Fish & Shellfish Immunology (2008) **25**, 271–280

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Regulation of rainbow trout (*Oncorhynchus mykiss*) interleukin-8 receptor (IL-8R) gene transcription in response to viral hemorrhagic septicemia virus (VHSV), DNA vaccination and chemokines

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Received 22 April 2008; revised 22 May 2008; accepted 25 May 2008
Available online 25 July 2008

KEYWORDS
Interleukin-8;
Receptor;
Rainbow trout;
Viral hemorrhagic septicemia virus;
CK-6;
DNA vaccine;
Poly I:C

Abstract An interleukin-8 receptor (IL-8R)-like sequence has been previously reported in rainbow trout (*Oncorhynchus mykiss*); however, no further studies to confirm its biological activity or regulation of expression have been performed. In this report, we have studied the regulation of the transcription of this receptor in response to different stimuli both in vivo and in vitro. We found that in response to a viral hemorrhagic septicemia virus (VHSV) infection, the levels of expression of IL-8R are suppressed in the head kidney, spleen and muscle, in contrast to what occurs in response to Poly I:C. These results might indicate a suppressive effect of the virus and a mechanism that enables it to elude the immune response. This response is no longer observed in vitro in the rainbow trout macrophage cell line RTS11, which has been shown to be resistant to VHSV complete replication, and where the virus produced no effect on the levels of mRNA expression of IL-8R. In these cells, as observed in vivo, Poly I:C significantly induced the expression of IL-8R, increase that came along with an increase in the chemotactic activity towards IL-8. In response to DNA vaccination, we found that the levels of mRNA expression are significantly increased only in the muscle at very early times post-vaccination. As an additional step to clarify whether this receptor is in fact being used by IL-8, we intramuscularly injected plasmids coding for different rainbow trout chemokines (IL-8 and other CC chemokines such as CK5B, CK6 and CK7A). Only plasmids coding for IL-8 and CK-6 were capable of significantly increasing the levels of transcription of IL-8R in the muscle. This effect was confirmed by the up-regulation of IL-8R mRNA production in head kidney leucocytes in response to recombinant IL-8 and CK-6.

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doi:10.1016/j.fsi.2008.05.009

Introduction

Chemokines are a superfamily of cytokines produced by different cell types that have, among other functions, chemoattractant properties. The chemokine superfamily is defined by the presence of four conserved cysteine residues and depending on the arrangement of the first two conserved cysteines in their sequence they are divided into four subfamilies: CXC (α), CC (β), C and CX_3C classes [1]. CXC chemokines can be further divided in those in which an ELR (Glu-Leu-Arg) motif is present at the N-terminus of their sequence and those in which this motif is absent. This ELR motif is responsible for receptor binding and activation of neutrophils, therefore CXC chemokines that lack this motif do not attract neutrophils [2,3]. Thus, the general rule is that CXC chemokines are attractant for neutrophils but not for monocytes/ macrophages, while on the contrary, CC chemokines are chemoattractant for monocytes/ macrophages but not for neutrophils [1].

Interleukin-8 (IL-8) is a CXC chemokine produced by numerous cell types. In mammals, it is known to be produced by macrophages/ monocytes, epithelial cells, neutrophils, fibroblasts, and endothelial cells upon infection or stimulated by cytokines such as IL-1β and tumor necrosis factor α (TNF-α) [4,5]. In mammals, IL-8 is known to act through two different receptors named CXCR1 and CXCR2 that share about 77% identity [6]. While the CXCR1 is only used by IL-8, CXCR2 can be catalogued as a ''shared'' receptor that may be used by IL-8 as well as by other CXC chemokines, although never by chemokines of different families [7]. Being a CXC chemokine with an ELR motif, IL-8 predominantly promotes the recruitment of neutrophils, and other cell types such as basophils, CD8+ T lymphocytes, and NK cells [5,8,9], suggesting the presence of both receptors in these cell types. A few studies have revealed however some attractant capacity of IL-8 for monocytes [10–13], and in fact, CXCR1 and CXCR2 to a larger extent are found in the surface of these cells.

In rainbow trout, *Oncorhynchus mykiss* [14], as in other fish species [15–17] with the exception of haddock [18], IL-8 does not have an ELR motif, although a conservative DLR motif is present. Human ELR mutated to DLR still retains neutrophil-attractant activity, thus suggesting that this fish DLR residue is responsible for the neutrophil attracting ability [19]. To confirm this, the neutrophil chemoattractant capacity of rainbow trout IL-8 was demonstrated indirectly, since the intramuscular injection of fish with an expression vector coding for IL-8 induced a massive neutrophil infiltration at the injection site [20]. Recently, the rainbow trout recombinant IL-8 was produced and used to establish the capacity of this chemokine to attract head kidney leucocytes, specially neutrophils, in vitro and in vivo [21]. Furthermore, a recent study [22] revealed the capacity of rainbow trout IL-8 to attract monocyte-like cells in established RTS11 monocyte–macrophage cultures. Concerning the regulation of rainbow trout IL-8 transcription, it has been demonstrated that viral infections such as those produced by viral hemorrhagic septicemia virus (VHSV) or infectious hematopoietic necrosis virus (IHNV) strongly induce IL-8 transcription in different organs [23,24]. In order to validate, however, the effect that the virus might have on the IL-8 biological activity, the effect that may be exerted on the receptor must also be taken into account. In 2002, Zhang et al. [25] reported the cloning of an IL-8 receptor-like sequence (IL-8R) in rainbow trout, with a 34.9% of amino acid identity to human CXCR1 and 36.99% to human CXCR2 which was constitutively expressed in the intestine, spleen, kidney, gill and white blood cells, but appeared weak in red blood cells and liver. To date, no additional studies have been performed related to this IL-8R, even though the elucidation of the mechanism of action of the receptor is essential for the understanding of the biological activity of IL-8. Therefore, in this work we have performed different studies concerning the regulation of transcription of rainbow trout IL-8R, the only IL-8R identified to date in fish, in response to various stimuli such as viral infection with VHSV, Poly I:C, DNA vaccination or different chemokines.

