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Abstract: There are still many details of how intestinal immunity is regulated that remain unsolved in teleost. Although leukocytes are present all along the digestive tract, most immunological studies have focused on the posterior segments and the importance of each gut segment in terms of immunity has barely been addressed. In the current work, we have studied the regulation of several immune genes along five segments of the rainbow trout (*Oncorhynchus mykiss*) digestive tract, comparing the effects observed in response to an infectious pancreatic necrosis virus (IPNV) infection to those elicited by oral vaccination with a plasmid coding for viral VP2. We have focused on the regulation of several mucosal chemokines, chemokine receptors, the major histocompatibility complex II (MHC-II) and tumor necrosis factor α (TNF- α). Furthermore, the recruitment of IgM⁺ cells and CD3⁺ cells was evaluated along the different segments in response to IPNV by immunohistochemical techniques. Our results provide evidences that there is a differential regulation of these immune genes in response to both stimuli along the gut segments. Along with this chemokine and chemokine receptor induction, IPNV provoked a mobilization of IgM⁺ and IgT⁺ cells to the foregut and pyloric caeca region, and CD3⁺ cells to the pyloric caeca and midgut/ hindgut regions. Our results will contribute to a better understanding of how mucosal immunity is orchestrated in the different gut segments of teleost.

Suggested Reviewers:

Modulation of genes related to the recruitment of immune cells in the digestive tract of trout experimentally infected with infectious pancreatic necrosis virus (IPNV) or orally vaccinated

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Abstract

There are still many details of how intestinal immunity is regulated that remain unsolved in teleost. Although leukocytes are present all along the digestive tract, most immunological studies have focused on the posterior segments and the importance of each gut segment in terms of immunity has barely been addressed. In the current work, we have studied the regulation of several immune genes along five segments of the rainbow trout (*Oncorhynchus mykiss*) digestive tract, comparing the effects observed in response to an infectious pancreatic necrosis virus (IPNV) infection to those elicited by oral vaccination with a plasmid coding for viral VP2. We have focused on the regulation of several mucosal chemokines, chemokine receptors, the major histocompatibility complex II (MHC-II) and tumor necrosis factor α (TNF- α). Furthermore, the recruitment of IgM⁺ cells and CD3⁺ cells was evaluated along the different segments in response to IPNV by immunohistochemical techniques. Our results provide evidences that there is a differential regulation of these immune genes in response to both stimuli along the gut segments. Along with this chemokine and chemokine receptor induction, IPNV provoked a mobilization of IgM⁺ and IgT⁺ cells to the foregut and pyloric caeca region, and CD3⁺ cells to the pyloric caeca and midgut/hindgut regions. Our results will contribute to a better understanding of how mucosal immunity is orchestrated in the different gut segments of teleost.

Keywords: infectious pancreatic necrosis virus (IPNV); DNA vaccine; rainbow trout; chemokines; digestive tract; leukocyte recruitment

Introduction

Although the structures and segments present in the digestive tract show significant differences among the diverse teleost species, a general division into three main segments has been established (Rombout et al., 2011). The first segment or foregut is where the food protein uptake takes place, with enterocytes acting as absorptive cells. This segment includes the esophagus and in some species, a defined stomach. The second segment is characterized by a strong uptake of macromolecules and enterocytes containing large supranuclear vacuoles. This segment includes the midgut and in some species such as salmonids, a variable number of pyloric caeca (pyloric appendages) near the pylorus. Fish caeca are an adaptation to increase gut surface area, contributing to a higher macromolecule uptake than that of the rest of the digestive tract. Finally, the third segment is the hindgut in which enterocytes are thought to have an osmorregulatory function, and includes an anal region that in certain species can constitute a proper rectum separated by valves.

Most previous studies related to immunological properties of the teleost digestive tract have focused on the last segments (Rombout et al., 2011), and very poor attention has been given to the other segments from an immunological point of view. In rainbow trout (*Oncorhynchus mykiss*), we have defined in a previous study that IgM⁺ and IgT⁺ cells are present all along the digestive tract, with the exception of the stomach (Ballesteros et al., 2013). IgM⁺ cells were mostly located in the lamina propria (LP) and as intraepithelial lymphocytes (IELs) in the pyloric caeca region. On the other hand, IgT⁺ cells were primarily localized as IELs. Furthermore, when fish were orally vaccinated with an alginate-coated DNA vaccine against infectious pancreatic necrosis virus (IPNV), a significant recruitment of B lymphocytes to the pyloric caeca region

