Identification of a novel CCR7 gene in rainbow trout with differential regulation in the context of mucosal or systemic infection

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ABSTRACT

In mammals, CCR7 is the chemokine receptor for the CCL19 and CCL21 chemokines, molecules with a major role in the recruitment of lymphocytes to lymph nodes and Peyer´s patches in the intestinal mucosa, especially naïve T lymphocytes. In the current work, we have identified a CCR7 homologue in rainbow trout (*Oncorhynchus mykiss*) that shares many of the conserved features of mammalian CCR7. The receptor is constitutively transcribed in the gills, hindgut, spleen, thymus and gonad. When leukocyte populations were isolated, IgM^+ cells, T cells and myeloid cells from head kidney transcribed the CCR7 gene. In blood, IgM^+ and IgT^+ B cells but not T lymphocytes were transcribing CCR7, whereas in the spleen, CCR7 mRNA expression was strongly detected in T lymphocytes. In response to infection with viral hemorrhagic septicemia virus (VHSV), CCR7 transcription was down-regulated in spleen and head kidney upon intraperitoneal infection, whereas upon bath infection, CCR7 was up-regulated in gills but remained undetected in the fin bases, the main site of virus entry. Concerning its regulation in the intestinal mucosa, the *ex vivo* stimulation of hindgut segments with Poly I:C or inactivated bacteria significantly increased CCR7 transcription, while in the context of an infection with *Ceratomyxa shasta*, the levels of transcription of CCR7 in both IgM^+ and IgT^+ cells from the gut were dramatically increased. All these data suggest that CCR7 plays an important role in lymphocyte trafficking during rainbow trout infections, in which CCR7 appears to be implicated in the recruitment of lymphocytes to mucosal tissues such as gills or intestine.

Keywords: chemokines, chemokine receptors, CCR7, rainbow trout, mucosal immunity, B lymphocytes.

1. Introduction

In rainbow trout (*Oncorhynchus mykiss*) twenty-two different genes coding for chemokines, or chemotactic cytokines, have been identified to date {Alejo, 2011 #4209}. In all vertebrates, chemokines can be further divided into subfamilies according on the position of conserved cysteines in their sequence, with the CC subfamily being the most numerous with twenty-eight members in mammals and eighteen in rainbow trout {Laing, 2004 #3921}. A high level of variation in the number of genes from this group in different fish species is evident, ranging from these eighteen genes present in trout to eighty-one that have been described in zebrafish (*Danio rerio)* {Nomiyama, 2008 #4234}. These differences indicate extensive duplication events that, together with the fact that chemokines are rapidly changing proteins {Bao, 2006 #4235; Nomiyama, 2008 #4234}, make the establishment of true orthologues between fish and mammalian chemokine genes very difficult. An attempt to group fish CC chemokines was made by Peatman and Liu {Peatman, 2007 #4236} who established seven large groups of fish CC chemokines through phylogenetic analysis: the CCL19/21/25 group, the CCL20 group, the CCL27/28 group, the CCL17/22 group, the macrophage inflammatory protein (MIP) group, the monocyte chemotactic protein (MCP) group and a fish-specific group.

Chemokines attract and modulate the immune function of the recruited cells through interaction with G protein linked chemokine receptors that form a family of structurally and functionally related proteins {Horuk, 1994 #4237}. Systematic searches for chemokine receptors in fish genomes and EST databases have identified twenty-six genes in zebrafish {DeVries, 2006 #4238; Nomiyama, 2008 #4234} for at least one hundred and eleven chemokine genes, as compared to the eighteen chemokine receptors

for forty-four chemokines known in humans. Although promiscuity in ligand binding is a known property of chemokine receptors, the large difference in chemokine and putative receptor numbers in zebrafish has suggested that fish chemokines may bind to receptors substantially different from known mammalian chemokine receptors {Nomiyama, 2008 #4234}. Interestingly, the pufferfish, which only encodes eighteen different chemokines as compared to the one hundred and eleven genes of zebrafish, still has a comparable number of putative receptor genes (twenty) {DeVries, 2006 #4238}. In rainbow trout, only the sequences of CCR9, CXCR4 and CXCR8 have been reported to date {Daniels, 1999 #4199; Zhang, 2002 #4082}. The CCR9 sequence was originally reported as CCR7, however, posterior analysis including the human CCR9 sequence identified it as a CCR9 homologue and it is now catalogued as such in the GenBank database (accession number NM_001124610).