Materials and methods

Cells and viruses

RTS11, a rainbow trout monocyte-macrophage-like cell line, originally isolated from a long-term spleen hematopoietic culture [26] was maintained at 18 °C in Leibovitz medium (L-15, Gibco, Invitrogen, UK) supplemented with penicillin (100 IU ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$) and 15% fetal calf serum (FCS, Gibco). Cells were grown at a high cell density and passaged at a 1:2 ratio approximately once a month as previously described [26].

VHSV (strain 0771) was propagated in the EPC cell line [27]. Cells were cultured at 20 °C in RPMI-1640 Dutch modified medium (Gibco) supplemented with antibiotics and 10% FCS. Virus was inoculated on EPC grown in L-15 with antibiotics and 2% FCS at 14 °C. When cytopathic effect was extensive, the supernatant was harvested and centrifuged to eliminate cell debris. Clarified supernatants were used for the experiments. The virus stock was titrated in 96-well plates according to Reed and Müench [28].

Recombinant rainbow trout chemokine production and chemokine expression plasmids

Recombinant rainbow trout IL-8 and CK-6 chemokines were produced in *Escherichia coli* cells and their biological activity determined through chemotaxis experiments as previously described [22]. Briefly, the coding sequences of both rainbow trout IL-8 and CK-6 were cloned into the pRSET A (Invitrogen) plasmid using designed primers in which EcoRI and BamHI restriction sites were included. The pRSETA vectors obtained produced fusion proteins in which an N-terminal 6× His-tag allowed the purification of the recombinant chemokines over a Ni-Sepharose affinity column. These constructs were used to transform BL21(DE3)pLysS chemically competent *E. coli* cells (Invitrogen) which were used for chemokine expression as previously described [22]. The protein concentration was determined by absorbance at 280 nm and the purity and size of the chemokines confirmed by SDS–PAGE.

The expression plasmids coding for different rainbow trout chemokines were obtained as previously described for

IL-8 [20]. PCR products encoding the entire open reading frame of IL-8, CK5B, CK6 or CK7A (including the stop codon) were obtained after designing the specific primers from the published sequences [14,29]. The non-purified PCR products (4 μl) were directly ligated into pcDNA3.1/V5-His-TOPO (Invitrogen) according to the manufacturer's instructions. The reactions were used to transform One Shot TOP10 *Escherichia coli* cells (Invitrogen). Clones containing a full-size insert were identified by PCR screening, and the proper orientation was verified by sequencing. The resulting constructs were designated as pIL8, pCK5B, pCK6 or pCK7A. An empty circularized pcDNA3.1/V5-His-TOPO plasmid was obtained and used as a control (pcDNA3).

Evaluation of the levels of expression of IL-8R in vivo

Experimental design

Rainbow trout (*Oncorhynchus mykiss*) of approximately 8—10 cm obtained from Lillogen (Leon, Spain) were maintained at the Centro de Investigaciones en Sanidad Animal (CISA-INIA) laboratory at 14 °C and fed daily with a commercial diet (Trow, Leon, Spain). Prior to experiments, fish were acclimatized to laboratory conditions for 2 weeks.

For the challenge experience with VHSV, trout were divided into two groups of 20 trout each. One group was infected by intraperitoneal injection with VHSV (100 μl of 1×10^8 $TCID_{50}$ ml^{-1} per fish). The other group was mock-infected with the same volume of L-15 medium. At days 1, 3 and 7 post-infection, two control and four VHSV infected trout were euthanized by overexposure to MS-222 and head kidney, spleen and muscle sampled.

In another experiment, 20 rainbow trout were either intramuscularly mock-injected with 100 μl of PBS or intramuscularly injected with Poly I:C (100 μl of PBS containing 500 μg of Poly I:C per fish). At days 1, 3 and 7, two control fish and four Poly I:C-injected fish were euthanized by overexposure to MS-222 and head kidney, spleen and a muscle section surrounding the injection site sampled.

The effect that DNA vaccination against VHSV might have on the expression of IL-8R was also studied. The pMCV1.4-G plasmid encoding the glycoprotein G gene of VHSV under the control of the long cytomegalovirus (CMV) promoter, previously described [30] was used for VHSV vaccination. Rainbow trout were divided into three groups of 20 trout each and intramuscularly injected with one of the following treatments: 100 μl of phosphate-buffered saline (PBS), 100 μl of PBS containing 0.5 μg of the empty pMCV1.4 plasmid DNA per fish, or 100 μl of PBS containing 0.5 μg pMCV1.4-G. At days 3 and 7, five fish from each group were euthanized by overexposure to MS-222 and head kidney, spleen and a muscle section surrounding the injection site sampled.