was observed. Furthermore, significant differences in the transcription of Ig genes and B cell transcription factors were only observed between vaccinated and control fish in this segment (Ballesteros et al., 2013). Hence, in this study, we wanted to compare the effects of oral DNA vaccination in the different gut segments to those elicited by a bath infection with IPNV. In this occasion, we have focused on the regulation of several chemokines and chemokine receptor genes that are related to mucosal responses, namely CK9, CK10, CK11, CK12, CCR7, CCR9 and CCR9B. We have also included the evaluation of transcription of the major histocompatibility complex (MHC-II) and tumor necrosis factor α (TNF- α) as indicators of antigen presentation and inflammation respectively. CK9, CK10, CK11 and CK12 chemokines are strongly expressed in rainbow trout mucosal tissues and are regulated in gills and skin in response to viral infections (Montero et al., 2011). On the other hand, the chemokine receptor CCR7 plays an important role in the recruitment of different leukocyte subtypes such as dendritic cells, T and B lymphocytes to the intestine in mammals (Jang et al., 2006; Okada et al., 2002; Wurbel et al., 2007). CCR7 has been recently identified in rainbow trout, where it also appears to be implicated in mucosal immunity (Ordas et al., 2012). In this species, CCR7 transcription was up-regulated in gut IgM⁺ and IgT⁺ cells from fish infected with an intestinal parasite in comparison to mock-infected controls (Ordas et al., 2012). Mammalian CCR9, in combination with other chemokine receptors, mediates the homing of antigen-secreting IgA⁺ cells to the mucosal LP (Hieshima et al., 2004). In rainbow trout, two CCR9 genes, designated as CCR9 (Daniels et al., 1999) and CCR9B (Dixon et al., 2013) were identified and are known to be differentially regulated (Dixon et al., 2013). Along with this evaluation of immune genes related to leukocyte mobilization, we have also studied through immunohistochemical techniques the actual recruitment of different leukocyte types to the different gut segments in

response to the IPNV infection. We have analyzed the presence of IgM⁺ and IgT⁺ cells, as well as cells expressing CD3, a marker for T lymphocytes along the digestive tract in control and infected fish. As previously described (Bernard et al., 2006), these CD3⁺ cells were located as IELs, however, our results provide the first evidence in teleost of a mobilization T IELs cell to the digestive tract in response to a viral infection.

Materials and Methods

2.1. Fish

Healthy specimens of rainbow trout (*Oncorhynchus mykiss*) of approximately 4-5 cm (approximately 2 g) were obtained from a local spring water farm with no history of viral disease. Fish were maintained at the Centro de Investigaciones Biológicas (CIB-CSIC) laboratory at 15°C with a re-circulating water system, 12:12 hours light : dark photoperiod and fed twice a day with a commercial diet (Skretting, Spain). Prior to any experimental procedure, fish were acclimatized to laboratory conditions for 2 weeks and during this period no clinical signs were ever observed. In addition, two pools of 5 fish were tested by standard methods to confirm the absence of any salmonid virus by isolation using BF cells (Alonso et al., 1999). The experiments described comply with the Guidelines of the European Union Council (2010/63/EU) for the use of laboratory animals and were previously approved by the local Ethics committee.

2.2. IPNV DNA vaccine

The DNA vaccine (pcDNA-VP2) was prepared as described previously (de Las Heras et al., 2008), by inserting the IPNV-VP2 gene into the pcDNA.3.1/V5/His-TOPO expression vector (Invitrogen, USA). The empty re-ligated plasmid was used as a control (pcDNA). The pcDNA-VP2 and pcDNA plasmids were coated with alginate, preparing the microspheres as previously described (de las Heras et al., 2010).

2.3. Virus propagation

The IPNV Sp strain obtained from the ATCC (ATCC VR 1318) was propagated in the BF-2 cell line from bluegill fry (*Lepomis macrochirus*, ATCC-CCL 91) with Leibovitz's medium (L15, Gibco, Spain) supplemented with 100 IU/ml penicillin G, 100 µg/ml streptomycin, 2 mM L-glutamine and 2% fetal bovine serum (FBS, Gibco, Spain) at 20°C. Supernatants from IPNV infected BF-2 cell monolayers were clarified by centrifugation at 1000 x *g* for 20 min after cytopathic effect was extensive. Clarified supernatants were used for the experiments. Viral titration was performed in 96 well culture plates. The infective titers were determined as the 50% infective dose in tissue culture (TCID₅₀/ml) according to the method described by Reed and Muench (Reed and Muench, 1938).

