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Cyprinid Herpesvirus 3 Il10 Inhibits Inflammatory Activities of Carp Macrophages and Promotes Proliferation of Igm$^+$ B Cells and Memory T Cells in a Manner Similar to Carp Il10

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Cyprinid herpesvirus 3 (CyHV-3) is the causative agent of a lethal disease of carp and encodes for an Il10 homolog (ORF134). Our previous studies with a recombinant ORF134-deleted strain and the derived revertant strain suggested that cyprinid herpesvirus 3 Il10 (CyHV-3 Il10 [cyhv3Il10]) is not essential for viral replication in vitro, or virulence in vivo. In apparent contrast, cyhv3Il10 is one of the most abundant proteins of the CyHV-3 secretome and is structurally very similar to carp Il10 and also human IL10. To date, studies addressing the biological activity of cyhv3Il10 on cells of its natural host have not been performed. To address the apparent contradiction between the presence of a structurally conserved Il10 homolog in the genome of CyHV-3 and the lack of a clear phenotype in vivo using recombinant cyhv3Il10-deleted viruses, we used an in vitro approach to investigate in detail whether cyhv3Il10 exerts any biological activity on carp cells. In this study, we provide direct evidence that cyhv3Il10 is biologically active and, similarly to carp Il10, signals via a conserved Stat3 pathway modulating immune cells of its natural host, carp. In vitro, cyhv3Il10 deactivates phagocytes with a prominent effect on macrophages, while also promoting proliferation of Igm$^+$ B cells and memory T cells. Collectively, this study demonstrates a clear biological activity of cyhv3Il10 on cells of its natural host and indicates that cyhv3Il10 is a true viral ortholog of carp Il10. Furthermore, to our knowledge, this is the first report on biological activities of a nonmammalian viral Il10 homolog. *The Journal of Immunology*, **2015, 195: 3694–3704.**

Throughout evolution, viruses have evolved to coexist with their host by developing strategies that help to avoid immune surveillance and elimination. For example, regulation of the host cytokine network by virus-encoded proteins is a common strategy to limit the ability of the host to clear the virus and thus helps to achieve successful establishment of infection (1). To this end, many viruses have acquired homologs of host cytokines or cytokine receptors, among which is IL10 (1, 2). Indeed, IL10-like open reading frames (ORFs) have been identified in multiple members of the Poxviridae and in members of the Herpesvirales, including human CMV and EBV (3–5). Almost all viruses for which IL10 homologs have been described are viruses infecting mammalian hosts, with only two reported exceptions, both in fish: anguillid herpesvirus 1 (Herpesvirales, Alloherpesviridae), infecting European eel (*Anguilla anguilla*) (6), and cyprinid herpesvirus 3 (CyHV-3, Herpesvirales, Alloherpesviridae), infecting common carp (*Cyprinus carpio*) (7). Thus, CyHV-3 is one of only few nonmammalian viruses known to express an Il10 homolog.

IL10 is one of the most important anti-inflammatory cytokines with a key role in the termination of inflammation and restoration of homeostasis. IL10 is a pleiotropic regulatory CyHV-3 Il10 (cyhv3Il10) cytokine that acts on different cell populations from both the innate and adaptive branches of the immune system. Structural analysis indicates that teleost fish, despite low sequence identity for Il10, possess a class II cytokine system surprisingly similar to that of humans (8). In a recent study, we showed that carp Il10 (cIl10) downregulates the inflammatory response of phagocytes, stimulates proliferation of subsets of memory T lymphocytes, and stimulates proliferation, differentiation, as well as Ab secretion by Igm$^+$ B lymphocytes (9). cIl10 acts through a conserved signaling pathway involving phosphorylation of Stat3, ultimately leading to an early upregulation of *socs3*, a cytokine-inducible negative regulator of cytokine signaling. Thus, mammalian IL10 and cIl10 share functional conservation of several biological activities.

ORF134 of CyHV-3 encodes for an Il10-like protein and is the second most abundant of all proteins in the CyHV-3 secretome (10). Furthermore, ORF134, here referred to as cyprinid herpesvirus 3 Il10 (CyHV-3 Il10 [cyhv3Il10]), is highly expressed in tissue of infected carp during the acute and reactivation phases of CyHV-3 infection and at lower levels during virus persistence at low temperature (11). It is likely that cyhv3Il10 helps to avoid immune surveillance and elimination by specifically targeting components of the carp immune system. However, in a recent study using infection with Il10-deleted and Il10-revertant recombinant CyHV-3 strains, we could not show that cyhv3Il10 is essential for replication in vitro or for virulence in vivo (10), indicating that the full potential of this viral gene product could not be revealed under the conditions used,

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Abbreviations used in this article: cIl10, carp Il10; cRPMI, complete RPMI; CyHV-3, cyprinid herpesvirus 3; cyhv3Il10, CyHV-3 Il10; HKL, head kidney leukocyte; LB, lysogeny broth; MHCI, MHC class I; MHCII, MHC class II; ORF, open reading frame; PBL, peripheral blood leukocyte; RT, room temperature; RT-qPCR, real-time quantitative PCR.

thereby leaving the exact immunobiological function of cyhv3Il10 unresolved. Also, a recent study in zebrafish embryos injected with CyHV-3 *il10* mRNA showed more lysozyme-positive cells, the effect of which could be abrogated by morpholino-mediated down-regulation of *il10 receptor-1* (*il10r1*), providing only indirect evidence for a biological role of cyhv3Il10 (11).

Given the apparent contradiction between the presence of an Il10 homolog in the genome and secretome of CyHV-3 and the absence of an obvious role for cyhv3Il10 in viral replication or virulence, in the present study, we investigated in detail the biological activity of cyhv3Il10 on cells of its natural host, the common carp. We demonstrate that, similar to cIl10, cyhv3Il10 acts through a conserved signaling pathway involving phosphorylation of Stat3, leading to an early upregulation of *socs3* expression in host cells. Furthermore, we studied the effect of cyhv3Il10 on cells of the innate as well as adaptive branches of the immune system of carp. Biological activity of cyhv3Il10 was examined by studying its ability to modulate oxygen and nitrogen radical production as well as proinflammatory gene expression in carp neutrophils and macrophages. We compared the biological effects of cIl10 and cyhv3Il10 by analyzing Mhc class I surface expression on carp leukocytes and by monitoring B cell and T cell proliferation. In summary, we describe activities of cyhv3Il10 on host (carp) immune cells, some of which are similar and some distinct from effects induced by cIl10. Our study demonstrates clear biological functions of cyhv3Il10 and, to our knowledge, is the first to provide evidence for direct biological activities of a nonmammalian viral Il10 homolog.

Materials and Methods

Production of concentrated supernatants from cells infected with CyHV-3 strains

A recombinant CyHV-3 FL strain deleted for the ORF134 (cyhv3Il10) and the derived revertant strain were produced using bacterial artificial chromosome cloning technologies as previously described (10). For clarity, throughout this study ORF134 is referred to as cyhv3Il10. To obtain cell culture supernatants enriched for, or depleted of, cyhv3Il10, CCB cells were infected with wild-type, cyhv3Il10-deleted, and cyhv3Il10-revertant recombinant CyHV-3 FL strains, or mock infected as described previously (10).

Production of recombinant cIl10 and cyhv3Il10

Recombinant cIl10 was produced as previously described (9).