In order to determine which chemokines might regulate the levels of expression of IL-8R, we also studied the effect of the intramuscular injection of fish with expression plasmids coding for different chemokines on the levels of expression of IL-8R in the muscle. For this, trout were divided into six groups and intramuscularly injected with one of the following treatments: 100 μl of PBS, 100 μl of PBS containing 0.5 μg of the empty pcDNA3 plasmid DNA per fish, or 100 μl of PBS containing 0.5 μg of the different expression plasmids (pIL8, pCK5B, pCK6, pCK7A) obtained as described above. At day 3, five fish from each group were euthanized by overexposure to MS-222 and a muscle section surrounding the injection site sampled.

cDNA synthesis

Total RNA of the different organs was extracted using Trizol (Invitrogen) as per manufacturer's instructions and stored at −80 °C until used. Five micrograms of RNA were used to obtain cDNA using the Superscript II reverse transcriptase (Invitrogen). Briefly, RNA was incubated with 1 μl of oligo (dT)12—18 (0.5 μg ml^{-1}) and 1 μl 10 mM deoxynucleotide triphosphate (dNTP) mix for 5 min at 65 °C. After the incubation, 4 μl of 5× first strand buffer (250 mM Tris—HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) and 2 μl 0.1 M dithiothreitol (DTT) were added, mixed and incubated for 2 min at 42 °C. Then, 1 μl of Superscript II reverse transcriptase was added and the mixture incubated at 42 °C for 50 min. The reaction was stopped by heating at 70 °C for 15 min, and the resulting cDNA stored at −20 °C.

In all cases, cDNA was obtained from individual samples. In the experiments in which the in vivo effect of VHSV and Poly I:C on the levels of expression of IL-8R were determined, the cDNAs obtained from the different individuals at each time point were pooled prior to the PCR analysis. In these two experiments, the cDNAs obtained from two controls at each time point, we pooled all together, since previous analysis demonstrated that there was no significant variation in the levels of expression of IL-8R in controls at different time points. In the experiments in which the effect of vaccination and plasmids coding for the chemokines was evaluated, the PCR was performed in individual cDNA samples.

PCR of IL-8R

For the evaluation of the levels of expression of the IL-8R previously identified by Zhang et al. [25], the CRF7 (5′-GGT GTT AGG AGA ATG TCT TG-3′) and CR2 (5′-CAG GGA CTG TTG ACT GAA GC-3′) primers designed in their study to amplify a 431 bp fragment were used.

All PCR amplification reactions were performed using 0.5 μl dNTP mix (10 mM each), 0.2 μl Taq polymerase (5 units μl^{-1}, Invitrogen), 2.5 μl Taq 10× buffer, 0.75 μl $MgCl_2$ 50 mM, 0.5 μl of each primer (50 μM) and 1 μl of cDNA in a final volume of 25 μl. A parallel PCR with primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed with all samples as a house-keeping gene to standardize the RT—PCR results using the conditions previously described [24].

All PCRs were carried out in a Perkin Elmer 2720 cycler. The amplification conditions consisted in a denaturing step of 94 °C for 5 min followed by 25—35 cycles of 94 °C 30 s, 58 °C 30 s and 72 °C 40 s followed by a final extension step of 72 °C 7 min. All amplifications were repeated at least twice to verify results. PCR products (8 μl) were visualized on a 1% agarose gel stained with ethidium bromide. Samples that were to be compared were run in the same agarose gel. A 100 bp ladder was used as a size marker. The intensity of the amplification bands was estimated using Image Gauge v. 4.0 software (Fujifilm). A semi-quantitative analysis of mRNA transcription of the IL-8R was performed and expressed as relative to the GAPDH

gene transcription (expression relative to GAPDH) estimated in the same sample by using the formula: intensity of the IL-8R band/intensity of the GAPDH band.

In some cases, a quantitative real-time PCR was optimized and performed to confirm the results obtained in some experiments. These PCRs were performed in a Light-Cycler System (Roche) using LightCycler FastStart DNA Master Sybr Green I following the manufacturer's instructions and the primers used for semi-quantitative PCR. A melting curve which allows to check the specificity of the amplification was always included at the end of run. Results were obtained using a standard curve generated from a cDNA with high levels of IL-8R gene expression included in every run.

Evaluation of the regulation of the IL-8R expression in vitro

Head kidney leucocytes were isolated following the method previously described [31]. Briefly, the anterior kidney was removed aseptically and passed through a 100 μm nylon mesh using Leibovitz medium (L-15, Gibco, Invitrogen, UK) supplemented with penicillin (100 IU ml^{-1}), streptomycin (100 μg ml^{-1}), heparin (10 units ml^{-1}) and 2% fetal calf serum (FCS, Gibco). The resulting cell suspension was placed onto 51% Percoll density gradients. The gradients were centrifuged at 500 × *g* for 30 min at 4 °C. The interface cells were collected and washed twice at 500 × *g* for 5 min in L-15 containing 0.1% FCS. The viable cell concentration was determined by Trypan blue exclusion. Cells were resuspended in L-15 with 5% FCS at a concentration of 1×10^6 cells $ml^{-1}$and disposed in 24-well plates (1 ml per well).

The following day, head kidney leucocytes were treated with different concentrations of recombinant rainbow trout IL-8 or CK-6, known to be active in chemotaxis experiments previously performed [22]. After a further 24 h of incubation, RNA was extracted from the cells using Trizol and the levels of expression of the IL-8R evaluated through semi-quantitative RT—PCR as described above for the in vivo experiments.

The levels of expression of the IL-8R mRNA were also evaluated in RTS11 cells infected with VHSV or exposed to Poly I:C. For VHSV infection, RTS11 cells were passaged and seeded in 24-well plates with L-15 medium supplemented with 15% FCS. After an overnight incubation at 20 °C, the medium was removed and 200 μl of L-15 medium with 2% FCS containing VHSV (1×10^6 $TCID_{50}$ ml^{-1}) added to each well. After 1 h of incubation at 14 °C, additional 800 μl of L-15 with 2% FCS were added to each well, and the cells were incubated at 14 °C for the different incubation periods. Non-infected controls treated in the same way were always included in the experiments. In the case of Poly I:C stimulation, RTS11 cells seeded in 24-well plates with L-15 medium supplemented with 15% FCS were exposed to Poly I:C at a final concentration of 30 μg ml^{-1}. Controls without Poly I:C were also included. The cells were then incubated at 20 °C for 6 or 24 h, until total RNA was extracted as described above.

