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Expression of immune-related genes of common carp during cyprinid herpesvirus 3 infection

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ABSTRACT: Fish herpesviruses and their hosts may have coevolved for 400 to 450 million yr. During this coexistence, the hosts have equipped themselves with an elaborate immune system to defend themselves from invading viruses, whereas the viruses have developed strategies to evade host immunity, including the expression of cytokine genes that have been captured from the host. Taking advantage of our experimental model for cyprinid herpesvirus 3 (CyHV-3) persistence in carp, we studied the gene expression of host and virus immune-related genes in each stage of infection: acute, persistent and reactivation phases. IFNγ-1, IFNγ-2, IL-12 and IL-10 host genes, and the CyHV-3 vIL-10 gene (khvIL-10) were highly significantly up-regulated in different phases of CyHV-3 infection. Similarly, host IL-1β was up-regulated in the acute phase of CyHV-3 infection. There was no significant difference in the expression of host TNFα-1 and MHC-II genes during all phases of CyHV-3 infection. Based on the expression profile of carp immune-related genes in each stage of CyHV-3 infection, we propose a possible interaction between carp IL-12, carp IL-10 and khvIL-10 during the course of viral infection. To our knowledge, this is the first report on the expression of cytokine genes during all phases (acute, persistent and reactivation) of CyHV-3 infection.

KEY WORDS: Cyprinid herpesvirus 3 · Koi herpesvirus · Immune response · Gene expression profiling · Persistent infection · Latent infection · *Cyprinus carpio*

INTRODUCTION

Koi herpesvirus (KHV; species *Cyprinid herpesvirus 3*, CyHV-3) is the aetiological agent of an emerging infectious disease (KHV disease, KHVD) associated with mass mortality of koi and common carp *Cyprinus carpio*. KHVD causes huge economic losses to the aquaculture industry worldwide (FAO 2010, OIE 2012). CyHV-3 is a linear double-stranded DNA virus with a genome size of 295 kb, 156 putative open reading frames (ORFs) and 2 terminal repeats of 22 kb each (Aoki et al. 2007). The virus belongs in the genus *Cyprinivirus* within the family *Alloherpesviridae*, order *Herpesvirales* (Davison 2010). Complete genome analysis of the Japan (J), USA (U) and Israel (I) strains of CyHV-3 indicated >99% identity in nucleotide sequence and suggested that 2 lineages (J and U/I) have arisen from a common ancestor (Aoki et al. 2007). However, more recent studies discovered an intermediate link between the 2 lineages (Bigarré et al. 2009, Kurita et al. 2009, Sunarto et al. 2011).

Fish herpesviruses and their hosts may have co-evolved for 400 to 450 million yr (Kumar & Hedges 1998, McGeoch & Gatherer 2005, Davison 2010). During this co-existence, the hosts have equipped themselves with an elaborate immune system to defend themselves from invading viruses and other pathogens. Host defence against herpesvirus infections includes activation of innate and adaptive immune responses. Cytokines, including interferons (IFNs), interleukins (ILs), tumor necrosis factors

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(TNFs), colony stimulating factors and chemokines (Savan & Sakai 2006), are not only important modulators of the innate immune response but they also integrate the innate and adaptive responses. At a very simple level, interleukin 10 (IL-10) may be seen as a pluripotent cytokine with both strong immunosuppressive and anti-inflammatory activities, but also with an important role in the growth and differentiation of B and T cells. IL-12, which promotes interferon gamma (IFNγ) production and has a profound impact on the development of both Th-1 cells and a cell-mediated immune (CMI) response, is negatively regulated by IL-10 (Holland & Zlotnik 1993).

The ability to establish a life-long latent infection is the hallmark of all known herpesviruses, including fish herpesviruses. Viral survival in an infected host depends to a large extent on evasion of the host immune system (Vossen et al. 2002). During coevolution, the host immune system acts in different ways to eliminate viruses, while viruses have developed strategies to evade host immunity, including the expression of cytokine genes that have been captured from the host (Hsu et al. 1990, Kotenko et al. 2000, Griffin et al. 2010). Infections with a number of different viruses are associated with elevated levels of IL-10 in the host (Slobedman et al. 2009), and this, in turn, appears to be associated with enhancement of the virus infection by suppression of parts of the host immune response. In fact, a number of viruses encode their own IL-10 homologue (vIL-10). An example is Epstein-Barr virus (EBV), where, during an infection, the vIL-10 may blunt the CMI response, thereby enhancing the opportunity for the virus to establish a latent infection (Kurilla et al. 1993).

