal 13 [*Anoplopoma fimbria*]	ACQ58237	2.8	0.0165
Chemokine CCL-C25a [*Danio rerio*]	BAF98240	2.8	0.0204
Immunoglobulin light chain [*Cyprinus carpio*]	BAA76502	2.8	0.0463
Tiarin protein-like [*Danio rerio*]	XP_001335383	2.7	0.0428
Taurine transporter [*Cyprinus carpio*]	BAA89537	2.7	0.0249
Cell division control protein 42 [*Oncorhynchus mykiss*]	ACO07740	2.7	0.0165
T cell antigen receptor alpha chain VJC region [*Cyprinus carpio*]	BAD88986	2.6	0.0185
Novel protein similar to vertebrate arachidonate 5-lipoxygenase (ALOX5) [*Danio rerio*]	CAX14764	2.6	0.0457
Novel protein similar to vertebrate NIMA-related kinase 5 (NEK5) [*Danio rerio*]	CAQ15143	2.6	0.0489
si:dkey-211e20.10 [*Danio rerio*]	XP_002666423	2.6	0.0415
Death associated protein 1b [*Danio rerio*]	AAI15169	2.6	0.0220
β-2-microglobulin precursor [*Salmo salar*]	ACI66331	2.6	0.0399
Id1 protein [*Danio rerio*]	AAH71286	2.5	0.0221
Zgc:153317 protein [*Danio rerio*]	AAI22237	2.5	0.0165
Short prolactin receptor S1 [*Cyprinus carpio*]	AAZ43193	2.5	0.0481
Nuclear protein 1-like [*Danio rerio*]	XP_002661973	2.5	0.0466
cytochrome c oxidase subunit VIIa polypeptide 2-like (Zgc:153177) [*Danio rerio*]	AAI24270	2.4	0.0451
Invariant chain like protein 2 [*Cyprinus carpio*]	BAC53768	2.3	0.0474
Ribosomal RNA processing 15 homolog [*Danio rerio*]	AAH46037	2.3	0.0466
CD3 γ/delta [*Cyprinus carpio*]	ABC97370	2.2	0.0221
Midkine a [*Carassius gibelio*]	ABS19628	2.2	0.0399
cysteine-rich protein 1 (Zgc:103738) [*Danio rerio*]	AAI53560	2.2	0.0165
HLA-DPA1 protein [*Danio rerio*]	AAH74082	2.1	0.0416
Bactericidal permeability-increasing protein/lipopolysaccharide-binding protein [*Cyprinus carpio*]	BAC56095	2.1	0.0442
Granulin [*Carassius auratus*]	ABD03952	2.1	0.0295

Table 3 (*continued*)