Chemotaxis experiments

The chemotactic capacity of RTS11 cells treated Poly I:C or infected with VHSV towards rainbow trout IL-8 was also determined and compared to that of untreated RTS11 cells. For this, RTS11 cells were pelleted at 500 × *g* for 15 min and adjusted to a concentration of 1×10^6 cells ml^{-1}. The cells were then treated with Poly I:C (30 μg ml^{-1}) or infected with VHSV as described above and respective controls were always included. After 6 h of incubation at 20 °C in the case of Poly I:C-treated cells and at 14 °C in the case of VHSV-infected cells, the chemotactic capacity towards recombinant IL-8 was assayed.

The chemotactic capacity was assayed in chemotaxis chambers introduced in 24-well plates (Costar-Corning Life Sciences). For this, 600 μl of L-15 medium supplemented with 15% FCS containing 150 ng ml^{-1} of recombinant rainbow trout IL-8 were placed in the wells. Controls with media alone were also included. After introducing the chemotaxis chambers in each of the wells, 100 μl of the RTS11 cells were loaded to the upper part of the chamber. The upper and lower chambers are separated by a 5 μm pore-sized polycarbonate filter. After 60 min of incubation at 20 °C, the number of cells that had migrated to the bottom of the wells was quantified by flow cytometry (FACS Calibur, Becton Dickinson). Cell number was determined at constant flow time (1 min) of the medium in the lower chamber. The migrating cells were analyzed based of forward and side light scatter parameters. Previous results [22] had shown that two subpopulations of non-adherent RTS11 cells distinguishable by flow cytometry that could be identified as immature monocyte- and mature macrophage-like populations are present in RTS11 cultures, and that only cells of the monocyte subtype specifically migrate towards IL-8. Thus, we only studied the number of monocyte-like cells (based on forward and side scatter parameters) that had migrated to the bottom of the wells in each case. All experiments were performed in duplicate, and were repeated twice.

Statistics

All data were statistically analyzed using Student's unpaired *t*-test and differences were considered statistically significant when $P < 0.05$.

Results

Evaluation of the in vivo effect of VHSV and Poly I:C on the level of expression of IL-8R

First, we determined the levels of expression of IL-8R in different tissues of rainbow trout previously infected with VHSV (Fig. 1A). The virus induced a significant decrease in the levels of expression of IL-8R in head kidney, spleen and muscle of infected trout when compared to the levels observed in non-infected controls. The results were evident through semi-quantitative RT—PCR (data not shown), but were also confirmed through real-time quantitative PCR (Fig. 1A), where we observed decreases in the levels of transcription of IL-8R of up to 10-fold in the case of the head kidney. In these same samples, previous studies revealed a significant up-regulation of the levels of expression of IL-8 in response to VHSV, especially in the spleen [24]; therefore, we can state that at the same time that

