CCR7 Is Mainly Expressed in Teleost Gills, Where It Defines an IgD $^+$IgM$^-$ B Lymphocyte Subset

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**CCR7 Is Mainly Expressed in Teleost Gills, Where It Defines an IgD⁺IgM⁻ B Lymphocyte Subset**

Rosario Castro,* Erin Bromage,† Beatriz Abós,* Jaime Pignatelli,* Aitor González Granja,* Alfonso Luque,* and Carolina Tafalla*

Chemokine receptor CCR7, the receptor for both CCL19 and CCL21 chemokines, regulates the recruitment and clustering of circulating leukocytes to secondary lymphoid tissues, such as lymph nodes and Peyer’s patches. Even though teleost fish do not have either of these secondary lymphoid structures, we have recently reported a homolog to CCR7 in rainbow trout (*Oncorhynchus mykiss*). In the present work, we have studied the distribution of leukocytes bearing extracellular CCR7 in naive adult tissues by flow cytometry, observing that among the different B lymphocyte populations, the highest numbers of cells with membrane (mem) CCR7 were recorded in the gill (7.5 ± 2% CCR7⁺ cells). In comparison, head kidney, spleen, thymus, intestine, and peripheral blood possessed <5% CCR7⁺ cells. When CCR7 was studied at early developmental stages, we detected a progressive increase in gene expression and protein CCR7 levels in the gills throughout development. Surprisingly, the majority of the CCR7⁺ cells in the gills were not myeloid cells and did not express membrane CD8, IgM, nor IgT, but expressed IgD on the cell surface. In fact, most IgD⁺ cells in the gills expressed CCR7. Intriguingly, the IgD⁺CCR7⁺ population did not coexpress memIgM. Finally, when trout were bath challenged with viral hemorrhagic septicemia virus, the number of CCR7⁺ cells significantly decreased in the gills while significantly increased in head kidney. These results provide evidence of the presence of a novel memIgD⁺memIgM⁻ B lymphocyte subset in trout that expresses memCCR7 and responds to viral infections. Similarities with IgD⁺IgM⁻ subsets in mammals are discussed.

The online version of this article contains supplemental material.

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The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; DD, degree-day; FF, first feeding; FSC, forward light scatter; HA, hemagglutinin A; LN, lymph node; mem, membrane; MHC-II, MHC class II; pAb, polyclonal Ab; PFF, prefirst feeding fry; P/S, penicillin/streptomycin; SSC, side scatter; TBT, Tris buffer with 0.2% Tween 20; VHSV, viral hemorrhagic septicemia virus.

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known yet for this receptor, its transcriptional regulation during pathogen encountering suggests a major role of the CCR7 in the mobilization of the lymphocytes to mucosal sites. In this study, we have characterized CCR7 at the protein level, showing that CCR7+ cells are mainly present in the gills. Furthermore, we have demonstrated that a major subpopulation of these CCR7+ cells in the gills constitute a novel memIgD+memIgM−B cell population, not previously reported in trout. These memIgD+memIgM− cells can also be detected at very low levels in other organs such as spleen, but not in PBLs. A possible homology to other subpopulations of memIgD+memIgM− cells found in mammals is discussed. Altogether, our results suggest a novel role for CCR7 in this subpopulation of B cells, not previously identified in other fish species.

Materials and Methods

Experimental animals

Female rainbow trout (Oncorhynchus mykiss) adults of ∼20–50 g were obtained from Centro de Acuicultura El Molino (Madrid, Spain) and maintained at the animal facilities of the Centro de Investigación en Sanidad Animal in a recirculating water system at 16°C, with 12:12-h light/dark photoperiod. Fish were fed twice a day with a commercial diet (Skretting).

Animal in a recirculating water system at 16˚C, with 12:12-h light/dark photoperiod. Fish were fed twice a day with a commercial diet (Skretting). Female rainbow trout eyed eggs (∼360 degree-days (DD)) postfertilization were also obtained from Centro de Acuicultura El Molino. Eggs were maintained in the same conditions as above and fed at 2 wk posthatching with a commercial diet. Individuals at different stages of the development were sampled for RNA extraction and immunohistochemistry as follows: eyed eggs (∼306, ∼354, and ∼402 DD), immediate posthatch fry ( hatch, ∼450 DD), first feeding fry (FF, ∼562 DD), at the stage of full dis- appearance of the yolk sac (first feeding [FF]; ∼674 DD), and fry 3 wk posthatching (FF, ∼786 DD).

Twenty-eight and 42 d after the first immunization, rabbits were boosted with an isotype-specific secondary Ab, Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Invitrogen), and with a mouse anti-β2-M PE-mAb (Miltenyi Biotec) for 30 min at 4˚C. After two washes in staining buffer, cells were coin- cubated with an isotype-specific secondary Ab, Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Invitrogen), and with a mouse anti-β2-M PE-mAb (Miltenyi Biotec) for 30 min at 4˚C. Cells were washed three times and analyzed on a FACSCalibur flow cytometer (BD Biosciences). Non-transfected cells and cells transfected with the empty plasmid were used as controls.