Putative gene product [Species of best BLAST hit]	Accession	Fold change	p-value
Alpha-1-microglobulin [*Ctenopharyngodon idella*]	ABW37743	2.1	0.0165
Ribosomal protein L34 [*Ictalurus punctatus*]	AAK95160	2.1	0.0461
Unnamed protein product [*Tetraodon nigroviridis*]	CAF92746	3.3	0.0165
Unnamed protein product [*Danio rerio*]	CAR80279	2.7	0.0165
Unnamed protein product [*Tetraodon nigroviridis*]	CAF95619	2.6	0.0391
Unnamed protein product [*Tetraodon nigroviridis*]	CAG10379	2.2	0.0408
Hypothetical protein isoform 1 [*Danio rerio*]	XP_002662640	5.6	0.0165
Hypothetical protein LOC799904 [*Danio rerio*]	NP_001077343	7.5	0.0489
Hypothetical protein LOC100170830 [*Danio rerio*]	NP_001124136	2.6	0.0325
Hypothetical protein LOC368902 [*Danio rerio*]	NP_001004525	2.0	0.0224
Line R3 < line K (14 genes)			
Lectin, galactoside-binding, soluble, (galectin 1)-like3 [*Danio rerio*]	AAH55581	0.2	0.0466
Nuclear cap-binding protein subunit 2 (Ncbp2 protein) [*Danio rerio*]	AAH83529	0.3	0.0165
Histidyl-tRNA synthetase 2, mitochondrial (putative) [*Danio rerio*]	AAH76141	0.4	0.0165
Sjogren syndrome antigen B (autoantigen La) [*Danio rerio*]	NP_955841	0.4	0.0466
C-type lectin [*Ctenopharyngodon idella*]	ACS16044	0.4	0.0287
Zinc transporter ZIP9 [*Danio rerio*]	NP_001013558	0.4	0.0287
CCT (chaperonin containing T-complex polypeptide 1) epsilon subunit [*Carassius auratus*]	BAA89277	0.4	0.0287
Immunoglobulin binding protein [*Danio rerio*]	AAT68067	0.4	0.0165
Similar to coxsackie adenovirus receptor-like [*Danio rerio*]	AAI62790	0.4	0.0401
Lamin-B receptor-like [*Danio rerio*]	XP_002664803	0.5	0.0403
Interferon γ 2b [*Cyprinus carpio*]	CAJ51089	0.5	0.0165
Dkc1 protein [*Danio rerio*]	AAH47840	0.5	0.0261
c-Myc [*Cyprinus carpio*]	BAA07129	0.5	0.0165
TPA: interleukin 13 receptor alpha 1 [*Danio rerio*]	CAI94933	0.5	0.0458
EBNA1 binding protein 2-like [*Danio rerio*]	AAT68147	0.5	0.0399
ATP synthase oligomycin sensitivity conferral protein [*Danio rerio*]	AAT68146	0.5	0.0462
Coiled-coil domain containing 43 [*Danio rerio*]	AAH57502	0.5	0.0343
Eukaryotic translation initiation factor 4A, isoform 1A [*Danio rerio*]	AAH48899	0.5	0.0165
Krr1 protein (Zgc:136398) [*Danio rerio*]	AAI17594	0.5	0.0221
nucleolar protein 1, 120 kDa [*Danio rerio*]	XP_001922354	0.5	0.0346
Unnamed protein product [*Tetraodon nigroviridis*]	CAG04699	0.5	0.0165

microfibrillar-associated protein 4 (MFAP4)) were also highly expressed in line R3. Recently it has been reported that MFAP4 genes may play similar roles during the innate immune response in fish as ficolin genes in mammals, which are known to be involved in recognition and binding of carbohydrates in pathogens and the activation of complement leading to opsonization, leukocyte activation, and direct pathogen killing (Niu et al., 2011). Metallothioneins have various functions and were also found to be involved in immune responses including inflammation reactions (Coyle et al., 2002; Mocchegiani et al., 2006). The higher expression of genes involved in complement activation in the more resistant R3 line could indicate for the role of this immune mechanism in the response against CyHV-3. It is known that activation of the
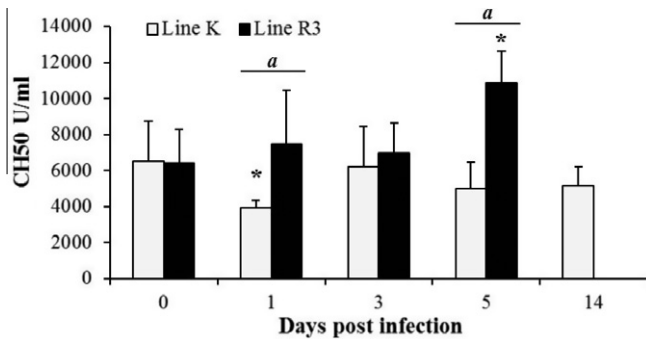
Fig. 2. Complement hemolytic activity in the classical pathway (CH50) analysis as measured in line K and R3. Data are shown as mean + SD of $n = 5$ fish. Symbol (∗) indicates statistical differences ($p \leqslant 0.05$) between infected and non-infected fish from the analyzed line. Symbol (a) indicates statistical difference ($p \leqslant 0.05$) between infected fish from 2 lines ($\overline{K}$ vs. R3) at the particular time point.

complement system can lead to neutralization of free viruses, phagocytosis of coated viral particles, lysis of virus-infected cells, and the generation of inflammation. Indeed, *in vitro* complement activation assay showed higher level of activation of complement in the classical pathway (CH50) in line R3 than in line K.