2.4. Experimental design

In order to compare the mucosal immune regulation elicited by IPNV infection with that provoked by oral DNA IPNV vaccination in the different segments of the digestive tract, rainbow trout were either orally vaccinated with alginate-coated pcDNA-VP2 or infected with IPNV by immersion in parallel experiments using corresponding control groups.

For the immunization, trout were divided into two different groups. One group was orally vaccinated with 10 µl of the vaccine microsphere suspension containing 10 µg of pcDNA-VP2, while a second group received 10 µg of the pDNA empty plasmid diluted in 10 µl of a microsphere suspension. Vaccination was performed with an automatic pipette with a 20 µl tip which was introduced into the mouth of each trout, supporting the tip end at the entrance of the digestive tract. For the IPNV bath infection, rainbow trout were transferred to 2 l of a viral solution containing IPNV Sp strain (5×10^5 TCID₅₀/ml). After 1 h of viral adsorption with strong aeration at 15°C, each experimental group was transferred to an individual water tank. Mock-infected groups were also transferred to 2 l tanks containing an equivalent amount of non-infected culture media. After 1 h of strong aeration, they were also moved to their corresponding tanks. The water-quality parameters were maintained at optimal levels and equal in all tanks.

In a preliminary experiment using this set-up, four fish infected with IPNV and four fish vaccinated with pcDNA-VP2 were euthanized with an overdose of MS-222 at days 1, 3 and 7 post-treatment and the esophagus, stomach, pyloric caeca, midgut and hindgut removed as described before (Ballesteros et al., 2013) and placed in Trizol reagent (Invitrogen) for posterior RNA extraction. In this preliminary experiment, unhandled fish sampled at day 0 were used as controls. This experiment was used to determine that 7 days was the time point at which most chemokine genes were

modulated in response to the different stimuli. Thus, the experiment was repeated once more sampling six fish from each of the experimental groups previously described at day 7 post-stimulation. In the case of the IPNV-infected and the mock-infected control groups, four additional trout were sampled for immunohistochemistry.

2.5 Gene expression analysis

Total RNA was isolated from these tissues using Trizol® reagent (Invitrogen) according to manufacturer's instructions. Five µg of RNA were used to obtain cDNA in each sample using the Super Script™ II kit (Invitrogen) and oligo (dT)₁₂₋₁₈ (25 pmol/µl) following manufacturer's instructions. The resulting cDNA was diluted and stored at -20°C.

Real-time PCR amplification was carried out in an iCycler iQ Real-Time PCR Detection System (Bio-Rad, Spain). The analysis of the immune genes was performed using Quantimix Easy Master Mix (Biotools, Spain) as described previously (Ballesteros et al., 2012a; Ballesteros et al., 2012b). All the primers used are shown in Table 1 and had already been optimized in previous studies (Dixon et al., 2013; Montero et al., 2011; Zou et al., 2002). The thermal profile used was 10 min at 95°C, followed by 40 amplification cycles (30 s at 95°C and 1 min at 60°C) and a dissociation cycle (30 s at 95°C, 1 min 60°C and 30 s at 95°C). After the run, the melting curve of each amplicon was examined to determine the specificity of the amplification. Elongation factor 1α (EF-1α) was used as house-keeping control gene. The expression of target genes was calculated as the relative values ($2^{-\Delta CT}$) or fold change relative to EF-1α expression according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

To analyze the transcription of the viral VP2 genes along the different gut segments, a Taq-Man probe and primers designed to the VP2 gene were used as described before (Rodriguez Saint-Jean et al., 2010). The probes were dual labeled with a reporter dye at the 5' end (FAM, 6-carboxy fluorescein) and a quencher dye at the 3' end (TAMRA, 6-carboxytetramthyrhodamine). In this case, β -actin was used as a reference house-keeping gene. Primers and probes were synthesized at Applied Biosystems and are also shown in Table 1. PCR amplification was performed in a final volume of 20 μ l by adding 1 μ l of cDNA, 10 μ l of iQ supermix (Bio Rad), 8 μ l of dH₂O and 1 μ l of a 20 $\times$ mix containing the forward primer (18 μ M), reverse primer (18 μ M) and probe (5 μ M). The cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The fluorescence output of each cycle was measured and recorded upon completion of the entire run, and a relative quantification of the transcripts was performed.

The data obtained were analyzed using the iQ5 optical system software version 2.0 (BioRad). All PCR reactions were performed in duplicate, expressing the results as the mean $\pm$ standard deviation.