Recombinant cyhv3Il10 was produced as follows. The mature form of cyhv3Il10 (Arg^{18}-His^{176}, GenBank, http://www.ncbi.nlm.nih.gov/genbank/ accession no. AJP55775.1) was ordered as a synthetic gene, codon optimized for expression in *Escherichia coli*, including additional BamHI (5′ end) and HindIII (3′ end) restriction sites, and delivered into pMA-T vector (Invitrogen, GeneArt). The generation of a codon-optimized synthetic gene was necessary, as the wild-type sequence substantially hindered bacterial growth. The gene was subsequently cloned between the BamHI and HindIII sites in-frame with the hexahistidine tag of a modified pET15b expression vector (Novagen) and transformed into *E. coli* BL21DE CodonPlus (Agilent Technologies). Bacteria containing the cyhv3Il10 plasmid were plated on lysogeny broth (LB) agar plates supplemented with ampicillin (50 μg/ml), chloramphenicol (50 μg/ml), and glucose (1%) and incubated overnight at 37°C. Half of the colonies were then transferred to 500 ml LB medium supplemented with ampicillin and chloramphenicol and incubated shaking at 200 rpm at 37°C until an OD_{600} of 0.6–0.8 was reached. Expression was induced by addition of isopropyl β-D-thiogalactoside (1 mM) and continued culturing for 4 h at 37°C. After incubation, bacteria were pelleted, resuspended in 40 ml 0.1 mg/ml lysozyme in 20 mM Tris-HCl, 500 mM NaCl and incubated for 30 min at room temperature (RT) to break down the peptidoglycan layer. Bacteria were lysed by adding 100 mM DTT, 50 mM EDTA, and 10% Triton X-100 (v/v) followed by four cycles of freeze-thawing. $MgCl_2$ was added to a final concentration of 0.05 M and released chromosomal DNA was digested by Benzonase (Novagen) treatment (2 U/ml) performed for 30 min at RT with slow rotation. Inclusion bodies were pelleted by centrifugation at 10,000 × *g* for 15 min, washed with 20 mM Tris-HCl, 500 mM NaCl, and 1% Triton X-100 (v/v) and solubilized by shaking at RT for 1 h in 10 vol 50 mM Tris-HCl, 10 M urea, and 25 mM imidazole. After clarification through a 0.2-μm filter, hexahistidine-tagged proteins were purified using Ni^{2+}-NTA agarose beads (Qiagen). Briefly, the solubilized inclusion bodies were incubated with 3 ml 50% Ni-NTA agarose beads slurry with rotation for 30 min at RT. The slurry was allowed to settle in a 12-ml column, washed with 5-column vol of cold (4°C) 20 mM Tris-HCl, 500 mM NaCl, 8 M urea, 25 mM imidazole, 1% (v/v) Triton X-100; 20-column vol cold 20 mM Tris-HCl, 500 mM NaCl, 8 M urea, 25 mM imidazole, 1% (v/v) Triton X-114; 5-column vol cold 20 mM Tris-HCl, 500 mM NaCl, 6 M urea, 40% (v/v) isopropanol; and 5-column vol cold 20 mM Tris-HCl, 500 mM NaCl, 8 M urea, 25 mM imidazole. Proteins were eluted with cold 20 mM Tris-HCl, 500 mM NaCl, 8 M urea, and 250 mM imidazole and refolded by rapidly diluting 10 times in 50 mM Tris-HCl, 0.1 mM oxidized glutathione, 0.5 mM reduced glutathione, and 0.5 M L-arginine followed by overnight incubation at 4°C with stirring. Finally, the refolded proteins were dialyzed against PBS, centrifuged at 10,000 × *g* to remove any precipitate, filtered sterilized, mixed with 20% (v/v) glycerol, and stored at −80°C until further use. All steps were carried out using endotoxin-free materials. The purity of the protein was assessed by SDS-PAGE analysis and the concentration was assessed using a NanoDrop 1000 spectrometer (Thermo Scientific).

An optimal stimulatory concentration of 0.5 U/ml recombinant cIl10, corresponding to 10 μg/ml recombinant protein, has previously been defined based on titration using a EPC–NF-κB–Luc cell line (9). This method proved unsuitable for the titration of cyhv3Il10 because cyhv3Il10 did not cross-react with the fathead minnow (EPC) cells, for which reason in the present study we show concentrations rather than units.

Production of recombinant carp Il2

Recombinant carp Il2a and Il2b were produced as follows. The nucleotide sequences encoding for the mature carp Il2a and Il2b peptides (GenBank, http://www.ncbi.nlm.nih.gov/genbank/ accession nos. KP771860 and KP771861, respectively) were cloned between the BamHI and HindIII sites of the pQE30-UA vector (Qiagen) in-frame with the N-terminal RGS-6xHis coding sequence. The mature protein includes amino acids Gln^{21}-Leu^{129} for Il2a and Gln^{21}- Gln^{122} for Il2b. Plasmids were transformed in M15 competent *E. coli* cells, and positive clones were selected on plates supplemented with ampicillin (100 μg/ml) and kanamycin (25 μg/ml) overnight at 37°C. One single colony was transferred to 20 ml LB medium supplemented with antibiotics and grown overnight at 37°C with shaking. The overnight culture was transferred to 1 l prewarmed Terrific Broth and incubated at 30°C with shaking. At OD_{600} of 0.6–0.8, protein production was induced with 1 mM isopropyl β-D-thiogalactoside and the culture was incubated for a further 4 h at 30°C. Bacteria were pelleted and lysed as described above. After centrifugation at 3360 × *g* for 20 min at 4°C, the proteins were isolated from the soluble fractions as described above with the exception that urea was omitted from the purification buffers and therefore refolding was not necessary.

Fish

European common carp (*Cyprinus carpio carpio* L.) were bred and raised in the central fish facility "Carus" at Wageningen University, the Netherlands, at 23°C in recirculating UV-treated tap water and fed pelleted dry food (Sniff, Soest, Germany) daily. R3xR8 carp, which are the offspring of a cross between fish of Hungarian origin (R8 strain) and of Polish origin (R3 strain), were used (12). All experiments were performed with the approval of the Animal Experimental Committee of Wageningen University. Fish immunized against the parasite *Trypanoplasma borreli* were obtained as previously described (9, 13). Briefly, fish were injected with 1×10^4 parasites per fish. Two weeks later, when parasitemia reached 1×10^6 to 1×10^7 parasites/ml blood, fish were injected once with 10 mg/kg melarsoprol (Arsobal), a drug for treatment of infections with human trypanosomes, previously shown to be effective against carp trypanosomes (9, 13). All experiments with immunized fish were performed 3 mo after parasite clearance.

Cell isolation and culture

For organ isolation, fish were killed with an overdose of tricaine methane sulfonate (Crescent Research Chemicals, Phoenix, AZ). Peripheral blood leukocytes (PBL), mid-kidney leukocytes, head kidney leukocytes (HKL), and total thymocytes were isolated as described previously (14, 15) and resuspended in the appropriate medium for the experiment as described below. Head kidney–derived mature macrophages (referred to as macrophages) were obtained upon 6 d culture of HKL as previously described (16).

MACS was used in combination with carp leukocyte-specific Abs to isolate carp neutrophils from mid-kidney leukocytes, and carp B cells from PBL as previously described (13, 17–19). The purity of the sorted

leukocytes was >98% as confirmed by flow cytometry using a BD FACSCanto A (BD Biosciences). After isolation, cells were washed in complete RPMI (cRPMI) with HEPES (RPMI 1640 with 25 mM HEPES [Lonza, Nalgene], adjusted to 280 mOsmol/kg and supplemented with 2 mM L-glutamine, 100 U/ml penicillin G, and 50 mg/ml streptomycin sulfate) and resuspended in the appropriate medium for the experiment as described below.

Generation of polyclonal Abs against cyhv3Il10 and cIl10

Mouse polyclonal Abs directed against the mature cyhv3Il10 (Arg^{18}-His^{176}) were produced by DNA immunization using a customized commercial service (Delphi Genetics). Affinity-purified polyclonal rabbit IgG anti-cIl10 was produced by immunization of rabbits with purified recombinant cIl10 according to a 3 mo standard protocol (Eurogentec).

Western blot analysis

To investigate whether cyhv3Il10 would trigger Stat3 phosphorylation, as confirmed previously for cIl10 (9), HKL (5×10^6) were incubated for 10 min with 10 μg/ml recombinant cyhv3Il10 or with 1:5 diluted cell culture supernatants from CCB cells infected with the above-described wild-type/recombinant/revertant CyHV-3 strains. HKL cell lysates were prepared and the cytoplasmic fraction (20 μg/lane) was assessed for the presence of phospho-Stat3 using a cross-reacting anti–phospho-Stat3 (Tyr^{705}) (3E2) mouse mAb (1:500, Cell Signaling Technology) as previously described (9). A cross-reacting anti–β tubulin rabbit Ab (1:1000, Abcam) was used to ensure equal loading. Proteins were visualized by chemiluminescence detection (Western Bright ECL Western blotting detection kit; Advansta) on x-ray films.

To confirm the presence of cyhv3Il10 and the absence of cIl10 in supernatants of CCB cells infected with the above-described wild-type/recombinant/revertant CyHV-3 strains, 20 μl supernatant was resolved on a 12.5% SDS-PAGE gel. One microgram recombinant cyhv3Il10 and cIl10 were included as controls for the respective Abs. Upon protein transfer to nitrocellulose membranes (Protran: Schleicher & Schuell BioScience) were blocked for 2 h at RT with 5% (w/v) nonfat dry milk in TBS-T (50 mM Tris, 0.15 M NaCl [pH 7.4], 0.2% (v/v) Tween 20) and incubated overnight at 4°C with a 1:500 dilution of mouse anti-cyhv3Il10 or 1:1000 dilution of rabbit anti-cIl10 in 5% nonfat dry milk TBS-T. HRP-conjugated goat anti-mouse or HRP-conjugated goat anti-rabbit Abs (1:1000, Dako) in 5% nonfat dry milk were used as secondary Abs (2 h at RT) and the proteins were visualized by chemiluminescence detection as described above.