In mammals, CCR7 is the receptor for both CCL19 and CCL21 {Forster, 2008 #4253}. CCR7 is highly expressed in naive T cells and is very important for their normal trafficking {Hwang, 2007 #4274}, since the expression of CCL21 in the luminal side of high endothelial venules facilitates their entrance in the lymph nodes {Gretz, 2000 #4240}. B lymphocytes on the other hand, express CCR7 at significantly lower levels, but CCR7 expression is increased upon engagement of the B cell receptor, thus facilitating T-B interactions within the lymph node {Okada, 2005 #4241}. CCR7 is also up-regulated in dendritic cells (DCs) during their maturation, leading them from their niches in peripheral tissues to the lymph nodes {Sanchez-Sanchez, 2006 #4242}. Apart from the maturing DC populations, a subset of DCs with an exclusive phenotype including CCR7 expression are thought to contribute to peripheral immune tolerance against self-antigens {Ohl, 2004 #4243}.

In gut-associated lymphoid tissue (GALT), the desensitization of CCR7 in wild-type mice {Warnock, 2000 #4254}, the genetic disruption of CCR7 {Forster, 2008 #4253}, or natural mutations in the CCR7 ligands CCL19 and CCL21 {Luther, 2000 #4256; Warnock, 2000 #4254}, all lead to a reduced homing of T cells into the Peyer´s patches present in the gut. Interestingly, B cell homing to these secondary lymphoid tissues has shown to be less CCR7 dependent {Forster, 2008 #4253; Okada, 2002 #4257}.

In the current study, we have identified and cloned a CCR7 homologue in rainbow trout. Phylogenetic analysis of the newly identified sequence reveals a closer sequence similarity to mammalian CCR7 sequences than the previous discovered trout chemokine receptor, confirming the change in ascription of that gene previously reported as trout CCR7 as CCR9. As in mammals, CCR7 is strongly transcribed in the thymus and moderately transcribed in gills, hindgut, spleen and gonad. When sorted leukocyte populations were examined for CCR7 transcription, the receptor was strongly detected in spleen T lymphocytes and at low levels in head kidney T cells, myeloid cells and some B cell populations. Surprisingly, T cells from blood showed undetectable levels of CCR7. In order to establish how different infection models regulated CCR7, we studied the levels of CCR7 transcription in response to viral hemorrhagic septicaemia virus (VHSV) intraperitoneal or bath infection as well as in response to *Ceratomyxa shasta*, a parasite with a strong tropism for the gut. Our studies reveal a major role of CCR7 in the mobilization of lymphocytes to mucosal sites such as gills or intestine, but not to the skin. Moreover, in the case of the *C. shasta*, we found the levels of transcription of CCR7 in both IgM^+ and IgT^+ B cells were strongly increased in response to the infection, revealing a major role of this chemokine receptor in the recruitment of B cells to the hindgut. Undoubtedly, understanding the mechanisms

through which chemokine receptors are regulated will be essential for the delimitation of the roles of the target chemokines and for understanding lymphocyte trafficking in fish.

2. Materials and Methods

2.1. Cloning of rainbow trout CCR7

The previously identified *Danio rerio* CCR7 sequence (GenBank Accession number NM-001098743.1) was used to search public EST databases for similar rainbow trout sequences. All sequences identified as chemokine receptor-like molecules were translated using the Clone Manager suite 7 program. Translated sequences were compared with mammalian CCRs using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), thus identifying two rainbow trout expressed tags (accession numbers CX721232 and CU065128) that encoded fragments of a CCR7-like molecule.

The sequences lacked a stop codon, therefore 3'RACE was performed to obtain the complete sequence using a SMART cDNA obtained from peripheral blood leukocytes (PBLs) and the primers indicated in Table 1. An overlapping fragment was amplified which contained the final segment of the CCR7 coding sequence and the 3´ untranslated region (UTR). Primers were then designed to amplify the full coding sequence.

2.2. *Sequence analysis*

Homology searching was performed using the basic local alignment tool program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). TMHMM was used to predict the protein structure. Multiple sequence alignments were carried out using the Clustal W program. The phylogenetic tree was created using the neighbor joining (NJ) method using the MEGA5 program and was bootstrapped 1000 times using the Jukes and Cantor model {Jukes, 1969 #4284}.

A previous sequence (Accession number AJ003159.1) had been identified as rainbow trout CCR7 {Daniels, 1999 #4199}, however posterior analysis in which the human CCR9 was included revealed a closer homology to CCR9 as verified in our study. This sequence is now identified in the GenBank as rainbow trout CCR9.

2.3. Fish

Healthy specimens of rainbow trout (*Oncorhynchus mykiss*) were obtained from Centro de Acuicultura El Molino (Madrid, Spain). Fish were maintained at the Centro de Investigaciones en Sanidad Animal (CISA-INIA) laboratory at 14ºC with a re-circulating water system, 12:12 hours L:D photoperiod and fed daily with a commercial diet (Skretting, Spain). Prior any experimental procedure, fish were acclimatized to laboratory conditions for 2 weeks and during this period no clinical signs were ever observed. The experiments described comply with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals.