We previously reported that CyHV-3 captured an IL-10 gene from a host and modulated it for the benefit of the virus (Sunarto et al. 2012). Whether the origin of the vIL-10 gene was carp or some other species is not known (Ouyang et al. 2013). We also demonstrated that carp IL-10 and KHV-encoded IL-10 (khvIL-10) function through the same IL-10R1 receptor. A cytomegalovirus-encoded IL-10 (cmvIL-10) has been described as having immune-inhibitory effects (Jenkins et al. 2004, 2008, Slobedman et al. 2009). However, little is known about the biological role of khvIL-10. Ouyang et al. (2013) reported that although khvIL-10 is among the most abundant proteins encoded by CyHV-3, it is not essential for either viral replication *in vitro* or for virulence *in vivo*.

Because of the global importance of CyHV-3, there have been great advances in our understanding of how the virus interacts with its host. In particular, studies of gene expression in carp during CyHV-3 infection have yielded insights into pathogenesis, the role of skin as a defence barrier (Adamek et al. 2013, Syakuri et al. 2013), the kinetics of cytokine gene expression (Ouyang et al. 2013), and differential immune responses toward CyHV-3 infection in resistant and susceptible carp lines (Rakus et al. 2012). However, all studies of gene expression were conducted during the acute phase of CyHV-3 infection. Taking advantage of our experimental model for persistent infection of CyHV-3 in carp (Sunarto et al. 2014), we now report the expression of immune-related genes of carp in each stage of CyHV-3 infection. These functional data provide evidence for a possible interaction between carp IL-12, carp IL-10 and khvIL-10 during the viral infection.

MATERIALS AND METHODS

Cell culture and virus isolate

The Indonesian CyHV-3 isolate (the C07 strain) used in this study was obtained from common carp suffering mass mortality in West Java, Indonesia, in 2007 (Sunarto et al. 2011). The virus was grown in cultures of the koi fin cell-line (KF-1) kindly provided by Professor R. P. Hedrick (University of California, Davis, CA, USA). The cells were maintained in Leibovitz L-15 medium (Life Technologies) supplemented with 10% foetal bovine serum (FBS; Thermo Trace), 2 mM L-glutamine (Invitrogen), 100 IU ml^{-1} penicillin (JRH Biosciences) and 100 µg ml^{-1} streptomycin (Sigma), and incubated at 25°C. A third passage virus was used for these experiments. The concentration of virus was estimated by determining the 50% tissue culture infective dose ($TCID_{50}$; Reed & Muench 1938).

Fish samples

An experimental model for acute, persistent and reactivation phases of CyHV-3 infection in carp has been described elsewhere (Sunarto et al. 2014). Briefly, 120 juvenile common carp (length = 12.1 ± 1.0 cm, mean ± SD) were distributed into 2 equal groups. Sixty carp were infected with CyHV-3 by immersion at a dose of 100 $TCID_{50}$ ml^{-1} for 2 h at 22°C. After exposure, fish were rinsed twice in fresh water and then separated into 2 groups: tank 1, in which fish were held at a water temperature of 22°C until there was evidence of clinical signs of KHVD with mortalities reaching 90% by 6 to 9 d post infection (dpi; acute phase), and tank 2, in which fish were

held initially at 22°C for 24 h and then the water temperature was decreased to 11°C over a period of 4 d, following which there was no evidence of disease (persistent phase). At 28 dpi, the water temperature in tank 2 was increased from 11 to 22°C in increments of 2 to 3°C d^{-1}, resulting in 45% mortality by Day 9 (reactivation phase). Two tanks of mock-infected carp from the same batch served as a control. Spleen was collected from 4 fish in the control group and from 4 fish at 7 (acute), 28 (persistent) and 37 (reactivation) dpi (Fig. 1). All samples were collected from fish that were euthanized, and the specimens were preserved in RNAlater and stored at −20°C until RNA extraction. No samples were collected from dead fish for this gene expression study. However, all fish were confirmed CyHV-3 positive by PCR. All experiments with carp were approved by the CSIRO Australian Animal Health Laboratory Animal Ethics Committee.