Oxygen radical production

To measure the production of oxygen radicals produced by carp macrophages and neutrophils, a real-time luminol-ECL assay was performed as previously described (20). Briefly, 50 μl luminol (10 mM [Sigma-Aldrich] in 0.2 M borate buffer [pH 9.0]) and 50 μl stimulus (1 or 10 μg/ml recombinant cyhv3Il10 or recombinant cIl10 and 0.1 μg/ml PMA in cRPMI) were added to the wells of a white 96-well plate with opaque bottom (Corning). Fifty microliters cell suspensions (1×10^6 neutrophils or 0.5×10^6 macrophages/well) in cRPMI was added and chemiluminescence emission was measured with a FilterMax F5 multi-mode microplate reader (Molecular Devices) after 45 min incubation at 27°C. This time point was already shown to correspond to the maximum release of oxygen radicals by neutrophils and macrophages under the same experimental conditions (9).

Nitrogen radical production

The production of nitrogen radicals was determined as previously described (21). Briefly, neutrophils (1×10^6 /well) or macrophages (0.5×10^6 /well) were seeded in 96-well plates (Corning) and stimulated with 20 μg/ml LPS in combination with 1 or 10 μg/ml recombinant cyhv3Il10 or recombinant cIl10 in a total volume of 150 μl complete medium: cRPMI supplemented with 1.5% pooled carp serum for carp neutrophils, or complete NMGFL-15 medium (16) for macrophages. Neutrophils were incubated for 96 h and macrophages for 24 h at 27°C.

Gene expression analysis by real-time quantitative PCR

To measure changes in gene expression, 5×10^6 cells were stimulated with LPS (50 μg/ml) in the presence or absence of 10 μg/ml recombinant cyhv3Il10 or cIl10 or 20% (v/v) cell culture supernatants from CCB cells infected with the above-described wild-type/recombinant/revertant CyHV-3 strains. A time point of 3 h after stimulation was found optimal to assess *socs3* expression whereas 6 h was optimal to assess proinflammatory gene expression (9). Total RNA was isolated using the RNeasy kit (Qiagen), including on-column DNase treatment, according to the manufacturer's instructions, and stored at −80°C. Prior to cDNA synthesis, 500 ng total RNA was subject to a second treatment with DNase I, amplification grade (Invitrogen), and cDNA was synthesized using random primers (300 ng) and SuperScript III first-strand synthesis for RT-PCR (Invitrogen). cDNA samples were further diluted 25 times in nuclease-free water prior to real-time quantitative PCR (RT-qPCR) analysis.

RT-qPCR analysis was performed with a Rotor-Gene 6000 (Corbett Research) using ABsolute qPCR SYBR Green mix (Thermo Scientific). The primers used for RT-qPCR are shown in Table I. Fluorescence data from RT-qPCR experiments were analyzed using Rotor-Gene analysis software version 1.7. The take-off value for each sample and the average reaction efficiencies for each primer set were obtained upon comparative quantitation analysis from Rotor-Gene software (23). The relative expression ratio of a target gene was calculated based on the average reaction efficiency and the take-off deviation of sample versus control and expressed relative to the *s11* protein of the *40s* subunit as reference gene.

MhcI (Ua1) surface expression

HKL (2×10^6) were seeded in 48-well plates (Corning) and stimulated with recombinant cyhv3Il10 or recombinant cIl10 (10 μg/ml) in 200 μl complete advanced DMEM/F-12 (Life Technologies) (with 2 mM L-glutamine, 100 U/ml penicillin G, 50 mg/ml streptomycin sulfate, 1% FCS, and 10^{-5} M 2-ME) for 2 and 4 d at 27°C in the presence of 5% CO_2. Cells were then washed in FACS buffer (0.5% BSA, 0.05% NaN_3 in PBS), incubated with 50 μl 1:100 dilution of polyclonal rabbit anti-carp Ua1 (24) and a 1:50 dilution of monoclonal mouse anti-carp Igm (WCI12) (25) or mouse anti-carp neutrophils (TCL-BE8 (26) in FACS buffer for 30 min on ice. Cells were subsequently stained with polyclonal swine anti–rabbit Igs-FITC (Dako) and polyclonal goat anti-mouse Igs-RPE $F(ab')_2$ (Dako) (1:100 in FACS buffer) for 30 min on ice. The expression in total HKL of MhcI (Ua1) membrane molecules, Igm^+ B cells, and neutrophils was monitored by flow cytometry using a FACSCanto A (BD Biosciences) and analyzed using Flowing software v2.5.1 (Turku Centre for Biotechnology, Turku, Finland).

Proliferation assays

Proliferation was monitored using CFSE labeling as described previously (9). Briefly, sorted Igm^+ B cells, total thymocytes, or HKL were washed and resuspended in 0.1% BSA in PBS and a final concentration of 10 μM CFSE/5×10^6 cells was added. Cells were incubated at 27°C for exactly 10 min, diluted five times with cold RPMI 1640 containing 10% FCS, incubated on ice for 5 min, and washed three times with cold RPMI 1640 containing 10% FCS. Cells were then resuspended in complete advanced DMEM/F-12.

To study the effect of cyhv3Il10 on the proliferation of Igm^+ B cells, 2×10^6 CFSE-stained sorted Igm^+ B cells were seeded in a 48-well plate (Corning) and stimulated with 10 μg/ml recombinant cyhv3Il10 or cIl10 in the presence or absence of LPS (50 μg/ml) or *Trypanoplasma borreli* parasite lysate (0.5 parasites/cell) for 6 d at 27°C, in the presence of 5% CO_2. Cells were then analyzed by flow cytometry on a FACSCanto A (BD Biosciences).

To study the effect of recombinant cyhv3Il10 or cIl10 on the proliferation of thymocytes, a pilot study was performed to optimize culture conditions for proliferation. Briefly, 2×10^6 CFSE-labeled total thymocytes were seeded in 48-well plates in a final volume of 200 μl complete advanced DMEM/F-12 and incubated for 3 and 6 d with different concentrations of pooled carp serum (0.5, 1, and 2% [v/v]). To some wells, both 0.5 μg/ml carp Il2a and 0.5 μg/ml carp Il2b were added 48 h prior to sample analysis. Proliferation was analyzed by flow cytometry and the optimal culture conditions were determined as 6 d incubation with 0.5% (v/v) pooled carp serum with the addition of 0.5 μg/ml both carp Il2a and Il2b 48 h prior to sample analysis. The effects of 10 μg/ml recombinant cyhv3Il10 or cIl10 on thymocyte proliferation were tested under these optimized culture conditions in the presence of Il10 throughout the culture period.

In our previous study on the biological characterization of cIl10 we observed a clear effect of cIl10 on proliferation of memory T cells (9). To investigate the effects of cyhv3Il10 on proliferation of memory T cells we used a similar experimental set-up (9). Briefly, CFSE-labeled HKL (2×10^6 cells/well) from *T. borreli*–immunized fish were stimulated with cyhv3Il10 in the presence or absence of *T. borreli* lysate (concentration equivalent to 0.5 parasite/cell) for 6 d at 27°C in the presence of 5% CO_2. After incubation, nonadherent cells were collected and washed in FACS buffer and fixed and permeabilized using the BD Cytofix/Cytoperm fixation/permeabilization kit (BD Biosciences) following the manufacturer's instructions. Cells were then washed in FACS buffer and incubated with 50 μl 1:100 dilution of anti-Zap70 rabbit mAb (99F2, Cell Signaling Technology) in FACS buffer for 30 min on ice. Following two washes, 50 μl 1:100 dilution of R-PE–conjugated goat anti-rabbit IgG (H+L) Ab (Invitrogen Molecular Probes) was added and cells were in-

cubated for 30 min on ice before the last washes and flow cytometry analysis using a FACSCanto A (BD Biosciences).

In all flow cytometry experiments, prior to each analysis, propidium iodide (5 μg/ml) was added to a fraction of each sample to monitor cell viability and to set a "live cells only" gate. Only samples with cell viability >90% were used, and 10,000 events within the live-cells-only gate were used for the analysis.