A further verification of the Ab specificity was blocking the anti-CCR7 pAb with the specific synthetic peptide used for immunization. For this, a 30 min blocking preincubation of the pAb with the specific peptide at 1:1, 1:5, and 1:10 molar ratios was performed before cell staining for flow cytometry. A preincubation of the pAb with the CCR7 peptide (EATQGAANSTQTDCC) at a ratio 1:5 was also performed as further control.

Flow cytometric analysis of trout leukocyte populations

The percentage of CCR7+ cells was analyzed in blood-depleted (buffer-perfused) naive fish as well as in PBLs. Fish were anesthetized with 30 mg/ kg benzocaine in water. Blood was extracted from the cardiac vein with a heparinized needle and diluted 10 times with Leibovitz medium (L-15; Invitrogen) supplemented with P/S, 10 U/ml heparin, and 2% FCS. Sub- sequently, a transcardial perfusion was conducted to remove the circulating blood from the tissues. Heart was cannulated through the ventricle into the bulbus arteriosus for perfusion with 30 ml teleost Ringer solution (pH 7.4), with 0.1% pronase, using a peristaltic pump at a constant flow rate of ∼5 ml/min, whereas the atrium was cut to drain the blood out of the circu- latory system. After perfusion, tissues (gill, thymus, spleen, head kidney, and midgut) stained for leukocyte FACS staining were placed in L-15 with 10 U/ml heparin, and 2% FCS. All tissues with the exception of the midgut were washed with a 100-μm nylon mesh, and the resulting cell suspensions were spun at 300 g at 4˚C for the second dis- cernment. The Percoll gradient was centrifuged at 500 × g for 30 min at 4˚C. Blood cell suspensions were placed onto a 51% Percoll density layer and centrifuged as above. The interface cells were collected, washed at 500 × g for 5 min in L-15 containing 0.1% FCS, and resuspended in L-15 with P/S and 2% FCS. Midgut was opened lengthwise, washed in PBS, and cut into small pieces. The midgut cell-extraction procedure started with one round agitation at 4°C for 30 min in L-15 medium with P/S and 5% FCS followed by an ag- gitation in PBS with 1 mM EDTA and 1 mM DTT for 30 min. Finally, gut tissues were digested with 0.15 mg/ml collagenase (Sigma-Aldrich) in L-15 for 1.5 h at 20°C. All leukocyte fractions were collected and pooled, washed three times, and an aliquot of 1 × 106 cells was used for the flow cytometric analysis.

Transfection of trout gill lymphocytes

The percentage of CCR7+ cells was analyzed in blood-depleted (buffer-perfused) naive fish as well as in PBLs. Fish were anesthetized with 30 mg/kg benzocaine in water. Blood was extracted from the cardiac vein with a heparinized needle and diluted 10 times with Leibovitz medium (L-15; Invitrogen) supplemented with P/S, 10 U/ml heparin, and 2% FCS. Subsequ- ently, a transcardial perfusion was conducted to remove the circulating blood from the tissues. Heart was cannulated through the ventricle into the bulbus arteriosus for perfusion with 30 ml teleost Ringer solution (pH 7.4), with 0.1% pronase, using a peristaltic pump at a constant flow rate of ∼5 ml/min, whereas the atrium was cut to drain the blood out of the circulatory system. After perfusion, tissues (gill, thymus, spleen, head kidney, and midgut) stained for leukocyte FACS staining were placed in L-15 with 10 U/ml heparin, and 2% FCS. All tissues with the exception of the midgut were washed with a 100-μm nylon mesh, and the resulting cell suspensions were spun at 300 g at 4˚C for the second discernment. The Percoll gradient was centrifuged at 500 × g for 30 min at 4˚C. Blood cell suspensions were placed onto a 51% Percoll density layer and centrifuged as above. The interface cells were collected, washed at 500 × g for 5 min in L-15 containing 0.1% FCS, and resuspended in L-15 with P/S and 2% FCS. Midgut was opened lengthwise, washed in PBS, and cut into small pieces. The midgut cell-extraction procedure started with one round agitation at 4°C for 30 min in L-15 medium with P/S and 5% FCS followed by an ag- gitation in PBS with 1 mM EDTA and 1 mM DTT for 30 min. Finally, gut tissues were digested with 0.15 mg/ml collagenase (Sigma-Aldrich) in L-15 for 1.5 h at 20°C. All leukocyte fractions were collected and pooled, washed three times, and an aliquot of 1 × 106 cells was used for the flow cytometric analysis.