Several genes which have functions in the MHC class I pathway such as invariant chain like protein 1, invariant chain like protein 2 and β-2 microglobulin precursor were more highly expressed in line R3 than in line K. Genes involved in antigen recognition by T cells, such as T cell antigen receptor α chain VJC region and CD3 γ/δ, were also highly expressed in the R3 line. The higher expression of genes involved in the MHC class I-restricted antigen processing in the more resistant R3 line might result in better cell-mediated cytotoxic responses in this line. The higher activation of CD8+ T cells in the R3 line was also supported by our RT-qPCR analysis of T cell markers. Thus, differences in the levels of activation of CD8+ T cells between the two carp lines might result in higher or lower resistance to CyHV-3.

Chemokine CCL-C25a and CCL-C25ab were also highly expressed in the R3 line. In mammals, the CC chemokine ligand 25 (CCL25), also known as thymus-expressed chemokine (TECK), was described to be primarily expressed in dendritic cells of the thymus and mucosal epithelial cells. CCL25 was shown to play a role in T cells development (Vicari et al., 1997) and it is thought to be one of the key factors in the development of the adaptive mucosal immunity by targeting T cells to specific locations of infection (Kunkel et al., 2000; Meurens et al., 2006, 2007). Recently, it was demonstrated that the skin of common carp is the major portal of entry of CyHV-3 and that at the early stage of the disease, the virus replicates in skin epithelial cells (Costes et al., 2009). Very recently, the anti-CyHV-3 properties of carp skin mucus were described (Raj et al., 2011).

Fourteen genes were more highly expressed in carp from line K than in line R3. These genes included immune-related genes such as: galectin1-like 3, Sjogren syndrome antigen B, C-type lectin, immunoglobulin binding protein, interferon γ 2b and interleukin 13 receptor α1.

4.3. The moderately susceptible R3 line present higher activation of CD8+ T cells in the first phase of CyHV-3 infection than the susceptible K line

Mammalian cytotoxic T cells (CD8+), as part of the adaptive immune system, recognize virus-infected cells by the binding of their T cell receptors (TCR) to MHC class I molecules loaded with viral peptides. The mechanisms of cell-mediated cytotoxicity are quite conserved from fish to mammals (Fischer et al., 2006; Nakanishi

et al., 2002; Utke et al., 2007) and T cell markers of common carp have been recently cloned and described (Forlenza et al., 2008a; Sun et al., 2007). This allowed us to use the expression of T cell markers to monitor the activity of cytotoxic T cells during the infection of carp with CyHV-3.

CD8 is known as a marker molecule for cytotoxic T cells. The expression of both duplicated common carp CD8α and CD8β genes were studied in terms of cytotoxic T cell activity during CyHV-3 infection. Among all the isoforms studied, only CD8β1 showed a tendency for up-regulation from day 1 to day 5 p.i. in the R3 line and at day 5 p.i. in the K line. Interestingly an up-regulation of other isoforms – CD8α1, CD8α2 and CD8β2 – could not be statistically confirmed. In the case of CD8β2, a down-regulation of the expression was detected in carp from line K at day 3 p.i. It is well established that on mammalian T cells, CD8 glycoproteins are expressed as either αα homodimers or as αβ heterodimers (Witte et al., 1999). Human cells can also express ββ homodimers (Devine et al., 2000) but in contrast to αα homodimers or αβ heterodimers they are unable to bind to MHC class I. Based on common carp cDNA analysis of the predicted glycoprotein structure and based on the conservation of essential structural motifs, Forlenza et al. (2008a) suggested the possibility for an expression of CD8ββ homodimers and a CDβ signaling function in common carp. In this case it is likely that common carp could have different CD8+ T cells subpopulations, these could be functionally slightly different. This might explain the activation of only the CD8β1 isoform after CyHV-3 viral infection. This is also in line with data reported by Forlenza et al. (2008a) which demonstrated a differential expression of CD8 isoforms in common carp during spring viraemia of carp virus (SVCV) infection.