Statistical analysis

Statistical analysis was performed using the statistical software R (3.0.2) (27). Analyses were performed on mean values or on proportions (for the proliferation assays) after testing the dataset for a normal distribution using the Shapiro–Wilk test for small samples. For normally distributed datasets, significant differences were evaluated using a Student *t* test. When the conditions for normal distribution were not met, nonparametric tests (Mann–Whitney–Wilcoxon) were used. In all cases, data were analyzed as paired data to eliminate the interference caused by the variability among individual fish. For gene expression analysis, relative expression ratios (R) were calculated as described, and transformed [ln(R)] values were used for statistical analysis. For multiple comparisons, one-way ANOVA followed by a Tukey test was used. In all cases, differences were considered significant at $p < 0.05$.

Results

cyhv3Il10 signals via a conserved pathway

We recently characterized the biological activity of cIl10 and showed that, similar to previously characterized fish Il10 molecules (28, 29), it signals through a conserved pathway including Stat3 phosphorylation and induction of *socs3* expression (9). Furthermore, through proteome analysis we showed that the ORF134 expression product, cyhv3Il10, is one of the most abundant proteins in the CyHV-3 secretome (10), but we failed to show a clear bioactivity of this molecule in vivo. First, we aimed to investigate whether the abundantly secreted cyhv3Il10 protein would activate the same conserved pathway as previously characterized for fish Il10 molecules, which would provide a first qualitative indication of its bioactivity, at least in vitro. To this end, HKL were stimulated with recombinant cyhv3Il10 or with cell culture supernatants obtained from CCB cells infected with wild-type or recombinant CyHV-3 strains. The cytoplasmic fraction of HKL was analyzed by Western blot using an anti–phospho-Stat3 Ab (Fig. 1A). Phosphorylation of Stat3 (seen as a double band at ~86 kDa) was completely absent in lysates of HKL stimulated with medium only, mock supernatants, and supernatants from CCB cells infected with the Il10-deleted strain. In contrast, pStat3 was clearly visible in lysates of HKL stimulated with supernatants from CCB cells infected with the Il10-revertant (Rev) and wild–type strains, as well as in supernatants from cells stimulated with recombinant cyhv3Il10 (Fig. 1A). As a control, absence or presence of cyhv3Il10 and cIl10 in the corresponding supernatants from CCB cells was confirmed by Western blot (Fig. 1B) using a mouse polyclonal Ab against cyhv3Il10 and an affinity-purified rabbit polyclonal Ab against cIl10. As expected, only supernatants from CCB cells infected with wild-type and Il10-revertant CyHV-3 strains were positive for cyhv3Il10, showing a band at ~18 kDa. cIl10 was absent in all CCB supernatants (Fig. 1B, *lower panel*), ruling out the possibility that endogenous CCB-produced Il10 could be responsible for the observed Stat3 phosphorylation.

Next, having confirmed the presence of cyhv3Il10 in supernatants of CyHV-3–infected CCB and their ability to induce Stat3 phosphorylation, we examined the regulation of *socs3* expression. Indeed, the same stimuli that induced phosphorylation of Stat3 in HKL also led to a significant upregulation of both *socs3* paralogs in a manner comparable to the cIl10 control (Fig. 1C). Relatively high amounts of cyhv3Il10 recombinant protein had to be used to achieve effects on *socs3* expression comparable to effects triggered by cyhv3Il10 present in supernatants from infected CCB cells; this is expected owing to the likely presence of improperly folded recombinant protein, which can lead to an overestimation of the concentration of bioactive cyhv3Il10. Collectively, our results not only show that cyhv3Il10 is produced and secreted by CyHV-3–

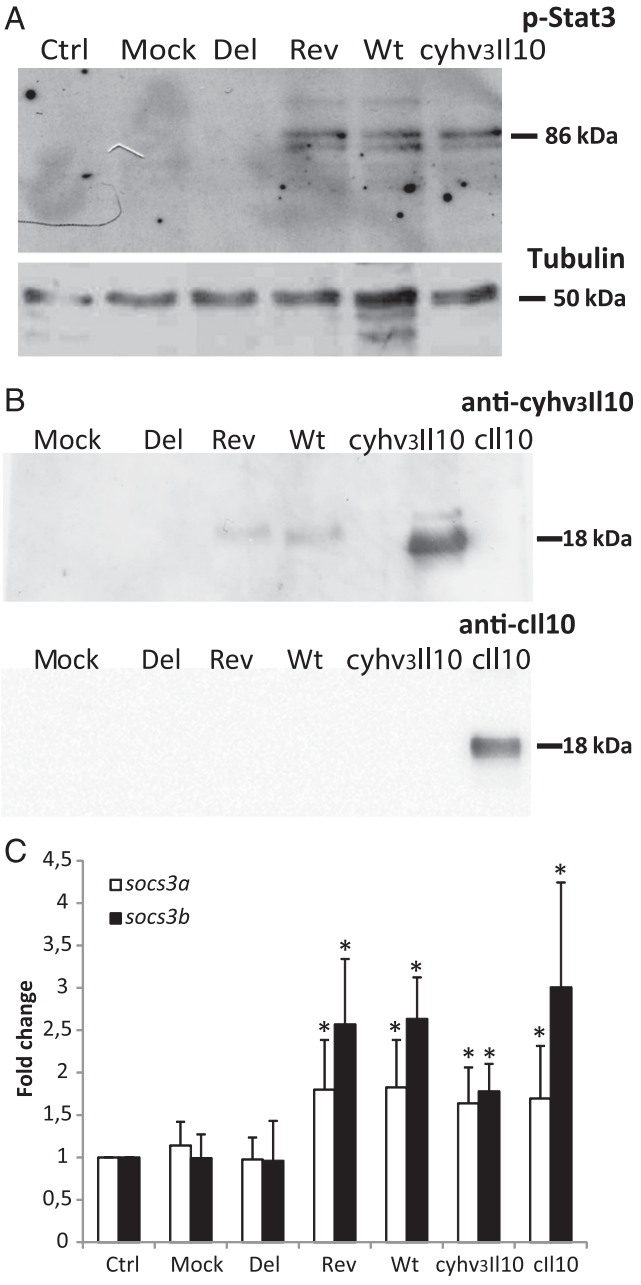

FIGURE 1. cyhv3Il10 signals via a conserved pathway. (**A**) Western blot analysis of Stat3 phosphorylation (86 kDa). HKL were incubated for 10 min with medium (Ctrl) or with supernatants (20% [v/v]) from mock-infected (Mock) CCB cells, or CCB infected with two different recombinant CyHV-3 strains: Il10-deleted (Del) and Il10-revertant (Rev), or CCB infected with wild-type CyHV-3 (Wt). Stimulation with recombinant cyhv3Il10 (10 μg/ml) was included as a positive control. An anti-tubulin Ab was used as a control (52 kDa). (**B**) Western blot analysis of cyhv3Il10 and cIl10 (18 kDa) in the CCB supernatants used as described for (A), with recombinant cyhv3Il10 and cIl10 as controls. (**C**) Gene expression analysis (fold change) of *socs3* paralogs (a and b). HKL were stimulated for 3 h with medium (Ctrl) or supernatants from infected CCB (20% [v/v]), recombinant cyhv3Il10, or recombinant cIl10 (10 μg/ml). Gene expression was normalized relative to the *s11* protein of the *40s* subunit as a reference gene and is shown relative to the unstimulated control (Ctrl). Data are means ± SD of $n = 3$ fish. Asterisks indicate significant differences relative to the unstimulated control.