2.4. Isolation of leukocytes

Blood was collected from the caudal vein using a heparinized syringe and immediately diluted in cold medium Mixed Isove's DMEM/Ham's F12 (Gibco) at a ratio of 1:1. Head kidney and spleen were aseptically removed and homogenized with an Elvehjem homogenizer to prepare single cell suspension. The purged and opened gut was cut into small pieces and vigorously shaken for 5 min in 30 ml of cold medium to free the cells from the tissue. Gills were cut into small pieces and after brief shaking in cold medium were homogenized with Elvehjem homogenizer. Prepared single cell suspensions of gut and gills were filtered through gauze. To discard the excess of mucus and cell debris, cells were centrifuged at 1800 x *g* for 5 min. Pellet was resuspended in fresh medium.

Single cells suspensions prepared in previous steps were layered onto an isotonic Percoll gradient (Biochrom AG) (r = 1.075g ml^{-1}) and centrifuged at 650 x *g* for 40 min. Cells at the interphase were collected, washed with PBS, resuspended in corresponding volume of medium to the final concentration of 4 x 10^6 cells ml^{-1} and kept on ice until further preparation.

2.5. Enrichment of the cells by magnetic cell sorting

Leukocytes isolated by density gradient centrifugation as described above were magnetically sorted according to instructions of manufacturer. Briefly, leukocytes were incubated for 30 min on ice with selected anti-trout IgM (1,14) {DeLuca, 1983 #4258} and anti-trout T cells (D30) mAbs. Following washing step, cells were resuspended in 160 µl of sorting buffer (PBS with 0,5% BSA and 2mM EDTA) plus 40 µl of goat-anti-mouse- IgG microbeads (Miltenyi Biotec, Germany) for 30 min. Finally, the

magnetically labeled leukocytes were resuspended in 2 ml of sorting buffer and applied to columns attached to the magnetic separator (MiniMACS, Miltenyi Biotec, Germany). Unlabeled leucocytes flowing through the column were discarded. After washing of the column with appropriate amount of buffer, column was detached from magnetic separator and labeled cells were flushed out using 1ml of buffer. Quality of the separation was assessed by the flow cytometry and only fractions exceeding 95% purity were used for preparation of RNA.

2.6. Rainbow trout infection with VHSV

For all *in vivo* infections with VHSV, the 0771 strain was used and propagated in the RTG-2 rainbow trout cell line as previously described {Montero, 2011 #4222}. All virus stocks were titrated in 96-well plates according to Reed and Müench {Reed, 1938 #409}.

Rainbow trout were divided in two groups of 20 trout each. Groups were injected intraperitoneally with either 100 µl of culture medium (mock-infected control) or 100 µl of a viral solution (1 $x10^6$ $TCID_{50}$ per fish). At days 1, 3, 7 and 10 post-injection, five trout from each group were sacrificed by overexposure to MS-222, and head kidney and spleen removed for RNA extraction. The experiment was repeated once to confirm the results.

In a further experiment, rainbow trout were challenged with VHSV through bath infection to determine if CCR7 played a role in the mobilization of cells to the fin bases, the main entry site and a primary replication area {Montero, 2011 #4222}. For this, 12 rainbow trout of approximately 4-6 cm were transferred to 2 L of a viral solution containing 5 $x10^5$ $TCID_{50}$ ml^{-1}. After 1 h of viral adsorption with strong aeration at

14ºC, the fish were transferred to their water tanks. A mock-infected group treated in the same way was included as a control. At days 1 and 3 post-infection, four trout from each group were sacrificed by overexposure to MS-222. The area surrounding the base of the dorsal fins as well as the gills were removed for RNA extraction from four fish in each group.

2.7. Hind gut in vitro stimulation

In order to establish if CCR7 transcription was regulated in the hindgut upon immune stimulation, hindgut segments of approximately 1 cm of length were removed from 3 naïve fish and placed in 24 well plates with 1 ml of Leibovitz medium (L-15, Invitrogen) supplemented with 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 5% FCS alone or supplemented with Poly I:C (10 mg ml^{-1}) or *Escherichia coli* inactivated for 5 min at 100ºC ($1x10^4$ bacteria ml^{-1}). After 24 h of incubation at 18ºC, RNA was extracted and the levels of CCR7 transcription determined.