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Sorting of leukocyte populations from trout

Gill leukocytes were sorted into either CCR7+ and CCR7− cells or IgD+ and IgD− cells using a BD FACSAria III (BD Biosciences) cell sorter. After sorting, 4 × 10^6 cells from each population were used to obtain total RNA and perform subsequent cDNA generation and real-time PCR using Power SYBR Green Cells-to-CT Kit (Life Technologies) according to the manufacturer’s instructions. Primers used for the transcriptional analysis of sorted populations had been previously designed and are listed in Table I.

Fluorescent microscopy

Gill leukocytes were seeded in eight-well culture slides coated with human fibronectin (BD Biocoat; BD Biosciences) for adherence. After 30 min, cells were fixed in 10% neutral buffered formalin for 15 min, washed three times in PBS, and stained with the anti-CCR7 pAb and/or the anti-IgD mAb overnight at 4˚C. Secondary Abs were then added (Alexa Fluor 488 donkey anti-rabbit IgG; Invitrogen) for 1 h at room temperature. Nuclei were stained with Hoechst 33342 (Pierce).

Gene expression analysis in early life stages

To analyze the levels of transcription of CCR7 in comparison with all known chemokine receptors at different stages of early trout development, total RNA was extracted from the samples using a combination of TRZol (Invitrogen) and RNAeasy Mini kit (Qiagen). One milliliter TRIzol was used to extract RNA from each sample and the procedure was then continued following the manufacturer’s instructions, performing on-column DNase treatment. Finally, RNA pellets were diluted in a 1:5 proportion with water and stored at −20˚C until used.

Two micrograms RNA was used to obtain cDNA in each sample using the Bioline reverse transcriptase (5 μl) and oligo(dT)12−18 (0.5 μg). One micromilliter TRZol was added to the RNA and the mixture was incubated for 1 h at room temperature. Nuclei were stained with Hoechst 33342 (Pierce). Samples were then homogenized through mechanical disruption in TRZol using a disruption pestle. A total of 200 μl/ml chloroform was then added and the suspension centrifuged at 12,000 rpm for 15 min. The clear upper phase was recovered, mixed with an equal volume of 100% ethanol, and immediately transferred to RNAeasy Mini kit columns (Qiagen). The procedure was then continued following the manufacturer’s instructions, performing on-column DNase treatment. Finally, RNA pellets were eluted from the columns in RNase-free water and stored at −80˚C.

Real-time PCR was performed with a LightCycler 480 System (Roche) using FastStart SYBR Green Master mix (Roche). Reaction mixtures containing 5 μl × SYBR Green Supermix, 300 nM gene-specific primers (Table I), and 2 μl cDNA template were incubated for 10 min at 95˚C, followed by 40 amplification cycles (30 s at 95˚C and 1 min at 60˚C). A melting curve for each PCR was determined by reading fluorescence every degree between 60˚C and 95˚C to ensure only a single product had been formed. The efficiency was calculated as E = 10^(−1/ΔCt), where ΔCt is the slope generated from the standard dilutions, when log dilution is plotted against Δ threshold cycle number. Differences in the gene expression level of the genes among the different stages of development were determined using the Pfaffl method (25), comparing the mean expression of each group to the mean expression of the earliest stage (∼306 DD).

Immunohistochemistry

Excised organs from adult fish and whole fingerlings were fixed in Bouin’s solution for 24 h. Fish at hatch, PFF, and FF were fixed in Davidson’s fixative for 24 h. All samples were embedded in paraffin (Paraplast Plus; Sherwood Medical) and sectioned at 5 μm. After dewaxing and rehydration, sections were subjected to an indirect immunocytochemical method to detect trout CCR7, IgM, or IgD. After a heat-induced epitope retrieval in Tris-EDTA buffer (pH 9) (800 W for 5 min and 450 W for 5 min in a microwave oven), the sections were preincubated for 10 min in 10% normal goat serum in TBT for 10 min. Then sections were incubated with primary Ab overnight at 4˚C. Rabbit anti-trout CCR7 pAb and mouse mAb anti-trout IgD were used at concentrations of 5 and 10 μg/ml, respectively. The anti-trout IgD mAb recognizes both the membrane and secreted forms of the IgD (22). A mouse anti-IgM mAb (kindly donated by Dr. Kurt Buchmann, University of Copenhagen, and Dr. Karsten Skjoedt, University of Southern Denmark) (26, 27) was used at a concentration of 10 μg/ml, also labeling the membrane and the secreted form of the IgM. Following this incubation, unbound primary Abs were washed off using TBT. The tissue was covered with Dako REAL detection System, alkaline phosphatase/RED, and rabbit/mouse (DakoCytomation) biotinylated secondary Ab and following the manufacturer’s instructions for staining. The specificity of the reactions was determined by omitting the primary Abs. Mayer’s hematoxylin (DakoCytomation) was used as nuclear counterstain, and mounting was conducted with Aquamount (Merck). Slides were examined with an Axioslab (Zeiss) light microscope.