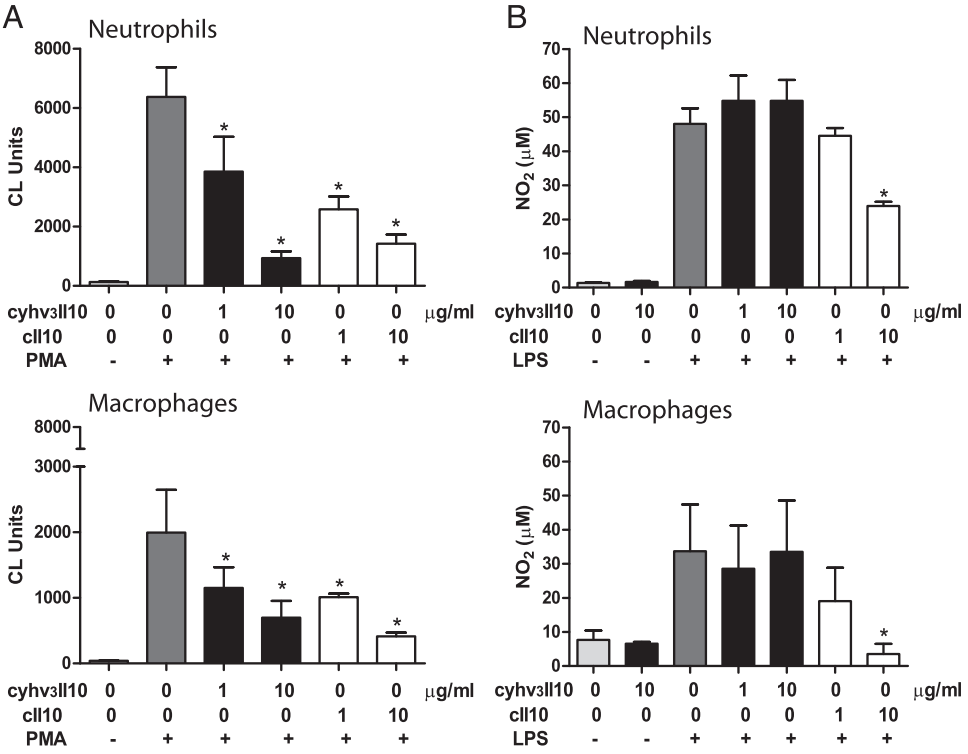

FIGURE 2. cyhv3Il10 inhibits oxygen but not nitrogen radical production by carp phagocytes. (**A**) Oxygen radical release by neutrophils or macrophages stimulated with PMA (0.1 μg/ml) alone or in combination with cyhv3Il10 or cIl10. Oxygen radical release was measured 45 min after stimulation with PMA and expressed as chemiluminescence (CL) units. (**B**) Nitrogen radical release by neutrophils or macrophages stimulated with LPS (20 μg/ml) alone or in combination with cyhv3Il10 or cIl10. NO (nitrite) was measured at OD_{450} at 96 h (neutrophils) and 24 h (macrophages) after stimulation with LPS and converted to micromolar concentrations using a nitrite standard curve. In all cases, bars represent mean ± SD of duplicate measurements from $n = 3$ fish. Asterisks indicate significant differences relative to the respective PMA- or LPS-stimulated group.

infected CCB cells, but also that the biologically active protein signals through a conserved pathway.

cyhv3Il10 inhibits oxygen but not nitrogen radical production by carp phagocytes

Because we are aware that cyhv3Il10 (ORF134) is not the only homolog present in the viral secretome with suspected immune function (7), and to avoid interference with contaminating CyHV-3– or CCB-derived molecules, subsequent experiments were performed with recombinant cyhv3Il10 rather than culture supernatants from CyHV-3–infected CCB cells. Recombinant cIl10 was included as a reference.

We recently showed that cIl10 is a potent inhibitor of radical production, especially oxygen radicals, by carp neutrophils and macrophages (9). To characterize the biological activity of cyhv3Il10, its effect on oxygen and nitrogen radical production by neutrophils and macrophages was measured. Production of oxygen radicals in unstimulated cells was not modulated by the recombinant protein alone (data not shown), but in PMA-stimulated cells, cyhv3Il10 very effectively inhibited the respiratory burst of both neutrophils and macrophages in a dose-dependent manner (Fig. 2A). The effect was not different from cIl10, suggesting that both cyhv3Il10 and cIl10 have a comparable effect on the production of oxygen radicals by phagocytes.

Production of nitrogen radicals in unstimulated cells was not modulated by the recombinant protein alone, and in LPS-stimulated cells cyhv3Il10 had no significant effects on nitrogen radical production by neutrophils or macrophages (Fig. 2B). This was in contrast to cIl10 that, at high doses, showed inhibitory activities on nitrogen radical production in particular of macrophages. Collectively, these results indicate that cyhv3Il10 has retained biological activities similar to cIl10 with respect to its ability to inhibit oxygen radical release, but different from cIl10 with respect to modulation of nitrogen radical release by phagocytes.

cyhv3Il10 downregulates the expression of proinflammatory genes in carp macrophages

We recently showed that cIl10 (9), similar to mammalian IL10 (30), is a potent inhibitor of the expression of proinflammatory genes in both neutrophils and macrophages. To characterize the biological activity of cyhv3Il10, the effects on the expression of several proinflammatory genes was measured in neutrophils or macrophages using cIl10 as a control (Fig. 3). The expression of *il1β*, *inos*, *tnfα*, and *il6* (Table I) was significantly downregulated in

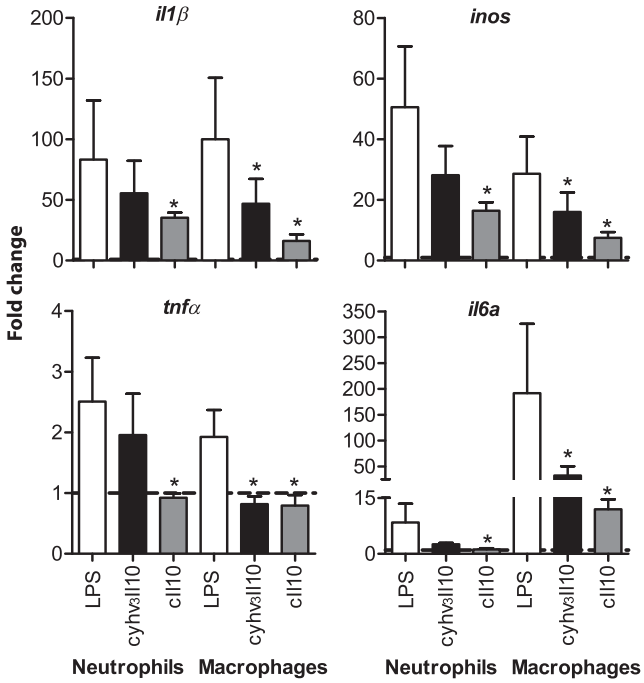

FIGURE 3. cyhv3Il10 downregulates the expression of proinflammatory genes in macrophages. Neutrophils and macrophages were stimulated for 6 h with LPS (50 μg/ml) alone or in combination with cyhv3Il10 or cIl10 (10 μg/ml). Gene expression (fold change) was normalized relative to the *s11* protein of the *40s* subunit as a reference gene and is shown relative to the unstimulated control (dashed line at y = 1). Data are presented as mean ± SD of $n = 3$ fish for neutrophils and $n = 4$ fish for macrophages. Asterisks indicate significant differences from the LPS-stimulated samples.

Table I. Primers used for real time-quantitative PCR

Primer	Sequence (5′→3′)	GeneBank Accession No.
*il1β*_FW	AAGGAGGCCAGTGGCTCTGT	AJ245635
*il1β*_RV	CCTGAAGAAGAGGAGGAGGCTGTCA	
*inos*_FW	AACAGGTCTGAAAGGGAATCCA	AJ242906
*inos*_RV	CATTATCTCTCATGTCCAGAGTCTCTTCT	
*tnfα*_FW	GCTGTCTGCTTCACGCTCAA	AJ311800 and AJ311801
*tnfα*_RV	CCTTGGAAGTGACATTTGCTTTT	
*il6a*_FW	CAGATAGCGGACGGAGGGGC	KC858890
*il6a*_RV	GCGGGTCTCTTCGTGTCTT	
*socs3a*_FW	CCTTCAGACGGACTCCAA	*Scaf* 42504.1 and 22777.1
*socs3a*_RV	CAAGGAAGGGGTCTCAAC	
*socs3b*_FW	CCGCTGGAGAAGGTGGAA	*Scaf* 15008.1 and 5193.1
*socs3b*_RV	CTGGAGGAACTCTTGGAGTG	
*40s*_FW	CCGTGGGTGACATCGTTACA	AB012087
*40s*_RV	TCAGGACATTGAACCTCACTGTCT	

Scaf refers to the scaffold number of the draft carp genome (Bioproject PRJNA73579) (22) from which the carp *socs3* homologs were identified (9).

a very similar manner by both cyhv3Il10 and cIl10 in macrophages. Although a similar trend could be observed, downregulation of expression of these genes in neutrophils was only significant when cIl10 was present whereas cyhv3Il10 failed to yield significant results. Collectively, these results suggest that cyhv3Il10 has retained a function similar to cIl10 with respect to inhibition of proinflammatory gene expression in macrophages in particular.

cyhv3Il10 does not affect MhcI surface expression

Mammalian IL10 decreases surface expression of MHC class II (MHCII) on monocytes (31) and MHC class I (MHCI) on melanoma cells (32) while increasing MHCII expression on B cells (33). The EBV IL10 homolog, however, has no effect on MHCII expression but decreases MHCI expression on B cells (34, 35). We recently showed that cIl10 downregulates *mhcI* and *mhcII* gene expression in neutrophils but not in macrophages (9). In the present study, we investigated the modulatory effect of cyhv3Il10 and cIl10 on the surface expression of MhcI (Ua1) on different leukocyte cell types of carp; regretably, no Abs are available to detect effects on surface expression of carp MhcII Ags. Neither cyhv3Il10 nor cIl10 showed a clear effect on MhcI surface expression of total HKL (Fig. 4A). However, when specific leukocyte populations were examined, differential effects could be observed. Neutrophils showed an evident reduction in MhcI surface expression after treatment for 2 and 4 d with cIl10, whereas cyhv3Il10 had no effect. Igm$^+$ B cells showed an increase in MhcI surface expression after treatment for 4 d with both cyhv3Il10 and cIl10.