2.8. Ceratomyxa shasta trout infection

Rainbow trout were infected with the metazoan parasite *Ceratomyxa shasta* and after 3 months of infection, gut lymphocytes were extracted as previously described {Zhang, 2010 #4205}. It has been previously shown that fish surviving to 3 months after initiation of the infection had obvious signs of inflammation in the gut mucosa, as shown by extensive infiltration of B lymphocytes. Both IgM^+ and IgT^+ B cells were isolated from the gut segments and sorted as described before {Zhang, 2010 #4205}.

2.9. cDNA preparation

Total RNA was extracted from trout tissues or isolated leukocyte populations using Trizol (Invitrogen) following the manufacturer´s instructions. Tissues were first homogenized in 1 ml of Trizol in an ice bath, while cells were directly resuspended in Trizol. Two hundred µl of chloroform were then added and the suspension was centrifuged at 12 000 x *g* for 15 min. The clear upper phase was aspirated and placed in a clean tube. Five hundred µl of isopropanol were then added, and the samples were again centrifuged at 12 000 x *g* for 10 min. The RNA pellets were washed with 75% ethanol, dissolved in diethylpyrocarbonate (DEPC)-treated water and stored at -80ºC.

RNAs were treated with DNAse I to remove any genomic DNA traces that might interfere with the PCR reactions. One µg of RNA was used to obtain cDNA in each sample using the Superscript III reverse transcriptase (Invitrogen). In all cases, RNAs were incubated with 1 µl of oligo $(dT)_{12\text{-}18}$ (0.5 µg ml^{-1}) and 1 µL 10 mM dinucleoside triphosphate (dNTP) mix for 5 min at 65ºC. After the incubation, 4 µL of 5x first strand buffer, 1 µL of 0.1 M dithiothreitol (DTT) and 1 µL of Superscript III reverse transcriptase were added, mixed and incubated for 1h at 50ºC. The reaction was stopped by heating at 70ºC for 15 min, and the resulting cDNA was diluted in a 1:10 proportion with water and stored at -20ºC.

2.10. *Evaluation of CCR7 gene expression by real time PCR*

To evaluate the levels of transcription of CCR7, real-time PCR was performed with an Mx3005P™ QPCR instrument (Stratagene) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures containing 10 µl of 2x SYBR Green supermix, 5 µl of primers (0.6 mM each) and 5 µl of cDNA template were incubated for 10 min at 95ºC, followed by 40 amplification cycles (30 s at 95ºC and 1 min at 60ºC) and a dissociation cycle (30 s at 95ºC, 1 min 60ºC and 30 s at 95ºC). For each mRNA, gene expression was corrected by the elongation factor 1α (EF-1α) expression in each sample and expressed as $2^{-\Delta Ct}$, where ΔCt is determined by subtracting the EF-1α Ct value from the target Ct as previously described {Cuesta, 2009 #4142}. The primers used were designed from the CCR7 sequence using the Oligo Perfect software tool (Invitrogen) and are shown in Table 1. All amplifications were performed in duplicate to confirm the results. Negative controls with no template were always included in the reactions.

3. Results

3.1. *Identification of rainbow trout CCR7*

Using the previously described zebrafish CCR7, public EST databases were searched and two overlapping EST sequences that correlated with different fragments of a CCR7-like sequence were identified (Accession numbers CX721232 and CU065128). Assembly of these two sequences gave a nucleotide sequence which encoded a 326 aa protein which closely resembled CCR7. Posterior phylogenetic analysis through neighbor joining trees with different teleost and mammalian CCR7 sequences

confirmed this identification. Once the complete sequence had been obtained through 3´RACE, it was used in a BLAST search of the GenBank database which revealed that it was highly similar to known teleost and other vertebrate CCR7 sequences (Table 2). The top 48 matches were CCR7 sequences from many different species, followed by other chemokine receptor sequences. The sequence similarity of this novel sequence and known CCR7 sequences from teleosts and mammals can be observed in the alignment shown in Fig. 1. Conserved features needed for CCR7 function such as seven transmembrane domains (predicted using TMHMM) {Krogh, 2001 #4280}, the DRY sequence in internal loop 2, which interacts with the G-protein signaling partner (Scheer et al. 1996), cysteines required for disulfide bond formation and regulation {Ai, 2002 #4204}, conserved aspartic acids and tyrosines in the N-terminus which interact with the ligand and serines and threonines in the internal C terminus which can be phosphorylated for regulating receptor function. Interestingly, while four of five residues, identified by Ott *et al*. {Ott, 2004 #4281} as important for CCR7 receptor activation, were conserved, the fifth asparagine at position 305 of the human CCR7 sequence is not conserved in either the teleost or duck sequences (although it was conserved in the trout CCR9 sequence). The teleost CCR7 sequences do have a conserved asparagine three amino acids before this location which may serve the same function, however.