Viral hemorrhagic septicemia virus in vivo infections

Rainbow trout were challenged with viral hemorrhagic septicemia virus (VHSV) through bath infection to determine whether the infection induced changes in the number of CCR7+ cells in the gills. The VHSV challenge was performed as previously described (28). Briefly, 12 fish of ∼200 g were transferred to 4 l viral solution containing 5 × 10^6 TCID50/ml VHSV strain 0771. After 1 h of viral adsorption with strong aeration at 14˚C, fish were transferred to their water tanks. A mock-infected group treated in the same way was included as control. At days 1 and 3 postinfection, six to seven trout from each group were sacrificed by overexposure to MS-222. Gills and kidney were sampled and processed for FACS analysis as described above. The spleen was also removed from these fish to evaluate viral transcription through real-time PCR using the same protocol as described for the evaluation of immune gene expression. Statistical differences between the number of CCR7+ cells in infected and mock-infected fish were determined using a Student t test (p < 0.05).

Results

Identification of CCR7+ cells using a specific anti-CCR7 pAb

The pAb specific to rainbow trout CCR7 generated recognized the CCR7 extracellular peptide via ELISA (titer >1:512,000) and was also screened for its ability to recognize the full-length CCR7 molecule in transfected cells. For this, HEK cells were transfected with a pDisplay-CCR7 construct that expresses the rCCR7 in the cell membrane together with an HA tag. Forty-eight hours posttransfection, the binding ability of the anti-CCR7 pAb to pDisplay-CCR7–transfected cells was assessed in cell extracts from transfected cells immunoprecipitated with the anti-CCR7 pAb. Fig. 1A shows a band of the precipitated protein, present only in pDisplay-CCR7–transfected cells and not in cells transfected with the empty pDisplay plasmid carrying the HA tag. These results reveal that both the anti-HA and the anti-CCR7 recognize the same molecule, demonstrating the specificity of the anti-CCR7 pAb. Additionally, the specificity of anti-CCR7 pAb was assessed by flow cytometry. The anti-CCR7 pAb recognized ∼5% of the transfected cells, all of which were HA positive (data not shown). Finally, the binding specificity of the pAb to CCR7 was also assessed by blocking the Ag recognition site with the peptide used for the immunization. After the blockage of the Ab, only a residual expression level of nonspecific binding could be detected (Fig. 1B), indicating a specific recognition. Furthermore, this blockage was not observed when an irrelevant peptide was used. This control in which the pAb was preblocked with the specific peptide was included in all further flow cytometry experiments, and the values obtained were subtracted from the actual samples.

Once the specificity of the anti-CCR7 pAb was verified, we characterized the number of CCR7+ cells in different rainbow trout leukocyte populations, as well as their transcriptional responses (Table I). Perfused blood-depleted fish were always used to avoid contamination of blood cells in the tissue samples. We evaluated the number of CCR7+ cells in five to eight individual fish and consistently found that the highest population of CCR7+ cells was observed in the gills (Fig. 2A, Table II), with a mean percentage of 7.5% of cells within the leukocyte gate expressing CCR7 on their surface. A mean percentage of 3.7% CCR7+ cells were observed in the spleen, 1.8% in kidney, 1.7% in thymus, and the smallest proportion of CCR7+ leukocytes were observed in midgut and PBLs (0.5%). The presence of CCR7+ cells in the gills, spleen, kidney, and thymus was also confirmed by immunohistochemistry, revealing a disperse distribution of CCR7+ cells inside the tissues (Fig. 2B).
CCR7 expression through early developmental stages

Because CCR7 is an homeostatic receptor that participates not only in leukocyte trafficking during immune responses, but also in organ development and homeostasis, the transcription of CCR7 was assessed at key stages of the early trout development, comparing them to the levels of transcription of all other known chemokine receptors in rainbow trout. All chemokine receptors were transcribed as early as 306 DD postfertilization (Fig. 3A). CCR7 mRNA levels started to significantly increase after hatching, continuously increasing at each posterior step analyzed. An increased expression was observed after hatching for CCR6 and after FF for CCR9 and CXCR1, whereas no significant differences were observed among the different developmental stages for CCR9B, CCR13, or CXC receptor CXCR4.

To study whether this CCR7 expression at early life stages was also present in the gills, we conducted immunohistochemistry with samples obtained at hatch, PFF, and FF stages. Although some CCR7 staining was already visualized at hatching, the staining for CCR7 increased through the different developmental stages in the gills, in accordance with the results obtained in the transcriptional analysis of the complete individual (Fig. 3B). Our results reveal that CCR7+ cells are present in the gills from early developmental stages.