2.6. Immunohistochemistry

Segments from the digestive tract obtained from control and IPNV-infected fish were fixed in Bouin's solution for 24 h, embedded in paraffin (Paraplast Plus; Sherwood Medical) and sectioned at 5 μ m. After dewaxing and rehydration, some sections were stained with hematoxylin–eosin in order to determine the levels of infiltration, apparent damages or pathological changes. A second set of sections was

subjected to an indirect immunocytochemical method for detection of trout IgM, IgT and CD3. The anti-IgM and anti-IgT monoclonal antibodies were kindly donated by Dr. Kurt Buchmann from the University of Copenhagen and Dr. Karsten Skjoedt from the University of Southern Denmark (Denmark) (Olsen et al., 2011; von Gersdorff Jorgensen et al., 2011). These antibodies recognize both the membrane and the secreted forms of the Igs. The anti-CD3 antibody was kindly provided by Dr. Erin Bromage from the University of Massachusetts Dartmouth (USA) (Boardman et al., 2012). Endogenous peroxidase was inhibited after rehydration of the sections by 10 min incubation in 3% H₂O₂ in PBS. After a heat induced epitope retrieval in Tris-EDTA buffer pH 9.0 (800 w for 5 min and 450 w for 5 min in a microwave oven), the sections were pre-incubated in two different blocking solutions consisting of 2% BSA (bovine serum albumin; Sigma-Aldrich) in TBT (Tris buffer with 0.02% tween 20) at room temperature for 30 min, and 10% normal goat serum in TBT for 30 min. Then, sections were incubated with primary antibody solution overnight at 4°C. Monoclonal mouse anti-trout IgM was used in a dilution of 1:150 while monoclonal mouse anti-trout IgT and anti-trout CD3 were added at a 1:300 dilution. Following this incubation, unbound primary antibodies were washed off using TBT. The tissue was covered with anti-mouse EnVision™ System HRP labelled secondary antibody (Dako) and left for a 30 min incubation period at room temperature. Subsequently, the tissue was washed three times with TBT and then incubated in AEC substrate [0.05M acetic acid buffer (pH 5) with 0.015% H₂O₂ and 0.4 g/l 3-Amino-9-ethylcarbazole (Alfa Aesar)] for 15 min and afterwards washed for 4 min in tap water. The specificity of the reactions was determined by omitting the primary antibodies. Mayer's haematoxylin (Dako) was used as nuclear counter stain, and mounting was conducted with Aquamount (Merck). Slides were examined with an Axiolab (Zeiss) light microscope.

2.7. Statistical analysis

Prior to statistical analyses, the normal distribution of the data was checked and confirmed using the Shapiro–Wilk test. Analysis of variance (factorial ANOVA) were run to determine whether the differentially expressed gene differed between the replicates for an individual gene, followed by Tukey’s multiple comparison test for differences between experimental groups. The Student’s *t* test was used also to compare some paired samples. All statistics were run in SPSS Version 15. *P* values less than 0.05 were considered significant.

Results

3.1. IPNV replication along the digestive tract

In a previous study, we had established that after oral vaccination with an IPNV DNA vaccine, VP2 transcription could be detected all along the trout digestive tract (Ballesteros et al., 2013). To verify, whether IPNV is also capable of replicating in all the different gut segments after a bath infection, we studied VP2 transcription through real time PCR in the samples obtained from the IPNV-infected group. VP2 transcription was detected along all the gut segments from day 1 to day 7 post-infection (Fig. 1). At day 1 post-infection, the VP2 mRNA levels were higher in the esophagus in comparison

to the other segments. The levels of VP2 transcription peaked at day 3, to decrease again at day 7. At both of these time points, however, no differences were observed in VP2 mRNA levels among the different segments.

3.2. Modulation of mucosal chemokines in the different gut segments

The main aim of our study was to compare the immune response elicited by IPNV bath infection in the different gut segments to that observed in response to oral DNA vaccination. As a preliminary step, we performed an experiment, in which four fish per group were sampled at different time points in the IPNV-infected and the pcDNA-VP2-vaccinated groups and CK9, CK10, CK11, CK12 and CCR7 mRNA levels studied in comparison to the levels observed in unhandled fish before treatment. Once the transcription of these genes was determined in the different segments (Supplemental Fig. 1), we established that 7 days post-stimulation was the adequate time point to study these responses in both the infected and the vaccinated groups, because it was at this time point when most of the significant gene modulations were observed in all segments. Therefore, the experiment was repeated and six fish per group were sampled in all experimental groups at day 7 post-stimulation exclusively.