Further phylogenetic analysis of the rainbow trout CCR7 with the other known teleost CCR7 sequences (Fig 2), as well as sequences of several human CCRs showed that rainbow trout CCR7 clustered with other teleost and mammalian CCR7 sequences far from the rainbow trout sequence previously identified as CCR7 (and now designated CCR9).

3.2. Distribution of CCR7 expression in naïve rainbow trout tissues and isolated leukocyte populations

A very specific expression pattern was observed for the trout CCR7 gene in tissues obtained from naïve fish as it was only detected in gills, hindgut, spleen, thymus and gonad (Fig. 3A). No transcription was detected in the skin, head kidney, liver or the brain. Surprisingly, when leukocyte populations were isolated with Percoll, some differences were observed, and for example gill and hindgut leukocytes from naïve fish showed undetectable levels of CCR7 transcription, despite the fact that CCR7 had been detected in the complete tissue (Fig. 3B). On the other hand, CCR7 transcription was detected in blood, spleen and head kidney leukocytes (Fig. 3B). The fact that head kidney leukocytes but not head kidney as a whole transcribes CCR7 may be due to activation of the cells through the isolation process. No CCR7 mRNA was detected in the RTS11 monocyte-macrophage cell line.

In those leukocyte populations in which CCR7 mRNA was detected, we sorted the main cell types and further analyzed CCR7 transcription (Fig. 3C). In blood, both IgM^+ and IgT^+ lymphocytes showed detectable levels of CCR7 transcription, which was not observed in blood T lymphocytes. In the spleen, however, T lymphocytes showed the higher levels of CCR7 transcription followed by IgM^+ cells. In this case, no CCR7 mRNA was detected in IgT^+ cells. Finally in head kidney, CCR7 transcription was detected in IgM^+ cells, T cells and myeloid cells but not in IgT^+ cells.

3.4. CCR7 transcription in response to VHSV infection

In order to determine if VHSV plays a role in lymphocyte trafficking during the course of a systemic viral infection such as VHSV, we first studied CCR7 transcription in the spleen and head kidney after infecting rainbow trout intraperitoneally with the virus. In the spleen, VHSV induced a significant down-regulation of CCR7 transcripts at both days 1 and 10 post-infection (Fig. 4A). In the head kidney, a significant increase of CCR7 transcription was detected at day 1 post-infection in response to the virus, however, as in the spleen, VHSV reduced CCR7 transcription at both days 3 and 7 post-infection. This experiment was repeated once, and very similar results were obtained.

It has been established that the fin bases constitute the main entry site for fish rhabdoviruses such as VHSV {Harmache, 2006 #4270}. Once the virus is internalized the dermis layer of the skin constitutes one of the primary replication sites within this area {Montero, 2011 #4222}. Although CCR7 had not been detected constitutively in the skin, we also studied whether VHSV exposure could induce a mobilization of leukocytes to this area that could be mediated by this receptor. However, throughout the complete experiment CCR7 remained undetected in the fin base area, thus suggesting that this molecule is not implicated in lymphocyte homing to the skin.

Although the gills do not constitute a major entry site for VHSV with only low {Brudeseth, 2002 #4275} or undetectable {Montero, 2011 #4222} levels of virus replication, a bath challenge with VHSV is able to trigger an effective immune response in this tissue with a strong up-regulation of many different chemokine genes at days 1 and 3 post-infection {Montero, 2011 #4222}. In this context, we have seen that the levels of transcription of CCR7 are strongly up-regulated in response to the virus, at day 3 post-infection (Fig. 5), when the levels of induction of chemokine genes is known to be high.

3.5. Transcription of CCR7 in the hind gut in response to immune stimuli

After having determined that CCR7 transcription was detected in hindgut but not in hindgut isolated leukocytes, we studied the effect of stimulating hindgut segments *in vitro* with Poly I:C or inactivated *E. coli* on CCR7 transcription (Fig. 5). Both Poly I:C and specially inactivated bacteria were capable of up-regulating the levels of transcription of this chemokine receptor.

3.6. Transcription of CCR7 in response to C. shasta infection

Infection of rainbow trout with *C. shasta*, a parasite with strong tropism for the gut, significantly up-regulated the levels of transcription of CCR7 in both sorted IgM^+ and IgT^+ cells in the gut (Fig. 6). In the spleen, a small increase was also observed in spleen IgT^+ cells in infected fish, however, IgM^+ cells did not show a significant increase in CCR7 transcription in response to the parasite infection. These levels of CCR7 transcription are relative to the levels of expression of EF-1α in an approximately equal number of sorted cells in each organ, and thus provide us an estimate of number of transcripts per cell. Therefore it reveals a specific role for CCR7 in the mobilization of B cells to the gut.