Table I. Oligonucleotides used for real-time PCR in this study

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F, Forward; R, reverse.
CCR7+ cells in the gills are mainly IgD+ cells

To determine which cells were expressing CCR7 in the gill in physiological conditions, we performed a double labeling of gill CCR7+ cells with specific leukocyte markers available for rainbow trout. Although memIgM+ cells in the gills were the major leukocyte type (∼15.5%), only ∼18% of these memIgM+ cells had CCR7 in the cell membrane (Fig. 4). A similar percentage was obtained for IgT cells that expressed CCR7 in the membrane. However, and despite the fact that memIgD constituted only ∼8.8% of the gill leukocyte population, 64% of these cells were expressing CCR7. Interestingly, 75% of the cells expressing CCR7 were memIgD cells. This experiment was repeated in five independent fish, and similar results were always observed, with some fish having all memIgD cells expressing CCR7, and memIgD cells accounting for most of the cells expressing CCR7 (80 ± 20% of memIgD cells express membrane CCR7 [memCCR7]). Myeloid cells were ∼14.3% of the gill leukocytes, but <15% of the cells expressed CCR7. Finally, only 4% of the CD8α cells in the gills expressed CCR7.

To confirm these results, we analyzed gene expression on the sorted CCR7+ population from the gills in comparison with the negative fraction. As expected, the transcripts for CCR7 were ∼10 times higher in sorted CCR7+ cells, but were also present in the negative fraction, suggesting that different cell types with no memCCR7 may have the potential to express CCR7 in response to different stimuli (Fig. 5A). The sorted CCR7+ cell fraction contained mRNA for memIgD as well as for secreted IgD, supporting the flow data. In contrast, although mRNA for total IgM was detected, the levels of transcription of the memIgM remained undetectable. Additionally, transcripts for the MHC class II (MHC-II) and the DC marker CD83 were also detected in gill CCR7+. In correlation with the flow data, no CD3 or CD8α transcripts were found in the CCR7+ sorted population, strongly suggesting that T cells in the gills do not express memCCR7 in physiological conditions.

As the majority of IgD+ cells were also CCR7+, we performed a transcriptional analysis of sorted IgD+ cells from the gills (Fig. 5A). We found that these cells transcribed CCR7 at levels ∼10 times higher than the negative fraction. These memIgD cells transcribed both memIgD as well as secreted IgD, suggesting that cells that still retain memIgD also secrete IgD. In contrast, sorted IgD+ mouse standard.
To verify that a subpopulation of cells in the gills was coexpressing CCR7 and IgD, we also performed immunofluorescence in gill leukocytes (Fig. 5B). Multiple CCR7+ cells and IgD+ cells were found among gill leukocytes. Although some single-positive cells were detected for both markers, many double-positive CCR7+ IgD+ cells were observed in concordance with the flow cytometry results.

Interestingly, when we compared the distribution of CCR7 in the gills to that of IgM and IgD by immunohistochemistry using serial sections from gill tissues, we observed that the distribution of IgM and IgD appeared very different (Supplemental Fig. 1). Apart from the strong IgM staining in the blood vessels of the secondary lamellae, IgM staining was visualized in the apical area of the primary lamellae as scattered cells. IgD staining was also observed in the apical area of the primary lamellae, but the distribution was more homogenous through the most exposed surface. Furthermore, positive IgD staining was also detected in and between secondary lamellae. When the distribution of CCR7 staining was compared, it resembled best that of IgD, being homogenously distributed through the primary lamellae as well as in and between secondary lamellae.

**CCR7 is strongly expressed in IgD+ cells with no memIgM**

To determine that IgD+ cells bearing CCR7 constitute a new B lymphocyte subset with no IgM in the cell surface, we performed a triple IgM/IgD/CCR7 staining in gill leukocytes. We simultaneously performed the same analysis in splenocytes, because this population is characterized by low expression of CCR7. IgD/IgM/CCR7-labeled cells were analyzed by FACS, and leukocytes were identified and gated on the basis of forward light scatter (FSC)/side scatter (SSC). First, CCR7 expression levels were determined in leukocytes from gills (Fig. 6A) and spleen (Fig. 6C) and compared against the expression of memIgD and memIgM. As shown in Fig. 6A, most of the IgD+ cells showed surface CCR7, and there were virtually no IgM+ cells expressing CCR7 (0.49% CCR7+IgM+ against 10.7% CCR7+IgM− cells). When memIgD and memIgM were plotted, we observed that almost no cells were double positive, but in contrast, memIgD+memIgM− and memIgD−memIgM+ were the major cell types (Fig. 6B). CCR7 expression levels were much lower in the spleen. Although mem-IgM+ splenocytes were very abundant in the spleen, just a very small fraction of this population expressed CCR7, which was rather expressed among the mem-IgD+ splenocytes, because almost one third of the mem-IgD+ cells expressed CCR7 (Fig. 6C). In this case, we could detect some mem-IgD+mem-IgM− cells, although still ~79% of the total number of mem-IgD+ cells were mem-IgM+ cells (Fig. 6D). Finally, to ascertain the contribution of the different B cell subsets to the expression pattern of CCR7 in these specific tissues, mem-IgD+ and mem-IgM+ populations in the gills (Fig. 6B) and spleen (Fig. 6D) were plotted to determine the CCR7 expression.
level in IgD\(^+\)IgM\(^-\), IgD\(^-\)IgM\(^+\), and IgD\(^+\)IgM\(^+\) B cell subsets. In the gills, cells bearing only memIgD showed the highest expression of CCR7, whereas cells bearing only memIgM did not express the receptor. Moreover, memIgD\(^+\)memIgM\(^+\) double-positive cells presented an intermediate level of CCR7 expression (Fig. 6B), suggesting that CCR7 expression was restricted to memIgD\(^+\)memIgM\(^-\) or memIgD\(^+\)memIgM\(^+\) cells and somehow excluded from memIgD\(^-\)memIgM\(^+\) cells. Overall, a similar scenario was found in the spleen, although CCR7 expression levels were lower (Fig. 6D). Cells bearing only memIgD contained a CCR7\(^+\) subpopulation, which presented the highest levels of CCR7 found in the spleen, memIgM-bearing cells, not expressing memIgD, did not express CCR7, whereas memIgD\(^+\)memIgM\(^+\) double-positive B cells showed an intermediate CCR7 expression level, which was consistent with the