Both TCRα1 and TCRα2 genes were up-regulated at day 1 p.i. in carp from line R3 but not from line K. On day 5 p.i. TCRα1 and TCRα2 genes were down-regulated in the R3 line carp. The down-regulation occurred even faster in the K line carp, which showed lower levels of TCRα2 on day 3 p.i. A high up-regulation of the transcription of both TCRα and TCRβ genes was observed at day 1 after DNA vaccination of Japanese flounder against the hirame rhabdovirus (HIRRV). However 7 days after vaccination the expression levels of these genes decreased and returned to the level of gene expression of non-vaccinated fish (Takano et al., 2004). Infection of common carp with SVCV revealed no significant changes of gene expression of both TCRα1 and TCRα2 genes after primary infection, whereas the two TCR genes were already up-regulated 6 h post-secondary infection and remained high until day 7 p.i. (Forlenza et al., 2008a).

The up-regulation of both TCR genes and the CDβ1 gene in the first phase of infection, which was observed in the R3 line but not in the K line, might indicate a higher activation of CD8+ T cells in this line, which could result in a better immune response against the CyHV-3 virus and finally could contribute to the higher survival rate.

4.4. Differential kinetics of expression of cytokine genes between carp lines R3 and K

IL-1β is one of the best described pro-inflammatory cytokines in fish. An up-regulation of IL1-β1 was described in various fish species during infection with ectoparasites (Forlenza et al., 2008b; Gonzalez et al., 2007; Sigh et al., 2004), with the blood parasite Trypanoplasma borreli (Saeij et al., 2003), bacteria (Mohanty and Sahoo, 2010), and viruses (Purcell et al., 2004; Tafalla et al., 2005). CyHV-3 infection clearly up-regulated IL-1β expression in carp from line K (at day 3 p.i.) and R3 (at day 5 p.i.). Faster up-regulation of IL-1β expression in carp from line K (peak at day 3 p.i.) compared to carp from line R3 (peak at day 5 p.i.) might be correlated with the difference in virus levels between the two lines. Indeed, the
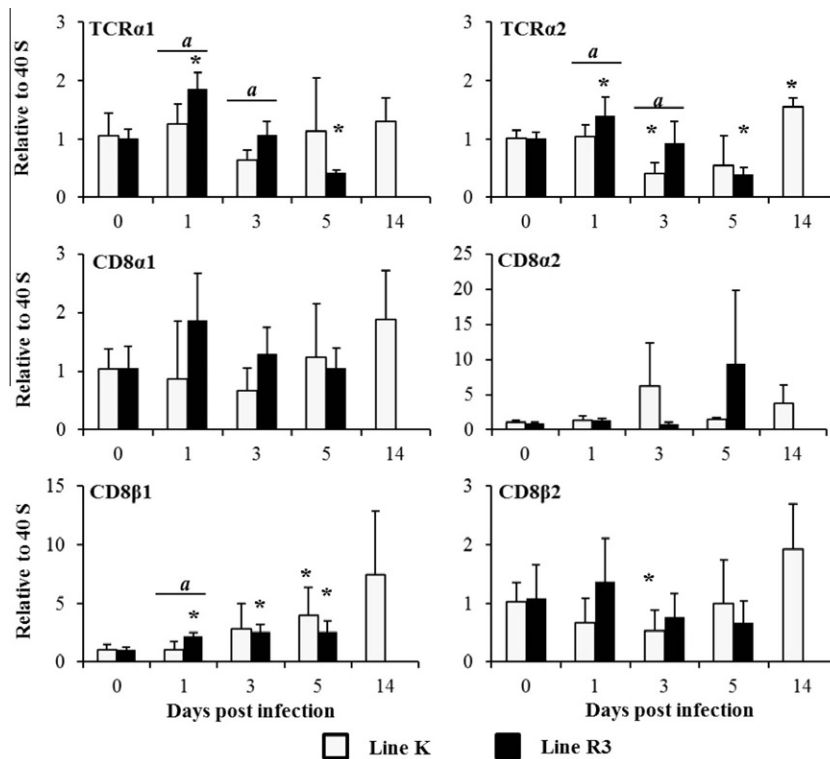
Fig. 3. Real-time quantitative PCR analysis of T cell markers. Gene expression was measured in the spleen of infected and non-infected (day 0) common carp individuals from two lines (Polish K and Polish R3) at different time points during the course of infection with CyHV-3. At day 14 p.i., the gene expression was not investigated in the carp from line R3. Gene expression was normalized relative to the expression of the gene encoding the S11 protein of the 40S subunit as a reference gene. Data are shown as mean + SD of $n = 4$ fish. Symbol (*) indicates statistical differences ($p \leqslant 0.05$) between infected and non-infected fish from the analyzed line. Symbol (_a_) indicates statistical difference ($p \leqslant 0.05$) between infected fish from 2 lines (K vs. R3) at the particular time point.