Nucleic acid extraction

Total DNA and RNA were extracted from RNAlater®-stabilised tissues using an AllPrep DNA/RNA extraction kit (Qiagen) according to the manufacturer's protocols. A 25 mg sample of tissue was homogenized using MagNA Lyser Green Beads (Roche) in RLT buffer (Qiagen). The DNA was bound to a cellulose membrane, eluted in 100 µl Buffer AE (Qiagen), and stored at −20°C. Following precipitation of the eluate with 70% ethanol, RNA was bound to a cellulose membrane by centrifugation of the precipitate in an RNeasy spin column at 8000 × *g* for 15 s. The DNA-free RNA was then washed with RPE buffer (Qiagen), eluted in 30 µl RNase-free water and stored at −20°C. Contaminating DNA in total RNA extracts was removed by on-column digestion with RNase-free DNase I (Qiagen).

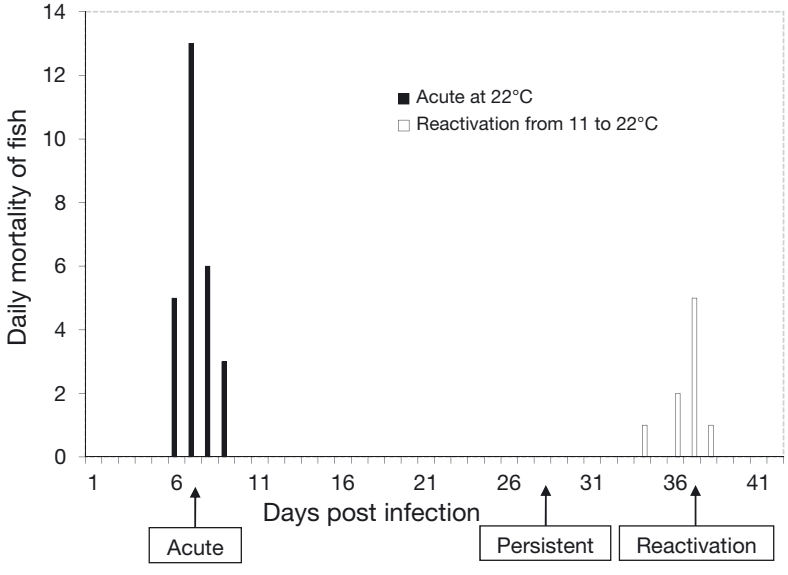

Fig. 1. Schematic time-line for the acute, persistent and reactivation phases of cyprinid herpesvirus 3 (CyHV-3) infection in common carp *Cyprinus carpio*. Carp were infected with CyHV-3 and kept at 22°C for 24 h. Infected fish were then separated into 2 groups: Tank 1 fish were held at a constant 22°C, resulting in 90% mortality within 9 dpi (acute phase, black bars). Water temperature in Tank 2 was reduced from 22 to 11°C from 1 dpi, held at that level until 28 dpi (no mortality observed during this period; persistent phase), then slowly raised to 22°C, resulting in 45% mortality by 38 dpi (reactivation phase, white bars). Samples were collected at 7, 28 and 37 dpi (arrows)

TaqMan qPCR amplification

A quantitative TaqMan real-time PCR (qPCR) targeting the CyHV-3 thymidine kinase (TK) gene was used for detection and quantification of CyHV-3 DNA. The forward primer TK F95 (5′-GCT GCA TCG CCG TCA AG-3′), reverse primer TK R162 (5′-GCT GTG CAT GGC CAC CTT-3′) and probe TK P113 (5′-ACG CCA TAG ACC AGC GCT ACA CCG-3′) were designed with Primer Express software version 3.0 (Applied Biosystems). The TK CyHV-3 probe was modified with FAM and TAMRA at the 5′ and 3′ ends, respectively (GeneWorks). The detection range of the assay, estimated by serial dilution of a plasmid bearing the TK gene, was 10^1 to 10^8 copies. The TaqMan qPCR was conducted using reagents supplied by Applied Biosystems, unless stated otherwise. The reaction mixtures were set up in triplicate and comprised 12.5 µl TaqMan Universal Master Mix, 1.25 µl each primer (18 µM) and probe (5 µM), 2 µl template DNA (100 ng $reaction^{-1}$), and 6.7 µl DNase-free water. A 7500 Fast Real-time PCR System was used with the following profile: 1 cycle at 50°C for 2 min and at 95°C for 10 min; 40 cycles at 95°C for 15 s and 60°C for 1 min. A plasmid bearing the TK gene was diluted in a 10-fold dilution series and used to generate a standard curve. Absolute quantification of viral DNA (viral copy number per 100 ng total DNA) was determined using 7500 Fast SDS software version 2.0.3 (Applied Biosystems).