FIGURE 5. (A) Transcriptional analysis of sorted CCR7\(^+\) and IgD\(^+\) cell populations from the gills. Data are shown as the mean gene expression relative to the expression of an endogenous control (EF-1\(\alpha\)) \pm SD obtained in three technical replicates from a pooled sample obtained from three individual fish out of three different pools analyzed. (B) Immunofluorescent detection of CCR7 and IgD in gill leukocytes using the anti-CCR7 pAb and the anti-IgM mAb. Arrowheads point to examples of single-positive cells, whereas arrows label CCR7\(^+\)IgD\(^+\) cells. The cell enlarged in the inset figure is indicated in a square. Scale bar, 200 \(\mu\)m.

FIGURE 6. FACS analysis of CCR7\(^+\)IgD\(^+\)IgM\(^-\) cells in rainbow trout. Characterization of CCR7\(^+\) cells in the gills (A, B) and the spleen (C, D) was performed by triple staining of gill and spleen leukocytes with a pAb against CCR7 together with mAbs against IgD and IgM. FSC/SSC leukocytes were gated in gill (A, left panel) and spleen (C, left panel) samples, and CCR7 fluorescence intensity was analyzed against gill (A, middle panel) and splenic IgD (C, middle panel), as well as gill (A, right panel) and splenic IgM (C, right panel). The presence of IgD and IgM B cell subsets was analyzed in the gills (B, left panel) and in the spleen (D, left panel). Then, IgD\(^+\)IgM\(^-\), IgD\(^-\)IgM\(^+\), and IgD\(^+\)IgM\(^+\) subpopulations were gated, and their fluorescence intensity for CCR7 was analyzed and plotted as a histogram for gills (B, right panel) and spleen (D, right panel) together with the fluorescence intensity obtained in the isotype controls (shaded histogram). Data shown correspond to a single representative fish of four to six individualized fish analyzed.
results observed in the gills. Together, these data suggest that memCCR7 expression in naive B cells is associated to the presence of IgD on the membrane of B cells, excluding the expression of the receptor from memIgM⁺ memIgD⁻ B cells. The results also indicate that memIgD⁺ memCCR7° B cells are found preferentially in the gills.

Effect of VHSV infection on the distribution of CCR7⁺ cells
To assess if an encounter with a pathogen can modify the distribution of the CCR7⁺ cell population in the gills, we performed a bath challenge with VHSV and then recorded the number of CCR7⁺ leukocytes at days 1 and 3 postinfection in both the gill and the kidney, a target organ for Ag presentation. We have previously established that at these days postinfection, VHSV induces the transcription of several chemokine genes in both gills and head kidney (28, 29). In some fish, the number of memIgD cells was also assessed. First, we assessed viral transcription in the spleen of these infected fish to ensure that fish exposed to the virus became infected. Transcription of the N gene was detected in all infected fish at day 3 postinfection. As shown in Fig. 7A, despite the high variability in CCR7⁺ numbers observed among individual fish, the percentage of CCR7⁺ cells in the gills significantly decreased in response to VHSV in comparison with the levels observed in mock-infected controls at day 1 postinfection. On the contrary, the percentages of CCR7⁺ significantly increased in the head kidney of infected animals in comparison with mock-infected controls at both days 1 and 3 postinfection. When we analyzed the number of memIgD⁺ cells in some of these samples, we observed that the level of memIgD significantly correlated to the level of memCCR7 in the gills (Fig. 7B). Based on these results and our previous findings that identified memIgD⁺ memIgM⁻ cells as the major cell type expressing CCR7, we can speculate that these cells are playing a role in Ag sensing and presentation. In contrast, although the number of CCR7⁺ cells increase in the head kidney in response to the infection, memIgD cells in these samples remained undetected. These results suggest two possible explanations for the increase in CCR7⁺ cells observed in head kidney in response to VHSV. Either activated CCR7⁺ cells from the gills are mobilized to the kidney while losing memIgD in response to activation, or the viral infection itself triggers memCCR7 expression in resident head kidney cells with no memIgD.