Firstly, we studied the transcription of CK9, CK10, CK11 and CK12 chemokines. The essential role that these four chemokines play in mucosal responses (gills and skin) has been previously demonstrated using a VHSV bath infection model (Montero et al., 2011). In the current work, in response to IPNV, CK9 transcription was up-regulated in all gut segments, although differences were only significant in esophagus, stomach, midgut and hindgut (Fig. 2A). The VP2 vaccine also produced an

up-regulation of CK9 transcription in the esophagus, the stomach and hindgut, but not in the midgut (Fig. 2A). On the other hand, pcDNA-VP2 significantly down-modulated CK9 transcription in the pyloric caeca (Fig. 2A). In the case of CK10, IPNV significantly up-regulated the chemokine mRNA levels in the esophagus, midgut and hindgut, whereas the oral vaccine up-regulated its transcription in the esophagus, the stomach and hindgut (Fig. 2B). CK11 transcription significantly increased in all gut segments in response to IPNV (Fig. 2C), but only in the esophagus, pyloric caeca and hindgut of vaccinated fish (Fig. 2C). Finally, CK12 mRNA levels increased only in the midgut and hindgut after IPNV infection (Fig. 2D), and only in the esophagus after oral IPNV vaccination (Fig. 2D).

3.3. Modulation of chemokine receptors in the different gut segments

We also studied the transcription of CCR7, CCR9 and CCR9B chemokine receptors, since these receptors are implicated in the recruitment of immune cells to the intestine in mammals (Okada et al., 2002; Wurbel et al., 2007). IPNV significantly increased CCR7 mRNA levels in all the gut segments except the stomach (Fig. 3A), whereas the pcDNA-VP2 vaccine only increased CCR7 transcription in the esophagus and the stomach (Fig. 3A). CCR9, on the other hand, was only significantly up-regulated in midgut and hindgut of virus infected fish (Fig. 3B). In response to the oral DNA vaccine, no significant up-regulations of CCR9 levels were detected, but the vaccine significantly down-modulated CCR9 transcription in the midgut (Fig. 3B). Finally, IPNV significantly increased the transcription of CCR9B in the midgut (Fig. 3A), whereas pcDNA-VP2 significantly increased CCR9B transcription only in the esophagus.

3.4. Modulation of MHC-II and TNF- α in the different gut segments

To study whether this modulation of chemokines and chemokine receptors correlated with an increased antigen presentation or with a pro-inflammatory response, the levels of expression of MHC-II and TNF- α were also assayed. MHC-II mRNA levels were quite high in all gut segments, and were significantly up-regulated in response to IPNV in the stomach and midgut (Fig. 4A). When fish were orally vaccinated, MHC-II transcription was only up-regulated in the esophagus and the stomach (Fig. 4B). In the case of TNF- α , IPNV increased its mRNA levels in all gut segments with the exception of the esophagus (Fig. 4B). Orally-vaccinated fish, on the other hand, had increased TNF- α mRNA levels in all gut segments except the pyloric caeca (Fig. 4B). A summary of the fold increases observed in infected and vaccinated groups in comparison to their corresponding control groups is shown in Fig. 5 for these and all of the other genes studied in this work.

3.5. IgM⁺ cell mobilization in response to IPNV along the digestive tract

In a previous study, we had examined the mobilization of IgM⁺ cells to an oral DNA vaccination with pcDNA-VP2 (Ballesteros et al., 2013). That study revealed that IgM⁺ cells were constitutively present in all gut segments with the exception of the stomach and in response to the vaccine were recruited mainly in the pyloric caeca region. Thus, in the current study, we evaluated the presence of IgM⁺ cells along the digestive tract in fish infected with IPNV in comparison to mock-infected fish. In the

foregut, most IgM⁺ cells were present in the LP (Fig. 6A), as previously described (Ballesteros et al., 2013). When fish were infected with IPNV, the number of IgM⁺ in this area increased, while some reactivity was also observed in the apical surface of the enterocytes. In the pyloric caeca region, accordingly with what was published before (Ballesteros et al., 2013), IgM⁺ cells were detected as IELs (Fig. 6C). In response to IPNV infection, the number of IgM⁺ cells strongly increased in this area (Fig. 6D). Finally, in the hindgut / midgut area, IgM⁺ cells were mostly localized in the LP (Fig. 6E), but in this case the number of cells was not consistently different in IPNV-infected fish (Fig. 6F).