4. Discussion

In the current study, we have identified a rainbow trout chemokine receptor sequence, which is a homologue to mammalian CCR7. It has been difficult to establish

true homologies between identified teleost CC chemokines and their mammalian counterparts, but it seems that the degree of sequence conservation is much higher for CC chemokine receptors that have been identified to date {Liu, 2009 #4246}. Although a previous rainbow trout chemokine receptor sequence had been reported as CCR7, it was discovered before the human CCR9 had been identified {Daniels, 1999 #4199}. A more recent analysis including all mammalian chemokine receptors has demonstrated that this sequence is in fact a CCR9 homologue, and as such it is now so designated in the Genbank database. Our phylogenetic studies with the new chemokine receptor as well as the former sequence confirm the ascription of this new gene as the true rainbow trout CCR7 homologue.

The expression pattern of this rainbow trout CCR7 also resembles that of mammalian CCR7, which is strongly expressed in thymus, intestine and lymph nodes and at low levels in spleen, kidney, lung and stomach {Yoshida, 1997 #4274}. In these species, CCR7 is expressed at high levels in T cells and at lower levels in B cells {Yoshida, 1997 #4274}. Surprisingly, in our experiments a very high level of CCR7 transcription was detected in spleen T cells, whereas it was detected in head kidney T cells at levels equivalent to IgM^+ cells and remained undetected in blood T cells. These differences among the levels of CCR7 detected in T cell populations from different organs should imply differences in immune role and trafficking for different trout T cell subpopulations not yet clear, but previously suggested {Takizawa, 2011 #4263}. In blood, CCR7 transcription was also detected in IgT^+ cells, a fish specific B cell population specialized in mucosal immunity {Zhang, 2010 #4205}, for which recirculation patterns and chemokine receptor expression cannot be interfered from mammalian homologues.

In mammals, CCR7 is also expressed in mammalian mature DCs. While CCR6 is expressed primarily on immature DCs in the periphery, upon pathogen encounter, these DCs mature and migrate to secondary lymphoid organs where they present pathogen antigen to T cells to initiate specific adaptive immune responses {Sallusto, 1998 #4259}. DCs can then respond to CCL19 and CCL21 causing them to migrate to secondary lymphoid tissues where they can present antigen to naïve T cells and activate them {Sanchez-Sanchez, 2006 #4242}. In most teleost species, the presence of professional antigen presenting cells has not been clearly established. Some markers for DCs such as CD83 {Ohta, 2004 #4245} or CD80/86 {Zhang, 2009 #4276} in rainbow trout have been identified, and very recently a cell population resembling DCs has been characterised in this species adapting mammalian protocols {Bassity, 2012 #4277}. This $CD83^+$ DC-like population was shown to present antigen in a more efficient way than IgM^+ lymphocytes, however whether they represent an exclusive professional antigen presenting cells independent of other antigen presenting cells such as B cells remains questionable, especially since IgM^+ cells have been shown to express significantly higher levels of CD80/86 than other leukocyte subtypes {Zhang, 2009 #4276}.

Once we had established the distribution of CCR7 in tissues and leukocyte subtypes in normal conditions, we then studied how this receptor was regulated in the context of *in vivo* infections. When VHSV was injected intraperitoneally, CCR7 transcription significantly decreased in both spleen and head kidney, although in this last organ at very early times post-infection, an increase in CCR7 was detected. These results may indicate that upon viral infection through the peritoneum lymphoid cells are mobilized from these organs. Using infectious pancreatic necrosis virus (IPNV) we had previously demonstrated that virus injection in the peritoneal cavity induced a

mobilization of $CD4^+$, IgM^+, IgT^+ and $CD83^+$ cells {Martinez-Alonso, 2012 #4278}. It seems therefore feasible that CCR7 could be involved in this viral-induced mobilization of spleen and head kidney cells to primary sites of viral encounter. In concordance to this hypothesis, lymph nodes from $CCR7^{-/-}$ mice are devoid of naïve T cells whereas the T cell populations from spleen (red pulp), blood or bone marrow are greatly expanded {Forster, 1999 #4255}. Furthermore, LPS injection in chicken decreased the splenic CCR7 mRNA content by approximately 100 times {Annamalai, 2011 #4286}. Although it has been established that, upon TCR activation, CCR7 is up-regulated in T cells to promote the recirculation of recently activated T cells to encounter activated B cells, experiments performed with LCMV, revealed that although CCR7 is up-regulated in cytotoxic $CD8^+$ T cell populations upon virus exposure, *in vivo* infection with this virus provokes a marked down-regulation of CCR7 {Sallusto, 1999 #4272}. It has been hypothesized that once activated in lymphoid organs, effector CD8 T cells are armed with hazardous molecules (*e.g.* perforin, CD95L) and the main function of these cells is to destroy virus-infected target cells or tumor cells in the periphery. Antigen-bearing DCs in lymphoid organs are also potential targets for the effector cells. However, a rapid elimination of professional antigen presenting cells may hamper a sustained T cell immune response and may lead to a premature decline of the response before antigen in the periphery is completely cleared. Therefore, exclusion of effector CD8 T cells from the white pulp of spleen may protect professional antigen presenting cells from cytotoxic T cell attack {Potsch, 1999 #4273}.