Discussion
The results from this study demonstrate that rainbow trout CCR7 is mainly expressed in the gills in physiological conditions. Within the gills, although we could detect a small percentage of IgM⁺ and IgT⁺ cells expressing CCR7, this receptor is mostly expressed in a subpopulation of B cells with memIgD and no memIgM that represents an important leukocyte subpopulation in gills (memIgD⁺ memIgM⁻). In fact, the presence of CCR7 in the cell surface of trout B cells was associated to the expression of memIgD, because B cells lacking IgD in the cell surface (memIgM⁺ memIgD⁻) had no CCR7 expression.

Although IgD was mostly ignored in fish for some time after its discovery (12), recent evidence demonstrated the presence of secreted IgD, pointing to a conserved role of IgD in fish immunity (22). In mammals, IgM and IgD are coexpressed on the surface of naive B cells, which, upon Ag binding, downregulate IgD expression, accounting for the gradual disappearance of IgD from the cell surface of activated cells that goes along with somatic hypermutation and class-switch DNA recombination to diversify the Ig gene repertoire (30). These mechanisms still remain to be demonstrated in fish B cells, which do not undergo class switch. Naive B cells in trout blood and spleen have shown to express both IgM and IgD in the cell surface, with no single-positive B populations detected (22), although these experiments were performed in American rainbow trout, known to exhibit some genetic and immunological differences in comparison with European salmonid strains (31). On the contrary, some subpopulations of B cells expressing only IgD in the cell surface have been reported both in mammals (18, 32, 33) and fish (17). In mammals, two types of memIgD⁺ memIgM⁻ cells have been described. A memIgD⁺ memIgM⁻ population present mainly in the upper aerodigestive mucosa arises in humans after active IgM-to-IgD class switch. These plasmablast-like cells that retain IgD in the membrane secrete highly mutated mono- and polyreactive IgD, providing a layer of mucosal protection by interacting with pathogens and are either retained locally or circulated in the blood, where they can account for up to 0.5–1% of circulating B cells (18, 32). The second type of memIgD⁺ memIgM⁻ make up to 2.5% of circulating B cells in humans. These naive B cells have Ab V region genes in an unmutated configuration, thus are fully mature cells that are autoreactive and functionally attenuated and therefore have been cataloged as a new type of anergic B cells (33). Clonal anergy is closely related to self-tolerance, and although different types of anergic cells exist, anergy is always associated with the absence of IgM (34). Although most B cells expressing surface Ig that bind autoantigens are eliminated throughout the development, some B cells escape these checkpoints and survive in the periphery as autoreactive B cells. However, their functionality is strongly reduced so they will no longer react to self-Ags (clonal anergy). If we take into account that clonal anergy is a way to inactivate B cells stimulated early in development when only autoantigens would be presented (35), it might make sense that equivalent cells are present in the gills of teleost fish continuously exposed to...
water-borne Ags to which fish should not react. In contrast, the presence of $\text{mem}^{\text{D}^+}\text{mem}^{\text{M}^-}$ in trout gills could be an equivalent population to the IgD-secreting plasmablasts found in upper respiratory tract of mammals (32), thus additional studies are needed to determine if these trout cells correspond to naive anergic cells or if they are class-switched cells with an Ag experience. Although in our studies trout $\text{mem}^{\text{D}^+}\text{mem}^{\text{M}^-}$ were mainly present in the gills and not in peripheral blood (data not shown), catfish PBLs contain a distinct $\text{mem}^{\text{D}^+}\text{mem}^{\text{M}^-}$ B cell population that can make up to 60–80% of peripheral blood B cells, depending upon the individual fish. These catfish $\text{mem}^{\text{D}^+}\text{mem}^{\text{M}^-}$ B cells resembled human activated IgM $\text{D}^+$ B cells in that they have plasmablast morphology, exhibit restricted IgL isotype usage, and produce a secreted form of IgD (17). In fact, all evidence from humans to fish show that IgD displays a considerable diversity in structure and abundance both within a single individual at different moments and between individuals and has been suggested to be the most evolutionarily dynamic Ig class among all vertebrate IgS (18). Because of this association of CCR7 with these IgD$^+$IgM$^-$ cells in fish, it would be interesting to study whether CCR7 is also expressed in all similar IgD-expressing mammalian B cells. Interestingly, IgD$^+$IgM$^-$CD38$^+$ B cells that are selectively derived from human nasopharynx-associated lymphoid tissue, also cataloged as tolerogenic because of their expression of Ig V-gene repertoires that may allow considerable cross-reactivity and autoimmunity (36), are known to express CCR7 (37). Of course, whether trout $\text{mem}^{\text{D}^+}\text{mem}^{\text{M}^-}$ are in fact homologs of human anergic B cells remains to be demonstrated. The function of anergic cells in mammals can be rescued when sufficiently stimulated, normally through an interaction with both CD40L and IL-4 (33). This activation leads to proliferation and differentiation into plasmablasts.