3.6. IgT⁺ cell mobilization in response to IPNV along the digestive tract

In the foregut, very few scattered IgT⁺ cells were observed in some sections (Fig. 7A). When fish were exposed to IPNV, more IgT⁺ cells could be detected (Fig. 7B) in the tissue samples, even though still very few positive cells were present in this segment. In the pyloric caeca region, many more IgT⁺ cells were observed, mainly as IELs (Fig. 7C). As in the case of IgM⁺ cells, IgT⁺ cells were also mobilized to this area in response to the viral infection (Fig. 7D). Although numerous IgT⁺ cells could be detected in the midgut/ hindgut region (Fig. 7E), the numbers were not affected by the infection with IPNV (Fig. 7F).

3.7. CD3⁺ cell mobilization in response to IPNV along the digestive tract

The presence of CD3⁺ cells was also evaluated along the digestive tract, using a specific monoclonal antibody against this T cell marker (Boardman et al., 2012). Only a few scattered CD3⁺ cells were found in the foregut in either control (Fig. 8A) or infected fish (Fig. 8B). On the other hand, many CD3⁺ cells were found in the pyloric caeca region, mostly as IELs (Fig. 8C). When fish were infected with IPNV, the number of CD3⁺ cells detected in this region strongly increased (Fig. 8D). This was also the case for the midgut /hindgut region. The number of CD3⁺ cells present in control fish (Fig. 8E) increased in response to IPNV (Fig. 8F) in this region.

4. Discussion

Although leukocytes can be found all along the digestive tract in rainbow trout (Ballesteros et al., 2013), most studies concerning its immune regulation have focused on the posterior segments exclusively (Rombout et al., 2011). In the current work, we have compared the effects of viral infection to those elicited by oral DNA vaccination along five segments of the digestive tract, focusing on studying the transcription of several genes important for mucosal immunity. First we studied the transcription levels of CK9, CK10, CK11 and CK12 chemokines. These chemokines are regulated in response to VHSV infection in mucosal tissues such as gills or skin (Montero et al., 2011). All of them were significantly regulated in response to both the virus and the DNA vaccine, although important differences were observed in these two groups and among the different segments. These results suggest on one hand that the capacity of the different segments to recruit leukocytes is different; and on the other hand, that the virus

and the DNA vaccine trigger different immune mechanisms. It might also be possible that the virus and the vaccine do not replicate the same way in the different segments. Upon oral DNA vaccination with the pcDNA-VP2 plasmid, transcription of VP2 can be detected in all segments, although at significantly higher levels in the first three segments (Ballesteros et al., 2013). However, when fish are infected with IPNV, viral transcription levels at day 7 were not significantly different among the different gut segments. Therefore, it seems that the epithelial cells in the different segments, mostly responsible for the production of chemokines, are sensing stimuli in different ways along the different segments. These differences between stimuli and among segments were also visible when the transcription of chemokine receptor genes, MHC-II or TNF- α was analyzed. Indeed IPNV replication is an event much more complex than vaccination, because the virus elicits at the same time a rapid immunological response and damages to the implicated tissues. Furthermore, other structural proteins different that VP2 are known to play an important role in viral pathogenesis (Pedersen et al., 2007). To date, the role of intestine in IPNV infection has been scarcely approached under molecular or physiological point of view. One of the few studies performed in this sense in salmon demonstrated that IPNV can translocate across the intestinal epithelium, eliciting rapid changes in active transport after only 90 min of exposure to IPNV, significantly modulating the barrier function of the intestinal epithelium in the midgut /hindgut region (Sundh et al., 2011). Interestingly, when we evaluated the effects of oral DNA vaccination on the transcription of Ig genes and B cell related transcription factors (Ballesteros et al., 2013), the only segment that significantly responded to the vaccine was the pyloric caeca region. In the current study, however, significant up-regulations of the different genes (chemokines, chemokine receptors, MHC-II or TNF- α) were observed in all the other segments, strongly suggesting that

cells different than B cells are playing an important role in mucosal immunity in these other segments. This seems the case even in the stomach, because even though no B cells are visible (Ballesteros et al., 2013), significant CK9, CK10, CK11 and CCR7 up-regulations were observed. In this work, we also report the identification of CD3⁺ T lymphocytes in the foregut, pyloric caeca and midgut/ hindgut area. A previous study had already established that T cells were present as IELs in the posterior rainbow trout gut segments (Bernard et al., 2006), however, this is the first report of CD3⁺ T cells in the foregut and pyloric caeca regions. In sea bass, the expression of TCR- β had already been demonstrated by PCR in the anterior segment of the digestive tract (Picchietti et al., 2011). Furthermore, it might be possible that additional cell types such as dendritic cells or NK cells, for which no specific markers are available yet in rainbow trout, are being recruited to the mucosa in response to these chemokines and through the action of these chemokine receptors. CCR7, for example, is strongly expressed in mature dendritic cells in mammals (Sallusto et al., 1998), where it is known to mediate their migration of to the digestive tract (Jang et al., 2006).