number of virus copies as measured at day 3 p.i., was significantly higher in the K line than in the R3 line, while at day 5 p.i. the virus level in the R3 line increased and was on the same level as in the K line (data not shown). The transcription level of IL1-β1 returned to initial values by day 14 p.i. The striking differences in the transcription of IL-1β between carp lines K and R3 on day 5 p.i. (more than 5-fold) indicate that a higher stimulation of the immune response could be an important factor explaining the differences between these two carp lines in the resistance to an infection with CyHV-3.

Another important pro-inflammatory cytokine is IL-12. In carp, a single gene for IL-12p35 and three highly distinct genes encoding for IL-12p40 (a–c) were identified (Huising et al., 2006). Recently, an up-regulation of the genes encoding for IL-12p35 and only the IL-12p40b isoform was revealed during an infection of carp with SVCV (Forlenza et al., 2008a). We observed a significant down-regulation of IL-12p35 expression after CyHV-3 infection in carp from the K line (at day 3 and 5 p.i.) but not in carp from the R3 line. In addition, we observed an up-regulation of IL-12p35 in carp from the R3 line at day 3 p.i., although this up regulation could not be confirmed as statistically significant owing to high individual variations in the transcription levels. The cytokine IL-12 stimulates the activity of cytotoxic T cells and NK cells as well as the secretion of IFNγ and TNF. A down-regulation of IL12-p35 expression in carp from the K line coincides with a decreasing level of T cell marker expression, which might indicate a lower cytotoxic response in this line.

In mammals, IL-10 is known to be an anti-inflammatory cytokine which regulates the inflammatory response, however the direct function of IL-10 in fish is less known. Some studies on IL-10 gene expression in fish also suggested its role in the down-regulation of the inflammatory response (Forlenza, 2009; Ingerslev et al., 2009; Raida and Buchmann, 2008a). We observed a relatively high up-regulation of the expression of IL-10 on day 5 p.i. and an elevated level was maintained until day 14 p.i. This up-regulation of IL-10 might reflect an activation of humoral immunity and an inhibition of the inflammatory response.

The mammalian IL-6 family of cytokines are major players in hematopoiesis, as well as in the neuroendocrine and immune systems, and have pro- and anti-inflammatory functions. The IL-6M17 gene was shown to be involved in teleost immune responses (Fujiki et al., 2003; Wang and Secombes, 2009). In rainbow trout IL-6 expression was up-regulated by both LPS and β-glucan stimulation *in vivo* (Lovoll et al., 2007) and after *Yersinia ruckeri* infection (Raida and Buchmann, 2008b) which suggests a role as a pro-inflammatory mediator. We observed an up-regulation and a much higher level in the expression of the IL-6M17 gene in carp from the R3 line compared to carp from the K line, although this difference was only statistically significant at day 5 p.i., owing to high individual variations in the R3 line. Nevertheless it could be speculated that the level of macrophage and T-cell activation (main IL-6 secreting cells) in the R3 line was much more efficient than that in the K line.