When the VHSV infection was performed in trout through bath exposure, we observed an up-regulation of CCR7 in the gills suggesting a CCR7-mediated recruitment of immune cells, but not in the fin bases, despite the fact that this is the main site of virus entry into the host {Harmache, 2006 #4270}. Once the virus is

internalized through the fin bases, the dermis layer of the skin constitutes one of the primary replication sites within this area {Montero, 2011 #4222}, and although the chemokine response in this area seems to be limited in response to the virus {Montero, 2011 #4222}, all evidence points to the fact that CCR7 is not implicated in recruiting lymphocytes to the skin. In bovine, γδ T cells in the skin are shown to recirculate from the blood through the skin back into the afferent and efferent lymph within in a CCR7-independent fashion {Vrieling, 2012 #4271}.

Excluding the skin, CCR7 does seem to play a role in the mobilization of lymphocytes to peripheral mucosal tissues such as the gills (in response to VHSV) or the hindgut. The implication of CCR7 in lymphocyte mobilization to the gut is foreseen in the up-regulation of CCR7 transcription upon immune stimulation *ex vivo*, and in a more physiological way in the strong increase in CCR7 transcription detected in sorted B cells that have been mobilized to the gut in response to *C. shasta* infection. In mammals, although B cell homing to secondary lymphoid tissues has shown to be less CCR7 dependent than T cell homing {Forster, 2008 #4253}, B cells from $CCR7^{-/-}$ mice do not migrate to the lymph node upon antigenic challenge and B cells are mobilized to the lymph node B-zone-T-zone boundary upon exposure to antigen in a CCR7-dependent manner {Okada, 2005 #4241}. In our model, whether other CCRs are playing a role in the recruitment remains to be investigated.

In conclusion, we have identified a CCR7 homologue in rainbow trout that is strongly transcribed in spleen T cells and moderately transcribed in IgM^+ cells and IgT^+ cells from blood. Infection with VHSV induced a down-regulation of CCR7 transcription in spleen and head kidney and an up-regulation of CCR7 transcription in the gills. CCR7 also seems to be strongly involved in the recruitment of lymphocytes to the gut, since B cells mobilised to the gut in response to *C. shasta* showed a strong up-

regulation of CCR7 mRNA levels. Finally, all evidence point to a lack of CCR7 involvement in skin lymphocyte homing.

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References

Figure legends

Fig. 1. Alignment of rainbow trout CCR7 with known teleost, mouse and human CCR7 sequences and the known trout CCR9 sequence. Transmembrane sequences of the proteins are indicated in yellow. Cysteines needed for disulfide bonds and regulation are indicated in blue while conserved tyrosines and aspartic acid residues required for ligand binding are indicated in grey. The DRY sequence plus conserved serines and threonines that are required for regulation and G protein binding are indicated in purple as are putative glycosylation sequences in the amino terminus of the tilapia, zebrafish human and duck sequences. Residues required for activation of the receptor identified by Ott *et al*. {Ott, 2004 #4281}, are indicated in green (red for the probable alternate in the teleost sequences). The published sequences used were: *Oncorhynchus mykiss* chemokine receptor (ccr9), mRNA Accession number: NM_001124610.1 (as an outgroup), *Oreochromis niloticus* C-C chemokine receptor type 7-like (LOC100692019), mRNA Accession number: XM_003454632.1, *Danio rerio* chemokine (C-C motif) receptor 7 (ccr7), mRNA Accession number: NM_001098743.1, *Anas platyrhynchos* CC chemokine receptor 7 gene Accession number: EU418503.1, *Mus musculus* chemokine (C-C motif) receptor 7 (Ccr7), mRNA Accession number: NM_007719.2, *Homo sapiens* chemokine (C-C motif) receptor 7 (CCR7), mRNA Accession number: NM_001838.3.