Accordingly to mammalian literature, it was expected that CCR7$^+$ cells in the gills would be mainly T cells. In mammalian GALT, the desensitization of CCR7 (38), the genetic disruption of CCR7 (5), or natural mutations in CCL19 and CCL21 (38, 39) lead to a reduced homing of T cells into the Peyer’s patches. Interestingly, B cell homing to these secondary lymphoid tissues has been shown to be less CCR7 dependent (5, 40). Moreover, T cells are known to be present in teleost gills, even though there is some controversy in relation to actual numbers because some authors report very high numbers of T cells in gills (41), whereas others have reported that only ∼4–9% of the gill leukocytes are in fact T cells (9). Due to the lack of specific markers for surface Ags that define specific subpopulations, we exclusively used an anti-CD8α Ab in combination with the anti-CCR7 pAb. But the fact that CD8$^+$ cells did not express $\text{mem}^{\text{CCR7}}$ and CD3 mRNA was not detected in CCR7$^+$ sorted cells strongly suggest that naive T cells in the gills do not express $\text{mem}^{\text{CCR7}}$ in physiological conditions. Additionally, we have established that CCR7 is modulated in the gills in response to a viral infection, suggesting an important role for these $\text{mem}^{\text{CCR7}}\text{mem}^{\text{IgD}^+}$ cells in the early stages of the mucosal immune responses. Additional studies should be performed to determine whether the cell types that express $\text{mem}^{\text{CCR7}}$ in response to the infection remain the same as those observed in physiological conditions in tissues different from the gills. In the gills, our results point to $\text{mem}^{\text{IgD}^+}$ as the major cell type also during infection because despite the decrease in the number of CCR7$^+$ cells in gills in response to VHSV, the number of CCR7$^+$ cells significantly correlates with the number of IgD$^+$ cells in both control and infected fish. Because VHSV infection of gill leukocytes either in vitro or in vivo did not decrease the levels of transcription of CCR7 (data not shown), the reduction in the number of CCR7 cells in the gills seems to be a consequence of the mobilization of CCR7$^+$ cells from the gills. One possible explanation is that these CCR7$^+$ cells are mobilized to the head kidney, an organ in close relation to the gills, where we observed significant increases in the number of CCR7$^+$ cells. However, in this scenario, $\text{mem}^{\text{IgD}^+}$ should be down-regulated upon activation because we did not detect significant numbers of $\text{mem}^{\text{IgD}^+}$ cells in infected head kidney. An alternative hypothesis is that other cell types that did not express $\text{mem}^{\text{CCR7}}$ in physiological conditions express $\text{mem}^{\text{CCR7}}$ in response to activation. This might be possible because even if there is a low number of $\text{mem}^{\text{CCR7}^+}$ cells in the nonstimulated head kidney, moderate levels of CCR7 mRNA were detected in sorted head kidney T cells and IgM$^+$ cells (19). Our results point to CCR7 as a major player in the early teleost immune response in mucosal tissues. This is also supported by previous results from our group that demonstrated that the infection with an intestinal parasite provokes a significant increase in CCR7 mRNA levels in IgM$^+$ and IgT$^+$ cells in the trout gut (19). The possible role of CCR7 in IgT$^+$ cells remains to be investigated at the protein level, because we did detect a small percentage of IgT$^+$CCR7$^+$ in the gills, and IgT transcripts were observed in CCR7 sorted cells. Although $\text{mem}^{\text{IgD}^+}$ was not found in blood IgT$^+$ B cells (21), it might be possible that $\text{mem}^{\text{IgD}^+}$ is found in IgT$^+$ cells from mucosal tissues. In mammalian lungs, CCR7 regulates normal pulmonary leukocyte homeostasis because the lack of CCR7 induces pulmonary hypertension involving perivascular infiltration of B and T lymphocytes (42). The reason for this is that CCR7 not only efficiently infiltrates leukocytes to extralymphoid tissues and sites of inflammation but also mediates the exit of leukocytes from these tissues through afferent lymph vessels (43), although again, most of these studies were focused on T cells (44).

In summary, we have studied the physiological distribution of CCR7$^+$ cells for the first time, to our knowledge, in teleost, revealing a major presence of CCR7$^+$ cells in the gills from the very early developmental stages. Furthermore, most of these gill CCR7$^+$ cells define a subpopulation of B cells with $\text{mem}^{\text{IgD}^+}$ and no $\text{mem}^{\text{IgM}^+}$, not previously identified in rainbow trout. Finally, the number of cells with $\text{mem}^{\text{CCR7}^+}$ is regulated in response to a viral infection both in gills and head kidney, revealing an important role of these CCR7$^+$IgD$^+$ cells at the initial phases of teleost mucosal immunity.

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References