Interestingly, our results showed no differences in IFNαβ gene expression in both lines. An up-regulation of this gene was not seen in the microarray analysis and it was not observed by RT-qPCR analysis either. Nevertheless our microarray analysis revealed up-regulation of several genes activated by the type I IFNs. In fish, multiple forms of IFNs which are differentially induced during stimulation has been described (Sun et al., 2009). Very recently new genes of common carp type I IFNs were cloned and showed different expression profiles during CyHV-3 infection (Adamek et al., in preparation). It could therefore be speculated that primers and probes used in our study do not amplify all IFN genes present in carp and involved in the immune response against CyHV-3.
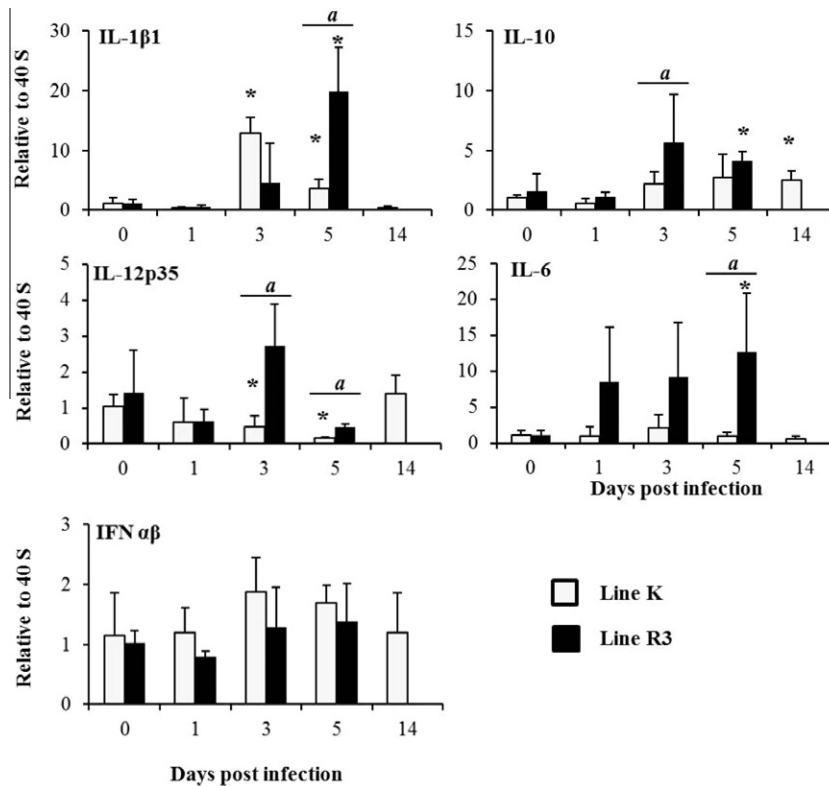
Fig. 4. Real-time quantitative PCR analysis of cytokine genes. Gene expression was measured in the spleen of infected and non-infected (day 0) common carp individuals from two lines (Polish K and Polish R3) at different time points during the course of infection with CyHV-3. At day 14 p.i., the gene expression was not investigated in the carp from line R3. Gene expression was normalized relative to the S11 protein of the 40S subunit as a reference gene. Data are shown as mean + SD of $n = 4$ fish. Symbol (∗) indicates a statistical difference ($p \leqslant 0.05$) between infected and non-infected fish from the analyzed line. Symbol (*a*) indicates a statistical difference ($p \leqslant 0.05$) between 2 lines of infected fish (K vs. R3) at a particular time point.

In conclusion, this is the first study of the genetic basis underlying the common carp immune response to the CyHV-3 virus. We identified a group of genes which were significantly regulated by CyHV-3 in both carp lines. We believe that these genes can serve as CyHV-3 infection markers for primary testing of different vaccines. Our study also revealed that differences in resistance to CyHV-3 between the two carp lines can be correlated with differentially expressed immune-related genes. The kinetics of expression of the immune-related genes indicate for a higher activation of cytotoxic T cells in the more resistant R3 line. Altogether, our results add to the understanding of the common carp immune response against the CyHV-3 virus and provide knowledge for developing future vaccines.

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