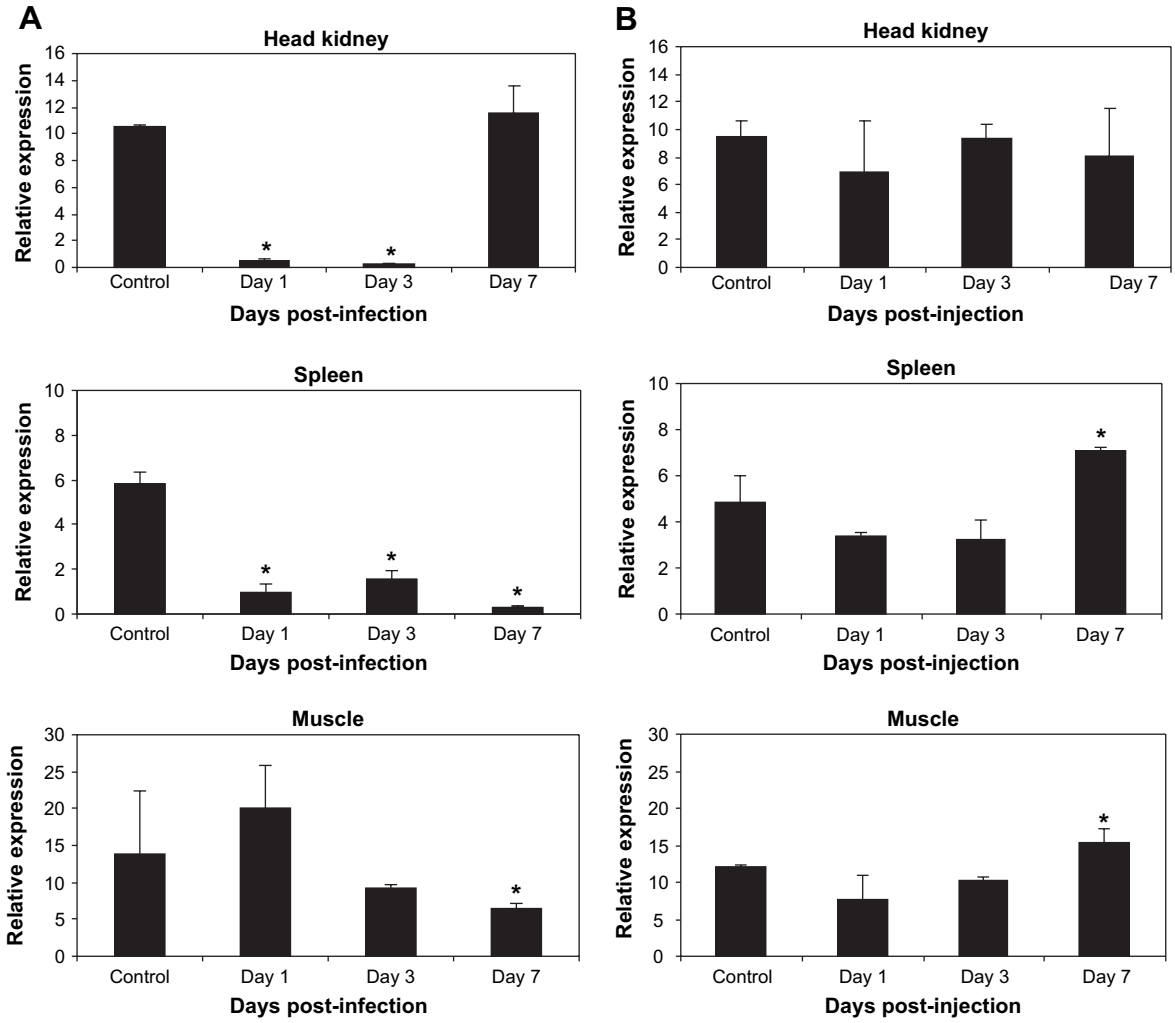

Figure 1 Quantitative real-time RT—PCR analysis of the expression of IL-8R mRNA in rainbow trout infected with VHSV (A) or treated with Poly I:C (B). The levels of transcription of IL-8R were evaluated in head kidney, spleen and muscle of fish that had been either been infected with VHSV intraperitoneally (1×10^7 $TCID_{50}$ per fish) or intramuscularly injected with Poly I:C (500 μg of Poly I:C per fish) after 1, 3 or 7 days and compared with their respective controls. RNA was extracted from all samples and the level of expression of IL-8R mRNA evaluated through semi-quantitative RT—PCR in cDNA pools of four individuals (data not shown). To confirm these results, a quantitative real-time RT—PCR was optimized and performed in these cDNA pools in duplicate. The results obtained through quantitative real-time RT—PCR are shown as mean expression determined with a standard curve generated using a standard cDNA with high levels of gene expression which was included in every PCR run. *Significantly different from respective controls as determined by unpaired *t*-test ($P < 0.05$).

VHSV induces an up-regulation of the transcription of IL-8, it down-modulates the transcription of its receptor.

In the case of injecting trout with Poly I:C, this decrease in the levels of expression was no longer visible (Fig. 1B). No significant effect of Poly I:C on the levels of expression of IL-8R were observed in the head kidney. In the spleen and in the muscle, however, a significant increase in the levels of transcription of IL-8R was observed and confirmed by real-time quantitative PCR analysis at day 7 post-injection.

Evaluation of the effect of VHSV and Poly I:C on the level of expression of IL-8R on the RTS11 cell line

Since RTS11 is a rainbow trout monocyte-macrophage-like cell line that has proved to be resistant to VHSV replication [32], we wanted to study how the transcription of IL-8R was affected by VHSV and Poly I:C in this cell line, to then compare it to the results previously observed in vivo. In this case, we found that the infection of RTS11 cells with VHSV did not have a significant effect on the levels of expression of IL-8R (Fig. 2). However, as observed in vivo, Poly I:C significantly induced the levels of transcription of IL-8R both after 6 and 24 h of incubation.

Chemotactic capacity of RTS11 previously treated with Poly I:C or infected with VHSV

We studied the chemotactic capacity towards IL-8 of RTS11 previously treated with Poly I:C for 6 h (in which the levels of transcription of IL-8R were very high), cells infected with