SYBR Green real-time RT-PCR and gene expression

Primers for detection of expression from host and virus immune-related genes are listed in Table 1. The primers were designed with Primer Express software version 3.0 (Applied Biosystems) and manufactured by GeneWorks. Expression of host and viral genes during each phase of infection was quantified by SYBR Green real-time reverse transcription-PCR (qRT-PCR) and normalised against the expression of 18S rRNA. As transcripts of 18S rRNA are more abundant than host or viral transcripts (Hashimoto et al. 2004, Kuchipudi et al. 2012), a limiting-primer strategy for 18S rRNA was used to allow detection of both transcripts simultaneously. Each reaction was set up in triplicate and comprised 12.5 µl One-Step RT-PCR Master Mix, 0.6 µl MultiScribe™ and RNase Inhibitor Mix, 1.25 µl each primer (2 µM for 18S rRNA and 18 µM for viral and other host transcripts), 2 µl template DNA (100 ng reaction^{-1}) and 7.4 µl RNase-free water. A 7500 Fast Real-time PCR System was used with the following profile: 1 cycle at 48°C for 30 min and 95°C for 10 min; 40 cycles at 95°C for 15 s and 60°C for 1 min; and 2 cycles of melting curve at 95°C for 15 s and 60°C for 15 s. Relative gene expression level was quantified based on comparative threshold cycle ($\Delta\Delta C_T$) using 7500 Fast SDS software version 2.0.3 and relative expression software tools in REST 2009 V2.0.13 (Pfaffl et al. 2002). Differences in expression between group means of control and treated samples were assessed for statistical significance by randomisation tests available in the REST software.

Table 1. Primers used for SYBR Green real-time RT-PCR assays

Gene	Primer	Sequence (5′→3′)
Interferon γ1 (IFNγ-1)	IFNγ-1 F	TGCACTTGTCAGTCTCTGCT
	IFNγ-1 R	TGTACTTGTCCCTCAGTATTT
Interferon γ2 (IFNγ-2)	IFNγ-2 F	TCTTGAGGAACCTGAGCAGAA
	IFNγ-2 R	TGTGCAAGTCTTTCCTTTGTAG
Interleukin 1β (IL-1β)	IL-1β F	AAGGAGGCCAGTGGCTCTGT
	IL-1β R	CCTGAAGAAGAGGAGGCTGTCA
Interleukin 10 (IL-10)	IL-10 F	CGCCAGCATAAAGAACTCGT
	IL-10 R	TGCCAAATACTGCTCGATGT
Interleukin 12 (IL-12)	IL-12 F	TGCTTCTCTGTCTCTGTGATGGA
	IL-12 R	CACAGCTGCAGTCGTTCTTGA
KHV-interleukin 10 (khvIL-10)	khvIL-10 F	GATGTTCATTGTGAGCACCTTTTT
	khvIL-10 R	CATGAACGAACTCGCCTCAA
Major histocompatibility complex class II (MHC II)	MHC II F	CTGATGCTGTCTGCTTTCACTGG AGCA
	MHC II R	GAGTCAGCGATCCGTGATAAAACAG
Tumour necrosis factor (TNFα-1)	TNFα-1 F	GAGCTTCACGAGGACTAATAG ACAGT
	TNFα-1 R	CTGCGGTAAGGGCAGCAATC
18S rRNA[a]	18S rRNA F	CGGCTACCACATCCAAGGAA
	18S rRNA R	GCTGGAATTACCGCGGCT

[a]The primers for the 18S rRNA gene were designed by Applied Biosystems

RESULTS

Spleen, kidney and thymus are considered the major lymphoid organs in fish (Rombout et al. 2005) and represent suitable sources for immune cells which respond to viral infection (Rakus et al. 2012, Ouyang et al. 2013). The spleen is also among the target organs in which CyHV-3 is most abundant during acute infection (Gilad et al. 2004). In this study, the kinetics of expression of a number of host immune-related genes was determined in spleens collected from carp during acute, persistent and reactivation phases of CyHV-3 infection. As shown in Fig. 1, 2 clear stages of CyHV-3 outbreaks were observed. The first stage between 6 and 9 dpi is due to an acute primary infection. The second stage, at 6 to 10 d post-reactivation (34 to 38 dpi), is believed to be due to viral reactivation. Spleens were collected from carp in all groups at each phase of infection, i.e. the acute (6–9 dpi), persistent (28 dpi) and reactivation (34–38 dpi) phases, and tested for the presence of viral DNA and the expression level of viral and host immune-related genes.