To quantify the above-described changes in MhcI surface expression, we extracted the mean fluorescence intensities of all populations shown in Fig. 4A and analyzed these values relative to the mean fluorescence intensities of the control samples (Fig. 4B). Quantitative analysis showed that MhcI surface expression of total HKL was upregulated to a marginal but statistically significant extent after treatment for 4 d with both cyhv3Il10 and cIl10. In contrast, MhcI surface expression was clearly downregulated in neutrophils after treatment for 2 and 4 d with cIl10, but not cyhv3Il10. Conversely, MhcI surface expression on Igm$^+$ B cells was upregulated after treatment for 2 and 4 d with cIl10, but not significantly affected by treatment with cyhv3Il10. Altogether, these results show that under the conditions studied, cyhv3Il10 does not have a prominent effect on MhcI protein expression whereas cIl10 exerts differential and cell type-specific effects on MhcI protein expression with possible consequences on Ag presentation.

cyhv3Il10 and cIl10 induce proliferation of Igm$^+$ B cells

Similar to their host homologs, some viral Il10s have the ability to increase B cell proliferation, a mechanism considered beneficial to the virus because this would lead to an increase in number of latently infected cells in the host (1). We recently showed that cIl10 increases Igm$^+$ B cell proliferation in PBL cultures from naive and immunized fish (9). In this study, we investigated the direct effect of cyhv3Il10 and cIl10 on the proliferation of CFSE-labeled Igm$^+$ B cells (Fig. 5). Incubation with cyhv3Il10 and cIl10 alone induced a significant increase in Igm$^+$ B cell proliferation (from 44 to 51–52%) with no differences between cyhv3Il10 and cIl10 (Fig. 5, *upper panels*) whereas LPS and parasite Ags alone had no effect on the total number of proliferating Igm$^+$ B cells. In combination with LPS, both cyhv3Il10 and cIl10 induced an even higher proliferation (~60%) than did the two cytokines alone (Fig. 5, *middle panels*). Interestingly, incubation with parasite Ags alone induced several rounds of proliferation (Fig. 5, *lower panel*, *left graph*), although the total percentage of dividing cells was not significantly different from the control. In the presence of cIl10, stimulation with parasite Ags led to an increased proliferation when compared with the parasite Ag alone, but not higher than stimulation with cIL10 alone (Fig. 5, *lower panel*, *middle graph*); cyhv3Il10 together with parasite Ags, however, was not able to restore the proliferation observed using the cytokine alone (Fig. 5, *lower panel*, *right graph*). Collectively, these results indicate that both cyhv3Il10 and cIl10 are able to directly increase Igm$^+$ B cell proliferation, but they also suggest that the proliferative effects of cyhv3Il10 on carp Igm$^+$ B cells are stimulus-dependent.

cIl10 inhibits Il2-induced thymocyte proliferation, whereas cyhv3Il10 has no effect on this activity

Mammalian IL10 induces thymocyte proliferation, whereas some viral IL10 homologs fail to do so, which is one of the characteristic differences between host and viral (EBV) (34) IL10s. The effect of fish Il10 on thymocyte proliferation has not been investigated previously. In this study, we characterize the activity of both cIl10 and cyhv3Il10 on in vitro cultures of carp thymocytes using Zap70 as a pan–T cell marker and CFSE to quantify cell division (Fig. 6). In the presence of Il2 alone, a very strong proliferation (66%) was observed. Strikingly, cIl10 significantly inhibited thymocyte proliferation, whereas cyhv3Il10 had no significant effect, again suggesting a cell-dependent effect of cIl10 and cyhv3Il10 activities.

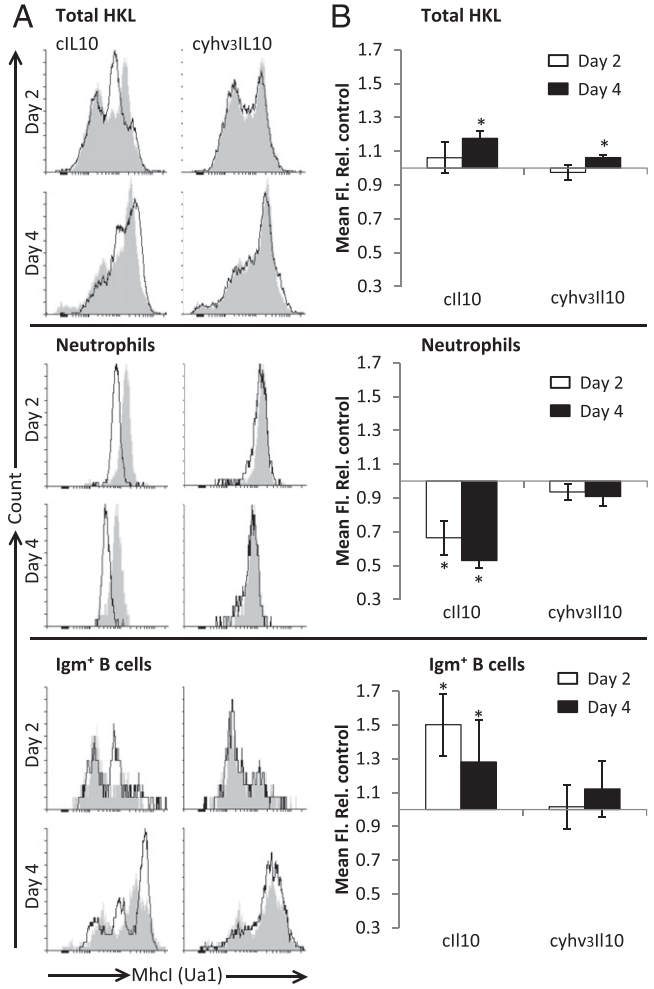

FIGURE 4. Carp Il10, but not cyhv3Il10, regulates MhcI surface expression in a cell type–dependent manner. Total HKL were incubated for 2 and 4 d with cIl10 or cyhv3Il10 (10 μg/ml) and then stained with anti-MhcI (Ua1) Ab in combination with anti-neutrophil (TCL-BE8) or anti-Igm (WCI12) Ab to identify the respective leukocyte populations by flow cytometry. (**A**) Overlay histograms showing count versus MhcI (Ua1) protein expression, where the gray histogram corresponds to the unstimulated samples and the white histograms to the samples stimulated with cIl10 or cyhv3Il10. Histograms are representative of $n = 4$ independent experiments. (**B**) Mean fluorescence intensity (FI) of the same samples displayed on the left, expressed relative to the unstimulated controls and shown as mean ± SD of $n = 4$ fish. Asterisks indicate significant differences with respect to the unstimulated control.