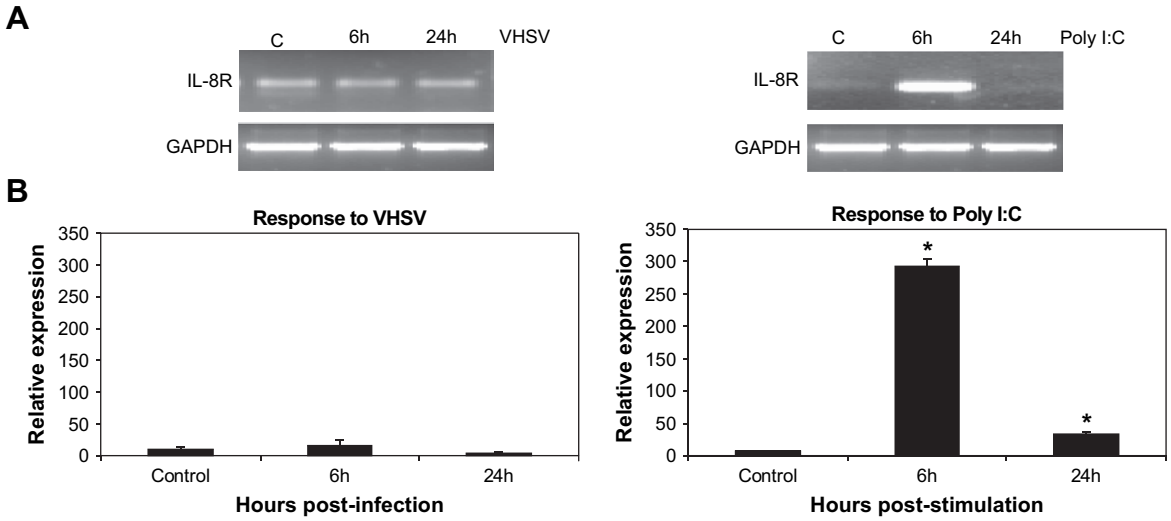

Figure 2 Expression of IL-8R mRNA in RTS11 infected with VHSV or treated with Poly I:C. RTS11 cells were either infected with VHSV (1×10^6 $TCID_{50}$ ml^{-1}) or exposed to Poly I:C (30 μg ml^{-1}). After 6 or 24 h of incubation at 14 °C or 20 °C, respectively, total RNA was extracted from the cells and the level of expression of the IL-8R gene evaluated in the different samples. (A) Stained gel showing the amplified products for the IL-8R gene in infected or stimulated cultures. (B) The IL-8R transcription was also evaluated by real-time PCR. Results are shown as mean expression determined using a standard curve generated from a cDNA with high levels of gene expression. *Significantly different from respective controls as determined by unpaired *t*-test ($P < 0.05$).

VHSV or untreated cells both with basal levels of IL-8R transcription, in order to establish if an increased IL-8R transcription could correlate with an increased chemotactic activity. As expected, RTS11 cells previously treated with Poly I:C had an increased chemotactic capacity towards IL-8 (Fig. 3), whereas the infection with VHSV that did not modify the levels of expression of IL-8R did not alter the chemotactic capacity of RTS11 cells. The number of migrated cells in RTS11 cultures treated with Poly I:C was significantly higher than that of untreated RTS11 cells not only in response to IL-8, but also in wells in which media alone was added, suggesting an influence of Poly I:C on random chemokinesis as well.

Expression of IL-8R in response to DNA vaccination against VHSV

In order to determine the effect that DNA vaccination might have on the levels of transcription of IL-8R, we studied the levels of transcription in the head kidney, spleen and muscle of fish intramuscularly vaccinated with a VHSV DNA vaccine (pMCV1.4-G) and compared it to the levels of expression obtained in response to the empty vaccine plasmid (pcDNA3) (Fig. 4). At day 3 post-vaccination, a significant increase in the levels of expression of IL-8R mRNA was observed in the muscle of vaccinated fish in comparison to the levels observed in controls, whereas no significant effect was observed in the spleen nor in the head kidney. At day 7 post-vaccination, this significant increase in the muscle was no longer visible.

Expression of IL-8R in the muscle of trout intramuscularly injected with plasmids coding for different rainbow trout chemokines

As a further step to clarify whether this receptor is in fact being used by IL-8, we intramuscularly injected plasmids coding for different rainbow trout chemokines (IL-8 and other CC chemokines such as CK5B, CK6 and CK7A) and evaluated the expression of IL-8R. Due to the previous results obtained in response to the VHSV DNA vaccine, where we only observed a significant induction of the levels of expression in the muscle at early times post-injection, in this case, we only evaluated the levels of transcription of IL-8R in the muscle at day 3 post-injection of the different plasmids. Before this experiment was conducted, we verified that the plasmids were expressed correctly upon intramuscular injection, by evaluating the transcription of the chemokine coded in the plasmid in the muscle. All chemokine constructs were efficiently expressed in the muscle upon injection (data not shown).

As expected, the injection of the expression plasmid coding for rainbow trout IL-8 induced a significant increase in the levels of expression of IL-8R in the injection site (Fig. 5). However, we also observed a significant increase in response to the plasmid which codes for the rainbow trout CC chemokine, CK-6. The other plasmids coding for other CC chemokines did not produce a significant effect on IL-8R transcription.

Expression of IL-8R in head kidney leucocytes exposed to IL-8 and CK-6 chemokines

Since the results obtained after the injection of the expression plasmids coding for the different rainbow trout chemokines pointed out to a role of both IL-8 and CK-6 in regulating the levels of expression of IL-8R, we studied the levels of expression of the receptor in head kidney leucocytes exposed to recombinant rainbow trout IL-8 and CK-6. The results obtained in vivo were confirmed and both IL-8 and CK-6 up-regulated the levels of transcription of IL-8R in head kidney leucocytes (Fig. 6).

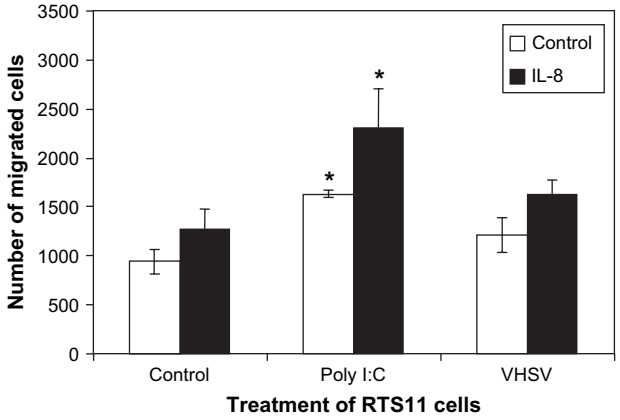

Figure 3 Chemotactic capacity of RTS11 cells previously treated with Poly I:C or infected with VHSV towards IL-8. RTS11 cells were either infected with VHSV (1×10^6 $TCID_{50}$ ml^{-1}) or exposed to Poly I:C (30 μg ml^{-1}), and incubated at 14 °C or 20 °C, respectively for 6 h. The chemotactic capacity towards IL-8 was assayed as described in the Section Materials and methods. Results are shown as the mean number of migrated monocyte-like cells with standard deviation for two wells of each treatment for one of two similar experiments. *Number of migrated cells significantly higher than that observed in response in RTS11 cells not treated with Poly I:C nor infected with VHSV determined by unpaired *t*-test ($P < 0.05$).