Viral DNA load in the spleen

High concentrations of viral DNA were detected (up to 10^7 copies per 100 ng total DNA) during acute and reactivation phases in spleens of infected carp (Fig. 2). However, the viral load was significantly lower ($p < 0.05$) during persistent infection, 10^3 copies per 100 ng total DNA. No viral DNA was detected in mock-infected fish.

Expression of host and viral interleukin genes

IL-12 was expressed at significantly higher levels ($p < 0.05$) during all phases of

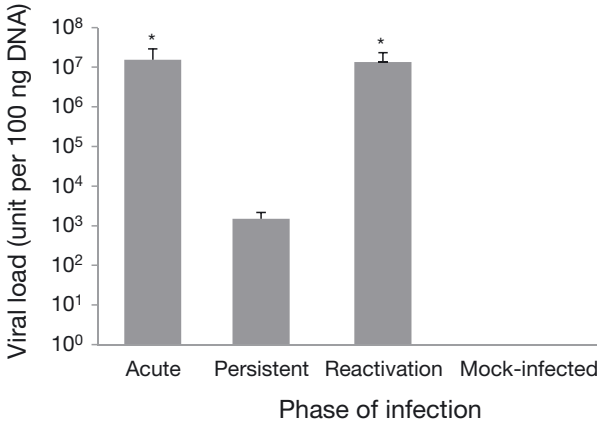

Fig. 2. Viral DNA load of cyprinid herpesvirus 3 (CyHV-3) in the spleens of common carp *Cyprinus carpio* during acute, persistent and reactivation phases of infection. The viral load was quantified using a TaqMan PCR assay for the CyHV-3 TK gene. Data are means ± SE of 4 individual fish per group. Asterisks (*) indicate a statistical difference ($p < 0.05$) between productive (acute and reactivation) and non-productive (persistent) phases of CyHV-3 infection

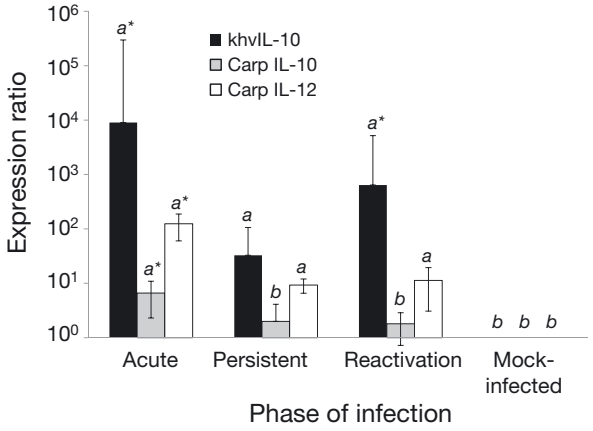

Fig. 3. Kinetics of expression of common carp *Cyprinus carpio* interleukin-12 (IL-12) and IL-10 and of koi herpesvirus interleukin-10 (khvIL-10) genes in the spleens of carp during the acute, persistent and reactivation phases of cyprinid herpesvirus 3 (CyHV-3) infection. Transcripts were quantified by SYBR Green qRT-PCR assays based on comparative threshold cycle ($\Delta\Delta C_T$) and normalised against expression of the 18S rRNA gene. Data are means ± SE of 4 individual fish per group. Different letters above bars indicate a statistical difference ($p < 0.05$) between infected and mock-infected fish. Asterisk (*) indicates a statistical difference ($p < 0.05$) between productive (acute and reactivation) and non-productive (persistent) phases of infection of CyHV-3 infection

CyHV-3 infection compared with mock-infected fish (Fig. 3). However, expression during the persistent and reactivation phases was significantly lower ($p < 0.05$) than during the acute phase. Carp IL-10 was significantly up-regulated ($p < 0.05$) during the acute phase of CyHV-3 infection, but not during the persistent and reactivation phases (Fig. 3). khvIL-10 was expressed at significantly higher levels during all phases of infection compared with mock-infected fish. In addition, expression of khvIL-10 was significantly higher in the acute and reactivation phases compared with the persistent phase ($p < 0.05$) of infected fish (Fig. 3).

IL-1β was significantly up-regulated ($p < 0.05$) in the acute phase of infection (Fig. 4A). There was little difference in expression levels of IL-1β between productive and non-productive phases.