Because we had previously established that IgM⁺ and IgT⁺ cells are recruited exclusively in the pyloric caeca region after oral DNA vaccination (Ballesteros et al., 2013), we now studied the homing of leukocytes to the different gut segments in response to IPNV infection. In this case, although the mobilization of IgM⁺ and IgT⁺ cells to the pyloric caeca was still massive, an infiltration of both types of B cells was also observed in the foregut. Surprisingly, homing of B cells to the posterior segments was not observed. Previous studies in rainbow trout have demonstrated homing of IgT⁺ cells to the posterior gut segments in response to the intestinal parasite *Ceratomyxa shasta* 3 months post-infection (Zhang et al., 2010). It seems probable that important differences in leukocyte homing are induced by different pathogens, whereas it might

also be possible that it takes longer for B cells to colonize the posterior segments. Interestingly, the homing of CD3⁺ cells in response to IPNV infection was also visible in the pyloric caeca and the midgut/foregut regions. Although a homing capacity has been established for mammalian intestinal T IELs (Buzoni-Gatel et al., 1999; Kim et al., 1997), this was not visualized in rainbow trout intramuscularly infected with VHSV (Bernard et al., 2006). Thus, our study constitutes the first report of teleost mucosal T cell homing in response to a viral infection.

In summary, IPNV bath infection in rainbow trout modulated the transcription of CK9, CK10, CK11, CK12, CCR7, CCR9, CCR9B, MHC-II and TNF- α in the digestive tract. Oral DNA vaccination against IPNV also modulated these genes, although important differences were observed concerning the gut segments where these modulations took place and the effects themselves. On the other hand, we have demonstrated that the IPNV infection mobilized IgM⁺ and IgT⁺ cells to the foregut and pyloric caeca regions, as well as CD3⁺ T cells to the pyloric caeca and midgut/ hindgut regions. These studies contribute to a better understanding of antiviral mucosal immunity in teleost.

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Figure legends

Fig. 1. VP2 transcription in the different segments of the digestive tract of IPNV infected fish. Fish were infected through immersion with IPNV Sp strain (5×10^5 TCID₅₀/ml) and sampled at days 1, 3 and 7 post-infection. Data are shown as relative transcription levels of VP2 normalized to the transcription of the house-keeping gene β -actin at different days post-infection.

Fig. 2. Mucosal chemokine modulation in response to IPNV infection or oral DNA vaccination. Left side: Trout were infected with IPNV Sp strain (5×10^5 TCID₅₀/ml) or mock-infected in the same conditions. Right side: Trout were orally vaccinated with 10 μ l of suspension of the vaccine microspheres containing either 10 μ g of pDNA-VP2 or 10 μ g of the pDNA empty plasmid diluted in PBS. In all cases, trout were sacrificed after 7 days and the different segments of the digestive tract removed for RNA extraction and analysis of immune gene transcription through real time PCR. E: esophagus; S: stomach; PC: pyloric caeca; M: midgut; H: hindgut. Levels of CK9 (A), CK10 (B), CK11 (C) and CK12 (D) transcription in the different segments were studied through real time PCR. Data are shown as the mean relative gene expression normalized to the transcription of the house-keeping gene EF-1 α $\pm$ SD (n=6). The relative significance of differences between treatment groups and their respective controls at each segment of the digestive tract was determined through a one-way ANOVA and is shown above the bars as * (up-regulations) or § (down-modulations).

Fig. 3. Mucosal chemokine receptor modulation in response to IPNV infection or oral DNA vaccination. The experimental design is described in the legend of Fig. 2. Levels of CCR7 (A), CCR9 (B), and CCR9B (C) transcription in the different segments were studied through real time PCR. E: esophagus; S: stomach; PC: pyloric caeca; M: midgut; H: hindgut. Data are shown as the mean relative gene expression normalized to the transcription of the house-keeping gene EF-1 α $\pm$ SD (n=6). The relative significance of differences between treatment groups and their respective controls at each segment of the digestive tract was determined through a one-way ANOVA and is shown above the bars as * (up-regulations) or § (down-modulations).