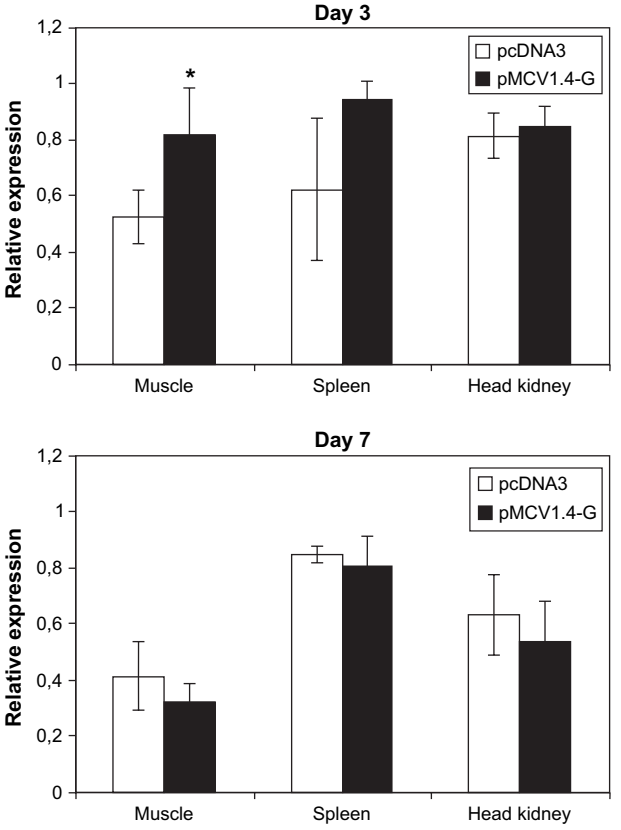

Figure 4 Semi-quantitative RT—PCR analysis of the levels of expression of IL-8R mRNA in rainbow trout intramuscularly injected with a DNA vaccine against VHSV (pMCV1.4-G) compared to the levels of expression observed in trout injected with the empty plasmid as a control (pcDNA3). The RNA was extracted from muscle, spleen and head kidney of vaccinated and control trout at days 3 and 7 post-vaccination. The levels of expression of IL-8R were evaluated through semi-quantitative RT—PCR in individual samples. Data are presented as mean relative expression ± SD for five individuals from each group. *Significantly higher that their respective controls as determined by unpaired *t*-test ($P < 0.05$).

Discussion

Some studies have focused on the regulation of the expression of IL-8 in response to viral infection and vaccination, since this chemokine is thought to play an important role in the first line of defence against viral pathogens. IL-8 was shown to be up-regulated in fish infected with either VHSV [24], IHNV [23] or in response to DNA vaccination [23]. However, no studies have been performed to date in fish concerning the regulation of the expression of its receptor, something that must be also taken into account if we want to study the real effect that the virus is having on the biological function of IL-8. Therefore, in the current work we approached these studies, focusing on the regulation of transcription of the rainbow trout gene previously identified as IL-8R [25], which is the only IL-8R identified to date in fish. These studies will also help to confirm that in fact this IL-8R with very low amino acididentity percentages to mammalian IL-8 receptors (34.9% of amino acid identity to human CXCR1 and 36.99% to human CXCR2) is in fact the receptor responsible for the binding of IL-8.

In this study, we have demonstrated that VHSV infection in rainbow trout induces a down-modulation of the levels of transcription of IL-8R, at very early times post-infection in the spleen and head kidney, and at later times post-infection in the muscle. VHSV is known to target these two hematopoietic organs at very early times post-infection, whereas it moves to the muscle at later times [33], thus the effect on IL-8R seems to be linked to viral replication. This down-regulation is no longer observed in response to Poly I:C, a dsRNA which in fact mimics the replication of the virus, and some up-regulation of IL-8R was even observed in this case, suggesting a viral interference in the regulation of IL-8R transcription. The mechanism through which VHSV is capable of down-regulating IL-8R gene expression is still unknown, but the down-regulation of chemokine receptors by viruses is a common pathogenic effect [34,35]. However, the chemokine presence in the cell surface may be regulated by two different mechanisms: altered gene expression or desensitization caused by phosphorylation-dependent internalization of the receptor upon ligand binding [36,37]. For example, human cytomegalovirus inhibits the migration of immature dendritic cells by down-regulating the presence of CCR1 and CCR5 on the cell surface, while the levels of expression are unaffected [34]. However, the levels of expression of CXCR2 but not of CXCR1 are down-modulated in children infected with human deficiency virus type 1 [35]. Recently, a new mechanism through which viruses inhibit the migratory capacity of dendritic cells without affecting the

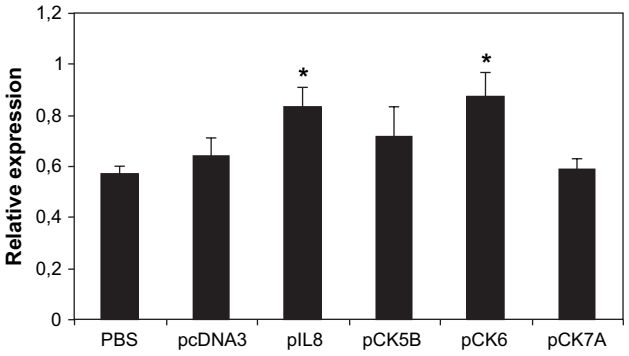

Figure 5 Semi-quantitative RT—PCR analysis of the levels of expression of IL-8R mRNA in rainbow trout intramuscularly injected with plasmids coding for different rainbow trout chemokines. Rainbow trout were intramuscularly injected with PBS, the empty pcDNA3 plasmid or plasmids coding for different rainbow trout chemokines (pIL8, pCK5B, pCK6, pCK7A). After 3 days, RNA was extracted from muscle samples, and the levels of expression of the IL-8R gene were evaluated through semi-quantitative RT—PCR in individual samples. Data are presented as mean relative expression ± SD for five individuals from each group. *Significantly higher that their respective controls as determined by unpaired *t*-test ($P < 0.05$).