Expression of host interferon gamma genes

IFNγ-1 was expressed at significantly higher levels ($p < 0.05$) in the spleen of carp during the productive (acute and reactivation) compared with the non-productive (persistent) phases of CyHV-3 infection (Fig. 4B). However, IFNγ-2 was only expressed at significantly higher levels ($p < 0.05$) in the spleen of carp during the reactivation compared with persistent phase of CyHV-3 infection (Fig. 4C). During the latter phase, the expression levels of IFNγ-1 and IFNγ-2 were comparable to those in mock-infected fish. In addition, IFNγ-1 expression during the acute phase was significantly higher ($p < 0.05$) than during other phases of infection (Fig. 4B).

Expression of other immune-related genes

There was no statistical difference in the expression of TNFα-1 and MHC-II genes in the spleen of carp during different phases of CyHV-3 infection, nor between infected and mock-infected fish (Fig. 5).

DISCUSSION

In this report, we document the expression of a number of immune-related genes, including host and viral cytokines, during the acute phase of CyHV-3 infection of carp. The kinetics of expression of immune-related genes of carp during the acute phase of CyHV-3 infection showed similar patterns to those previously reported (Rakus et al. 2012, Ouyang et al. 2013). However, some differences were also noted. For example, Ouyang et al. (2013) reported that TNFα-1 was significantly up-regulated in infected fish at 6 and 8 dpi, while we observed that the

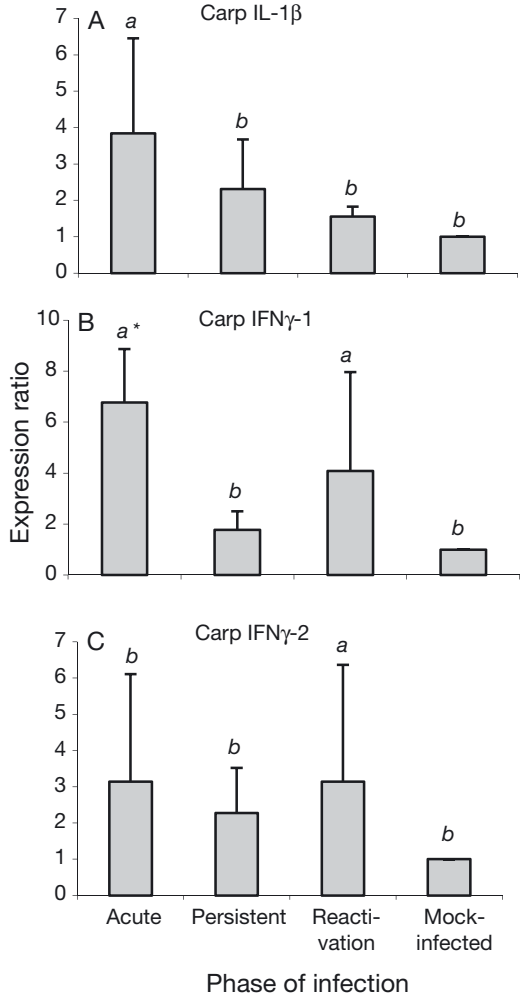

Fig. 4. Kinetics of expression of interleukin-1β and interferon gamma genes in the spleens of common carp *Cyprinus carpio* during the acute, persistent and reactivation phases of cyprinid herpesvirus 3 (CyHV-3) infection. Methods and statistics as described in Fig. 3

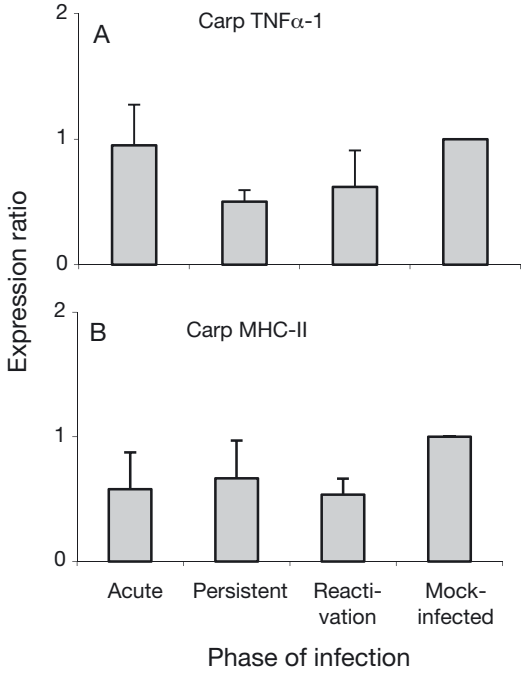

Fig. 5. Kinetics of expression of other immune-related genes in the spleens of common carp *Cyprinus carpio* during the acute, persistent and reactivation phases of cyprinid herpesvirus 3 (CyHV-3) infection. Transcripts were quantified as described in Fig. 3. Data are means ± SE of 4 individual fish per group. There was no statistical difference in gene expression between infected and mock-infected fish, nor between productive (acute and reactivation) and non-productive (persistent) phases of CyHV-3 infection