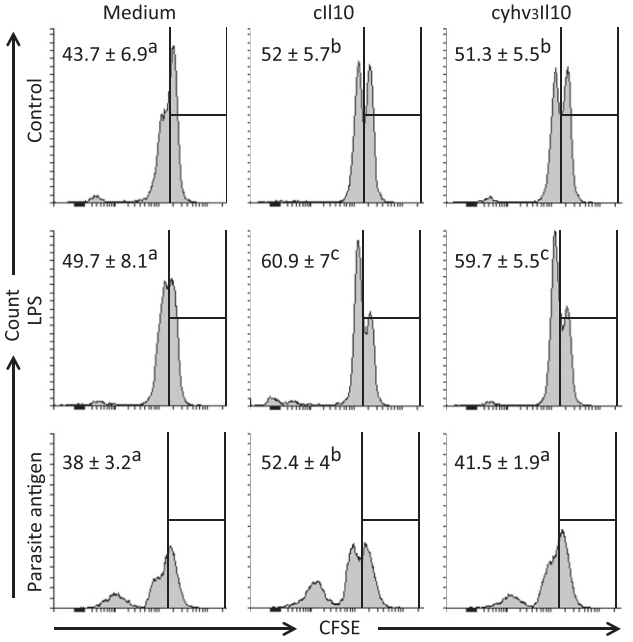

FIGURE 5. cyhv3Il10 and cIl10 directly induce proliferation of Igm$^+$ B cells. Sorted Igm$^+$ B cells were labeled with CFSE and stimulated for 6 d with cyhv3Il10 or cIl10 (10 μg/ml) alone or in combination with LPS (50 μg/ml) or parasite Ags (equivalent to 0.5 parasite/cell). The gate for undivided cells was set as to include 100% of the events at day 0 of the cell culture. Numbers represent the mean percentage of dividing Igm$^+$ B cells ± SD of $n = 4$ fish. Different letters indicate significant differences between groups.

cyhv3Il10 promotes survival and proliferation of memory T cells

Mammalian IL10 exerts differential effects on different T cell populations, inhibiting proliferation and cytokine synthesis of Th1 and Th2 (directly) and Th17 (indirectly), and acting as a growth factor to increase proliferation of specific subsets of CD8$^+$ T cells (30). We recently showed that cIl10 can induce survival and proliferation of memory carp T cells in vitro in the presence of the immunizing Ags (9). In this study, we examined the effect of cyhv3Il10 on memory T cells using a similar experimental set-up. HKL from carp that survived an infection with the parasite *T. borreli* (immunized) were incubated in vitro with cyhv3Il10 alone or in combination with parasite Ags. Recombinant cIl10 was also included as a control. Effects of cyhv3Il10 on survival and proliferation of T cells were monitored by flow cytometry using Zap70 as a pan–T cell marker (Fig. 7A) and CFSE to quantify cell division (Fig. 7B). At day 0, T cells accounted for ∼12% of the total HKL population (Fig. 7A), with all cells being CFSE$^+$. In the absence of any stimulus, the percentage of surviving T cells decreased to ∼2% at day 6. Stimulation with cyhv3Il10 or cIl10 alone did not significantly promote survival of T cells, whereas parasite Ags alone did, an effect that was further enhanced when parasite Ags were combined with cyhv3Il10 or cIl10.

To determine whether the above-described effects on T cell survival could be ascribed to cyhv3Il10 effects on proliferation, corresponding CFSE profiles were determined (Fig. 7B). Although some proliferation was observed in the unstimulated and also in the cIl10- or cyhv3Il10-stimulated samples (day 6), the presence of parasite Ags, in particular when combined with cyhv3Il10 or cIl10, induced a very strong proliferation. Parasite Ag and

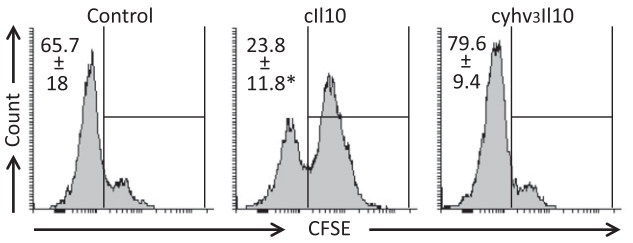

FIGURE 6. Proliferation of thymocytes is inhibited by cIl10 but not cyhv3Il10. Carp thymocytes were labeled with CFSE and stimulated for 6 d with cIl10 or with cyhv3Il10 (10 μg/ml). Thymocytes were cultured in medium containing 0.5% (v/v) pooled carp serum, with 0.5 μg/ml carp Il2a and Il2b added during the last 2 d of culture, prior to flow cytometric analysis. The gate for undivided cells was set as to include 100% of the events at day 0 of the cell culture. Numbers represent the mean percentage of dividing thymocytes ± SD of $n = 3$ fish. Asterisks indicate significant differences from the control group.

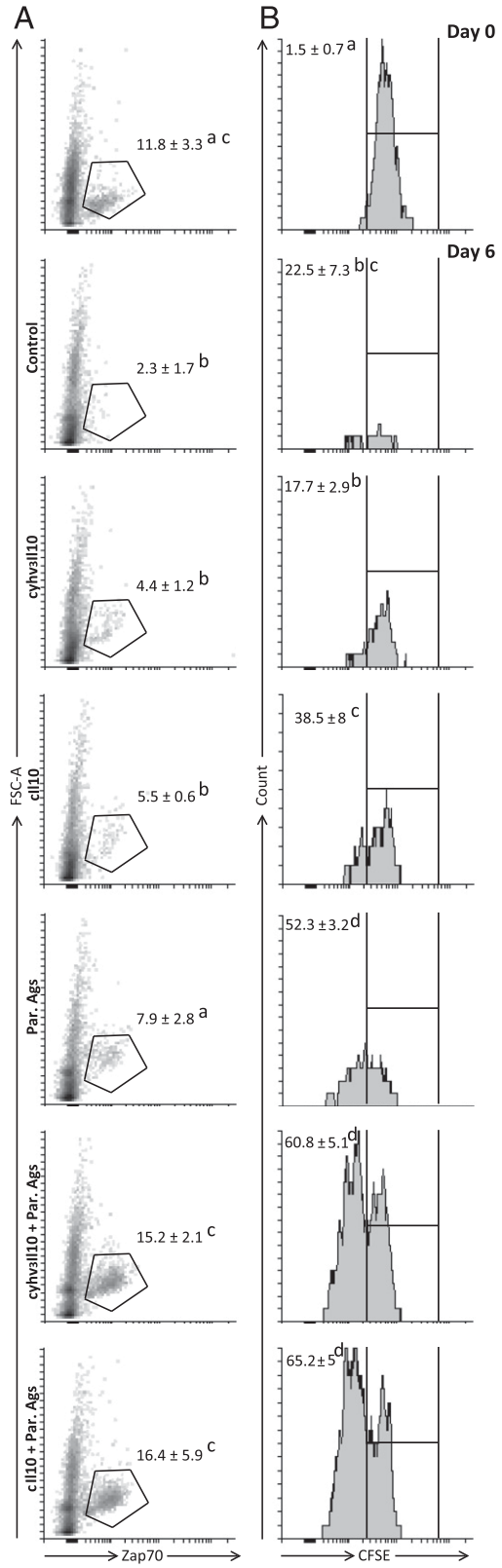

FIGURE 7. cyhv3Il10 promotes both survival and proliferation of memory T cells. CFSE-labeled HKL from fish immunized with parasite (*T. borreli*) Ags were stimulated with cyhv3Il10 or cIl10 (10 μg/ml) alone or in the presence of parasite Ags (0.5 parasite/cell) for 6 d. T cells were identified with an anti-Zap70 Ab, and their proliferation was monitored by CFSE analysis. (**A**) Density plots of the samples at day 0 (*upper left*) and at day 6 of culture. Numbers indicate the mean percentage of Zap70$^+$ T cells ± SD from $n = 4$ fish. (**B**) Count versus CFSE histograms of the corresponding Zap70$^+$ T cell population. The gate for undivided cells was set as to include 100% of the events at day 0 of the cell culture. Numbers represent the mean percentage of dividing T cells ± SD of $n = 4$ fish. Different letters indicate significant differences between groups.

cyhv3Il10 or cIl10, alone or combined, had no effect on survival and proliferation of T cells from naive fish (data not shown). Collectively, these results indicate that cyhv3Il10 exerts stimulatory effects on mature memory T cells in a manner analogous to cIl10 (9). Further characterization of the T cell population would be needed to identify exactly which T cell subpopulations are modulated in the presence of cyhv3Il10.

Discussion

In the present study, we investigated the biological activities of cyhv3Il10 on carp phagocytes and lymphocytes and show that the lack of a clear phenotype observed in our previous study (10) during infection with recombinant cyhv3Il10-deleted viruses cannot be ascribed to the lack of biological activity of this viral homolog. In fact, we demonstrate that cyhv3Il10 shares the signaling pathway and several prototypical activities with cIl10, its host homolog, including the ability to inhibit proinflammatory responses of macrophages and induce proliferation of Igm+ B cells and memory T cells. However, cell type–dependent effects of cyhv3Il10 were also noted; in contrast to cIl10, cyhv3Il10 had no significant inhibitory effect on the inflammatory activities of carp neutrophils, no modulatory effect on MhcI surface expression, and no direct effect on the proliferation of naive thymocytes. Collectively, we have shown that cyhv3Il10 shares several immunosuppressive as well as immunostimulatory activities with cIl10, but it does not mimic the full repertoire of cIl10 activities. To our knowledge, this is the first study describing direct effects of cyhv3Il10 on both innate and adaptive immune cells of its natural host, the common carp, and the first study to describe biological activities of a nonmammalian viral Il10 homolog, establishing a true orthology between cyhv3Il10 and cIl10.