expression levels of the receptor in the cell surface has been elucidated using vaccinia virus [38]. In this work, vaccinia infection resulted in a deficiency of extracellular signal-regulated kinase-1 and a disturbance of intracellular calcium mobilization, indicating a viral interference with signalling events downstream of the surface chemokine receptors. In fish, much more work needs to be performed to determine whether an additional IL-8R is present in fish as in mammals [7], and what are the effects of VHSV on the location of the receptors in the cell surface, but until antibodies against these receptors are obtained this work remains difficult. In any case, the down-modulation of IL-8R transcripts provoked by VHSV would impair the migration of sensitive immune cells to the site of infection with the consequent decrease in the local immune response.

We also wanted to determine the effect that DNA vaccination might have on IL-8R, since it was already demonstrated that IL-8 was up-regulated in response to DNA vaccines [23], the only effective method for controlling rhabdovirus infections in fish [39], regardless of the fact that the mechanism through which resistance is conferred is still unknown. The levels of IL-8R were increased in the muscle at early times post-vaccination, demonstrating that the vaccine mediates the attraction of immune cells to the injection site through IL-8 by both controlling the levels of IL-8 and of its receptor.

Finally, the effect that plasmids coding for different chemokines might have on the levels of expression was also studied, in order to further investigate the relations among different chemokines. Plasmids coding for IL-8 and CK-6 altered the levels of expression of IL-8R, and this regulatory capacity was also confirmed using recombinant chemokine products in leucocyte cultures. The fact that IL-8 increased the levels of expression of IL-8R was expected, pointing out the use of IL-8R by IL-8. The correlation observed between levels of expression of IL-8R and chemotactic capacity towards IL-8 found in RTS11 cells treated with Poly I:C also gives weight to the hypothesis that IL-8R is in fact being used by IL-8. Although it is generally accepted that CXC chemokines such as IL-8 are attractant for neutrophils but not for monocytes—macrophages, there are some exceptions to this general rule, and some effects of IL-8 on monocytes have been observed in mammals [10—13]. In rainbow trout, we have previously demonstrated that IL-8 is chemotactic for the subpopulation of RTS11 that could be catalogued as monocyte-like cells in the basis of their flow cytometric profile [22]. CK-6, however, also increased the levels of expression of IL-8R, and a possible use of IL-8R by CK-6 is not expected since in mammals one of the two IL-8 receptors, CXCR2, may be used by different chemokines than IL-8 but only by CXC chemokines [7]. Therefore, in this case, an indirect effect seems more probable and may be attributed to the fact that CK-6 increases the levels of expression of IL-8 (data not shown) that then could up-regulate IL-8R transcription.

In conclusion, we have determined that while the infection with VHSV decreases the level of transcription of IL-8R, Poly I:C increases it, suggesting a suppressive effect of the virus on this immune function, which might constitute a mechanism that enables it to elude the immune response. The fact that this viral suppression is no longer observed in the RTS11 cell line resistant to VHSV gives weight to this hypothesis, although much more work must be performed to determine the mechanism through which this suppression takes place. We have also determined that DNA vaccination significantly increased IL-8R transcription only in the muscle at very early times post-vaccination. Finally, as a demonstration of the fact that this receptor is being used by IL-8, we have determined that IL-8 controls the expression of IL-8R, and that when the levels of expression of IL-8R are significantly increased, the migratory capacity towards IL-8 is also increased. Furthermore, CK-6 indirectly regulates IL-8R expression as well. How and why the CC chemokine CK-6 modulates IL-8R transcription must still be studied in depth.

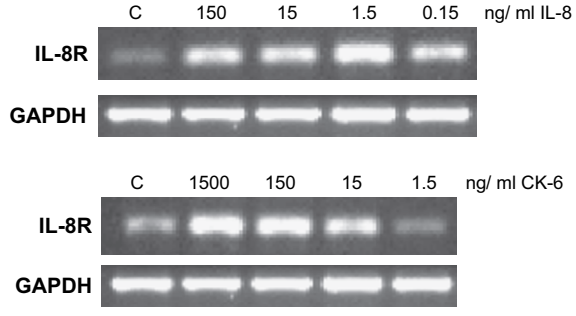

Figure 6 Semi-quantitative RT—PCR analysis of the levels of transcription of IL-8R in rainbow trout head kidney leucocytes in response to different concentrations of recombinant IL-8 and CK-6. After 24 h of incubation with the different chemokine concentrations, RNA was extracted and the levels of expression of IL-8R evaluated through semi-quantitative RT—PCR. The figure shows a stained gel with amplified products for the IL-8R and for their respective GAPDH products in a representative experiment.

Acknowledgments

This work was supported by grants AGL2004-07404-C02 and AGL05-00339ACU from the Ministerio de Educacion y Ciencia (Spain) and by the EPIZONE European Network of Excellence. The technical assistance of Esther Sanchez and Lucia Gonzalez is greatly appreciated.

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