expression of TNFα-1 at a similar stage of infection was comparable to those of mock-infected fish. We also report the first comparative expression of these genes during persistent and reactivation phases of infection, and we propose how the products of these various genes may interact during infection of carp by CyHV-3.

Herpesvirus survival at each stage of its life cycle — acute, persistent and reactivation phases — depends on evasion of the host immune response. The fact that herpesviruses persist for life indicates that they have evolved successful strategies to thwart host immunity (Vossen et al. 2002, Powers et al. 2008, Ressing et al. 2008). One such strategy is for the virus to acquire a host IL-10 gene. Jenkins et al. (2004) found that human cytomegalovirus (HCMV) has a cmvIL-10 gene that may help HCMV avoid recognition during the acute phase of infection. Like the host IL-10, it is proposed that cmvIL10 promotes an antibody response, but also suppresses the CMI response by suppressing IL-12 and other cytokines such as IL-2, TNFα and IFNγ. While specific antibody would mop up extracellular virus, even at this early stage of infection some virus will have already gained the sanctuary of a host cell. For long-term virus survival, it is therefore important that the virus begins to down-regulate the CMI response.

A potentially similar strategy involving a viral-encoded IL-10 (khvIL-10) has also been observed for CyHV-3 infection in carp (Sunarto et al. 2012). The current study focused on a possible association between host IL-12 and IL-10, and khvIL-10. The host genes are significantly up-regulated (compared with mock-infected fish) during some stages of CyHV-3 infection (Fig. 3), and the results are consistent with a previous report that these cytokines are interconnected and normally play a crucial role

against viral infection (Holland & Zlotnik 1993). However, in the case of infection by CyHV-3, the activities of these host cytokines appear to have been modulated by khvIL-10 (Fig. 3). While IL-12 is significantly up-regulated during all phases of infection, the levels of expression are approximately an order of magnitude lower in the persistent and reactivation phases. Given the low levels of expression of carp IL-10 during these phases, it seems unlikely that host IL-10 was associated with negative regulation of IL-12 expression. A more likely explanation is that the significantly higher expression of khvIL-10 not only suppresses host IL-10, but is also responsible for the decreased activity of host IL-12 during the persistent and reactivation phases. In the case of IL-10, carp IL-10 and khvIL-10 function via the same IL-10R1 receptor (Sunarto et al. 2012).

Based on the data in the current study (Fig. 3), we propose a possible interaction between carp IL-12, carp IL-10 and khvIL-10 following infection of carp by CyHV-3. We suggest that viral khvIL-10 is produced early in the productive (acute and reactivation) phases of infection, and that it may delay and blunt the host CMI response by suppressing expression of host IL-12. This would allow the virus to establish an intracellular infection that could potentially lead to a persistent infection (Kurilla et al. 1993). Even if khvIL-10 promoted the production of specific antibodies in the host by simulating host IL-10 activity and provoking a Th-2-dominated immune response (de Lemos Rieper et al. 2011), much virus would already be cell-associated, and therefore unaffected by a specific humoral response. Of course, these are only observations on a possible association among these 3 cytokines. Confirmation of a relationship would require further work (see later discussion).

In addition to these changes, we demonstrated that IL-1β expression was also significantly up-regulated ($p < 0.05$) during the acute phase of infection. However, the expression level decreased during the persistent infection, and reactivation of infection did not increase the expression level of IL-1β. The results are consistent with previous reports by Rakus et al. (2012), Adamek et al. (2013) and Syakuri et al. (2013), who observed that the IL-1β gene was highly expressed at 3 and 5 dpi, but significantly decreased at 14 dpi to levels comparable to those at 0 dpi.