Fig. 2. Evolutionary relationships of trout CCR7 with other known teleost CCR7 sequences inferred using the Neighbor-Joining method {Saitou, 1987 #4282}. The numbers shown next to the branches represent support for the cluster in a bootstrap test (1000 replicates) {Felsenstein, 1985 #4283}. The evolutionary distances were computed

using the Jukes-Cantor method {Jukes, 1969 #4284}. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 {Tamura, 2011 #4261}. The published sequences used were the same as in Figure 1.

Fig. 3. Constitutive levels of transcription of CCR7 in different tissues or leukocyte populations. The amount of CCR7 mRNA in a pooled sample from 2-4 naïve individuals was estimated through real time PCR in duplicate. CCR7 transcription was evaluated in gills, gut, skin, head kidney (HK), spleen, thymus, liver, gonad and brain (A), in complete leukocyte populations isolated form blood, spleen, head kidney (HK), gills, hindgut or RTS11 cells (B) or from sorted leukocyte populations from blood, spleen and HK using specific monoclonal antibodies (C). Data are shown as the mean gene expression relative to the expression of endogenous control EF-1α ± SD.

Fig. 4. Levels of transcription of CCR7 in the spleen (A) or head kidney (B) of rainbow trout infected intraperitoneally with VHSV or mock-infected with 100 µl of culture medium. At days 1, 3, 7 and 10 post-injection five trout from each group were sacrificed, RNA pooled and the levels of expression of CCR7 studied through real-time PCR in duplicate. Data are shown as the mean chemokine gene expression relative to the expression of endogenous control EF1-α ± SD. *Relative expression significantly different than the relative expression in respective control ($p<0.05$).

Fig. 5. Transcription of CCR7 in gills obtained from VHSV infected fish in comparison to mock-infected individuals. After 3 days of bath challenge with VHSV, the levels of transcription of CCR7 were assayed in four fish per group by real-time PCR. Data are shown as the mean chemokine gene expression relative to the expression of endogenous

control EF1-α. Open squares show valued in individual samples while black squares show the mean for each group.

Fig. 6. Levels of CCR7 transcription in hindgut segments stimulated in vitro with LPS or Poly I:C. Hindgut segments from different trout were treated in vitro with either Poly I:C (10 μg ml^{-1}) or inactivated *E. coli* ($1x10^4$ bacteria ml^{-1}). After 24 h of incubation at 18ºC, RNA was extracted and the levels of CCR7 transcription determined. Data are shown as the mean chemokine gene expression relative to the expression of endogenous control EF-1α ± SD of three independent cultures.

Fig. 7. Levels of transcription of CCR7 in IgM+ or IgT+ cells sorted from the spleen or gut of fish infected with *Ceratomyxa shasta* or mock-infected. After 1 month of infection, leukocytes were isolated from the gut and spleen of three animals in each group and IgM^+ and IgT^+ cells sorted using specific antibodies. The levels of transcription of CCR7 were individually assayed in these populations in duplicate through real time PCR. Data are shown as the mean chemokine gene expression relative to the expression of endogenous control EF-1α ± SD of three independent fish. *Relative expression significantly different than the relative expression in respective control ($p<0.05$).

Table 1. Primers used for the CCR7 cloning and real time RT-PCR expression.

Gene	Name	Sequence (5´-3´)	Notes
CCR7	RACE-F	CCTGAGGTGCTGCCTCAACCCCTTTG	3´RACE
CCR7	RACE-nF	CCTCCTGAAGCTGCTGAAGGATCTGG	3´RACE
CCR7	FULL F	CACCATGGCTACAGAGTTCATCACTGATTTCAC	Full sequence
CCR7	FULL-R	TTAGGGGGAGAAAGTGGTTGTGGTCT	Full sequence
CCR7	CCR7-RT-F	TTCACTGATTACCCCACAGACAATA	real time
CCR7	CCR7-RT-R	AAGCAGATGAGGGAGTAAAAGGTG	real time
EF-1α	EF1-RT-F	GATCCAGAAGGAGGTCACCA	real time
EF-1α	EF1-RT-F	TTACGTTCGACCTTCCATCC	real time

Table 2. The top five rainbow trout CCR7 blast search hits using the nucleotide coding sequence to search the nucleotide database by Blastn {Altschul, 1990 #4279}.

Species	Genbank Accession	E Value	% Identity
Oreochromis niloticus	XM_003454632.1	2e-169	74%
Oryctolagus cuniculus	XM_002719359.1	7e-62	67%
Danio rerio	NM_001098743.1	1e-58	73%
Danio rerio	BX546447.8	1e-58	73%
Taeniopygia guttata	XM_002193969.1	6e-57	66%