Although the protein sequences of cyhv3Il10 and cIl10 share only limited (26.9%) sequence identity, sequence similarity is much higher (67.3%) and the predicted three-dimensional structure and position of amino acids crucially important for the presumed interaction with the Il10 receptor (Il10r) are highly conserved (36), suggesting a conservation of signaling by these two orthologous molecules, at least in carp. A previous study conducted in zebrafish embryos showed that downregulation of the *il10r1* via a morpholino approach abrogated the response to both cyhv3Il10 and zebrafish Il10 (11). Although this study already pointed to an involvement of the Il10 receptor complex in cyhv3Il10 signaling, it did not confirm conservation of the signaling pathway downstream of the receptor nor did it investigate the direct activity of cyhv3Il10 on specific leukocyte cell types. Furthermore, the larval zebrafish model does not easily allow for an investigation of biological activities of cyhv3Il10, which would be difficult to study even in adult zebrafish, considering that this species is not a natural host for CyHV-3, and infection of zebrafish with CyHV-3 has never been reported. In contrast, carp is the natural host of CyHV-3 and can be infected with CyHV-3 experimentally in a reliable and controlled manner (10). Our results show that both supernatants from cells infected with CyHV-3, thus containing large quantities of the cyhv3Il10 protein, and recombinant cyhv3Il10 induce phosphorylation of Stat3 at position Y705. Not all viral IL10 proteins signal via JAK1-induced phosphorylation of STAT3. For example, CMV IL10 signals via PI3K-mediated STAT3 phosphorylation, which, in contrast to the JAK1-induced STAT3 phosphorylation

common to human IL10, occurs at a different position (S727) in the STAT3 sequence (37). Furthermore, stimulation of carp leukocytes with cyhv3Il10 leads to a subsequent upregulation of *socs3*, confirming conservation of the signaling pathway between mammalian IL10 (38), cIl10 (9), and cyhv3Il10.

The observation that both carp and cyhv3Il10 activate the same signaling pathway in carp leukocytes suggests a conservation of function of these two molecules. Although it is not uncommon for viral IL10 molecules to share several immunosuppressive and/or immunostimulatory properties with IL10 molecules of their host, viral IL10 molecules do not always mimic the entire repertoire of activities. For example, EBV IL10 shares with human IL10 the inhibitory effect on proinflammatory cytokine production (34, 39–41), deactivation of macrophages (42), and stimulation of proliferation, differentiation, and Ab secretion by B cells (33, 43, 44), but it fails to upregulate Mhc class II expression on murine B cells (33) and is less effective in inhibiting T cell proliferation (45). Alternatively, CMV IL10 mimics the complete repertoire of activities of human IL10 (1).

We recently showed that cIl10 exerts a very potent inhibitory effect on carp phagocytes as shown by a strong inhibition of oxygen radical production and downregulation of proinflammatory gene expression in carp macrophages and neutrophils (9). Although cyhv3Il10 does not share with cIl10 the potent downregulation of proinflammatory gene expression in carp neutrophils and inhibition of production of nitrogen radicals, we show that cyhv3Il10 does share with cIl10 the inhibition of production of oxygen radicals by carp phagocytes and downregulation of expression of proinflammatory genes by carp macrophages in particular. Deactivation of macrophages is one of the most important and best documented activities retained by virus-encoded IL10s. For example, Orf virus (ORFV, *Parapoxvirus ovis*) IL10 inhibits TNFα, IL8, and IL1β synthesis in activated ovine, murine and human macrophages (46–48). Similarly, ovine herpesvirus 2 IL10 inhibits IL8 production by LPS-activated macrophages (49); EBV IL10 inhibits oxygen radical and PGE_2 production by human monocytes (42, 50) and CMV IL10 inhibits TNFα, IL1α, IL6 and GM-CSF in LPS-stimulated monocytes (51). In conclusion, cyhv3Il10 shares with several other viral IL10s the ability to deactivate host macrophages.

Given the ability of (mammalian) viral IL10 homologs to modulate Ag presentation by regulating MHCI and MHCII surface expression levels (31–35), we analyzed the effect of cIl10 and cyhv3Il10 on MhcI (Ua1) expression on carp leukocytes. We show that whereas cIl10 differentially regulated surface expression of MhcI on different cell types, decreasing Ua1 expression on neutrophils and increasing Ua1 expression on B cells, cyhv3Il10 did not regulate surface expression of MhcI to the same extent. It cannot be excluded that cyhv3Il10 regulates surface expression of MhcI molecules other than those of the Ua lineage (52). Alternatively, reduced abilities of viral IL10s to regulate MHCI surface expression in comparison with host IL10s are not uncommon. For example, EBV IL10 fails to mimic the total range of human IL10 activities due to a 1000-fold lower affinity to the IL10R1 (45). If indeed cyhv3Il10 would have a lower affinity than cIl10 for the Il10r, it could explain our observation. This also suggests that, similar to the mammalian (viral) IL10 molecule, also the fish (viral) Il10 molecule might present at least two functional epitopes, the conservation of which might influence its biological activity owing to the differential affinity of the molecule for the Il10 receptor complex. In carp, functional analysis of receptor preferences and affinity analysis of cIl10 versus cyhv3Il10 are complicated by the fact that duplicated copies of each Il10 receptor molecule, that is, cytokine receptor family member b7 (Crfb7) and Crfb4 (29, 53), exist in the duplicated carp genome (unpublished data). Furthermore, given the high structural and sequence similarity with Crfb4 (29), the possibility that also Crfb5 could be involved in the formation of the Il10 receptor complex cannot be excluded. These complications have left the questions on receptor preferences and affinity analysis of cIl10 versus cyhv3Il10 currently unsolved.

Viral IL10s, such as EBV IL10 (43, 44) or CMV IL10 (54), directly stimulate B cell proliferation. In the present study we compared the activities of cIl10 and cyhv3Il10 on sorted Igm^+ B cells and observed that both Il10 molecules efficiently promoted B cell proliferation, in particular when costimulated with LPS. The collective data suggest that cyhv3Il10, similar to mammalian viral IL10s, promotes B cell survival and proliferation, possibly increasing the number of latently infected cells in circulation. Our data would therefore support the observation described in a recent study showing that CyHV-3 establishes latency in Igm^+ B cells from koi carp (55).

Proliferation of thymocytes, stimulated by IL2 and IL4, is further enhanced by human and murine IL10 whereas EBV IL10 lacks this activity (56). We show that, in clear contrast to mammalian IL10, cIl10 strongly inhibited Il2-induced thymocyte proliferation, whereas cyhv3Il10 had no significant effect on this activity. Owing to the fact that the carp thymus expresses low to moderate levels of *il10r1* (9), it is possible that the differential activity of cIl10 and cyhv3Il10 can be attributed to differences in binding affinity to the Il10 receptor.

Different from the contrasting effect on naive thymocytes, both cIl10 and cyhv3Il10 promoted proliferation of mature memory T cells, possibly $CD8^+$, when used in the presence of the immunizing Ag (Ref. 9 and the present study). Future studies should address the effect of cyhv3Il10 on subpopulations of T cells to fully characterize the effect of Il10 on the proliferation of naive T cells.

Our previous study using a recombinant ORF134-deleted strain and the derived revertant strain suggested that cyhv3Il10 is not essential for viral replication in vitro, or virulence in vivo (10). However, the observation that cyhv3Il10 is one of the most abundant proteins expressed during the acute phase of infection, together with the biological activities of cyhv3Il10 shown in the present study, clearly points to a biological relevance of this viral homolog. It is well possible that to appreciate the full biological effects of cyhv3Il10 in vivo, experimental conditions should be adjusted to mimic a more natural environment. Collectively, this study clearly demonstrates that cyhv3Il10 (ORF134) is a functional ortholog of carp and mammalian IL10 and confirms the potential of cyhv3Il10 to modulate the host immune response at various levels and contribute to virus persistence.

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Disclosures

The authors have no financial conflicts of interest.

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