This is the first report of the expression of IFNγ genes in carp during CyHV-3 infection. IFNγ-1 and IFNγ-2 were slightly, but significantly, up-regulated during the acute and reactivation phases, but not during the persistent phase of CyHV-3 infection. In particular, expression of IFNγ-1 was significantly lower in the persistent and reactivation phases compared with the acute phase (Fig. 4B). Such a finding would be consistent with the observed suppression of IL-12 expression in these phases (Trinchieri 1997). Unlike mammals, which have a single IFNγ gene, fish have 2 IFNγ genes, i.e. IFNγ-1 and IFNγ-2 (Igawa et al. 2006, Stolte et al. 2008). These genes have different abilities to induce downstream gene expression by binding to different receptor complexes (Aggad et al. 2010). IFNγ signals mainly through the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway to achieve transcriptional activation (Hu & Ivashkiv 2009). Previous reports indicated that type I interferon (IFNα and IFNβ) showed a different response to CyHV-3 infection. There is no difference in expression of the IFNαβ gene in the spleen of carp during CyHV-3 infection (Rakus et al. 2012). However, IFNα-1 and IFNα-2 were highly up-regulated in the skin and gut of carp during CyHV-3 infection (Adamek et al. 2013, Syakuri et al. 2013).

TNFα is an important factor in the early immune response, as it is expressed within hours of viral infection, but then rapidly declines to below basal levels (Paludan et al. 2001). In the current study, samples were collected from moribund fish well into the course of infection at 6 to 9 dpi, at 28 dpi during persistent infection or during the reactivation phase. The timing of these samples possibly accounts for why TNFα gene expression in carp during all phases of CyHV-3 infection was slightly lower than that in mock-infected fish. Our results also indicated that CyHV-3 infection slightly decreased the expression of carp MHC II in each phase of infection, although this down-regulation was not statistically significant. The results are similar to those reported for HCMV infection in humans (Jenkins et al. 2008). Down-regulation of MHC hinders the ability of T lymphocytes to recognize and eliminate virus-infected cells (Griffin et al. 2010).

The results from this short study lay the foundation for further investigation of the roles of, and relationships between, immune-related genes of carp and virus during infection by CyHV-3 (in particular carp IL-10 and -12, and khvIL-10). One possible means for extending the results might be through the use of a khvIL-10-deficient mutant CyHV-3 (Ouyang et al. 2013) to reveal the relative importance of host versus virus IL-10 in the different phases of infection. While Ouyang et al. (2013) explored the effects of khvIL-10 only during the acute phase of infection, they also

flagged their intention to study the effects of khvIL-10 during latency. The development of an IL-10-deficient carp (possibly through the use of RNA interference technology) would also be an interesting approach for the study of host and virus immune-related genes.

The data from the current study provide some insight into the pathogenesis of CyHV-3 in carp, including the active role of the virus in modulating the anti-viral host immune response. In particular, this study revealed that, following a CyHV-3 infection, the subsequent khvIL-10-influenced cytokine profile of the host may cause a Th-2-dominated response (especially characterized by suppression of IL-12 and IFNγ-1 in the persistent and reactivation phases) instead of an expected Th-1 response. While khvIL-10 may not affect replication *in vitro* or virulence *in vivo* (Ouyang et al. 2013), we believe that the data from the current study provide preliminary evidence that khvIL-10 is very influential in virus survival in infected carp by promoting a Th-2 immune response that only really becomes manifest beyond the acute phase of infection.

More detailed recent studies have revealed broader implications for pathogen-modulated host immune responses. These studies revealed how infection of a mammal with a helminth worm, also favouring a Th-2 immune response, can actually either enhance the chances of a dual viral infection (Osborne et al. 2014) or cause reactivation of a persistent viral infection (Reese et al. 2014). Understanding these relationships may have important implications in the prevention and treatment of some important infectious diseases of fish, especially in the face of dual infection with other pathogens. For example, recent research has reported that rainbow trout expressed IL-12 in response to the myxozoan parasite *Tetracapsuloides bryosalmonae*, as well as bacterial (*Yersinia ruckeri*) and viral (viral haemorrhagic septicaemia virus) infections (Wang et al. 2014). Knowing that a particular cytokine(s) may correlate with induced protection could be important for future vaccine development (Wang & Secombes 2013). In addition, unravelling the underlying strategies of viral evasion would be an important step toward identification of novel molecular targets for developing therapeutic and antiviral vaccines (Donnelly & Kotenko 2006). For example, vaccination against vIL-10 has been shown to significantly enhance innate immunity and restrict replication of HCMV (Eberhardt et al. 2013), and this approach may be worthy of investigation in the case of CyHV-3 infections of carp.

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