604

605 **Fig. 4.** MHC-II and TNF- α modulation in response to IPNV infection or oral DNA
606 vaccination. The experimental design is described in the legend of Fig. 2. Levels of
607 MHC-II (A) and TNF- α (B) transcription in the different segments were studied
608 through real time PCR. E: esophagus; S: stomach; PC: pyloric caeca; M: midgut; H:
609 hindgut. Data are shown as the mean relative gene expression normalized to the
610 transcription of the house-keeping gene EF-1 α $\pm$ SD (n=6). The relative significance of
611 differences between treatment groups and their respective controls at each segment of
612 the digestive tract was determined through a one-way ANOVA and is shown above the
613 bars as *.

614

615 **Fig. 5.** Heat map depicting relative expression profiles of regulated genes in the
616 segments of the digestive tract from rainbow trout. Fish were infected with IPNV or
617 vaccinated with the pcDNA-VP2 plasmid and processed after 7 days. Levels of relative
618 expression are represented by a range of colours from the lower (grey) to the higher
619 (red).

620

621 **Fig. 6.** IgM⁺ cell detection in the different gut segments in IPNV-infected fish and
622 mock-infected fish. Immunohistochemical detection of trout IgM⁺ cells in the foregut
623 (A, B), pyloric caeca (C, D) or midgut/ hindgut region (E, F) of mock-infected fish (A,
624 C, E) or IPNV infected fish (B, D, F). Trout were infected with IPNV as described in
625 the legend of Fig. 1 and sampled at day 7 post-infection. Arrows indicate examples of
626 positive cells. Bar: 200 μ m.

627

628 **Fig. 7.** IgT⁺ cell detection in the different gut segments in IPNV-infected fish and mock-
629 infected fish. Immunohistochemical detection of trout IgT⁺ cells in the foregut (A, B),
630 pyloric caeca (C, D) or midgut/ hindgut region (E, F) of mock-infected fish (A, C, E) or
631 IPNV infected fish (B, D, F). Trout were infected with IPNV as described in the legend
632 of Fig. 1 and sampled at day 7 post-infection. Arrows indicate examples of positive
633 cells. Bar: 200 μ m.

634

Fig. 8. CD3⁺ cell detection in the different gut segments in IPNV-infected fish and mock-infected fish. Immunohistochemical detection of trout CD3⁺ cells in the foregut (A, B), pyloric caeca (C, D) or midgut/ hindgut region (E, F) of mock-infected fish (A, C, E) or IPNV infected fish (B, D, F). Trout were infected with IPNV as described in the legend of Fig. 1 and sampled at day 7 post-infection. Arrows indicate examples of positive cells. Bar: 200 µm.

Table 1. Oligonucleotides and probes used for real time PCR in this study.

Gene	Primer	Sequence (5'-3')
EF-1 α	rtEF1 α F	GATCCAGAAGGAGGTCACCA
	rtEF1 α R	TTACGTTCGACCTTCCATCC
CK9	rtCK9 F	GGCTCTTATGGGAAGTGTG
	rtCK9 R	CTGGGATTGGCACAACAG
CK10	rtCK10 F	ATTGCCAAGATCCTCTTCTGTGTTC
	rtCK10 R	CCTGAGGCTGGTAACCTATGACAAC
CK11	rtCK11 F	CCTTTGAGCATACTAATGCGAGTGG
	rtCK11 R	GTCTGCACAATACTTCTCCCATTG
CK12	rtCK12 F	GACATCGATGCCACTGTGTT
	rtCK12 R	GGAGATGGTTCGCTCCAGAC
CCR7	CCR7 4F	TTCACTGATTACCCACAGACAATA
	CCR7 4R	AAGCAGATGAGGGAGTAAAAGGTG
CCR9	CCR9 F	TCAATCCCTTCCTGTATGTGTTTGT
	CCR9 R	GTCCGTGTCTGACATAACTGAGGAG
CCR9B	CCR9B F	AATATTTCCAACGTCTGAAACAGGA
	CCR9B R	CTCACCCAGGACTTATCACACATTC
MHC-II α	rtMHCII α F	ACACCCTTATCTGCCACGTC
	rtMHCII α R	TCTGGGGTGAAGCTCAGACT
TNF- α	TNF2 Fw	TGCTGCTCCATGTGTGGTGC
	TNF2 Rev	AGGGACGGGGAGCCTTGAT
VP2	VP2 F	GCCAAGATGACCGAGTCCAT
	VP2 R	TGACAGCTTGACCCTGGTGAT
	VP2 probe	CCGACCGAGAACAT
β -actin	Actin-F	GGCCGTGTTGTCCCTGTAC
	Actin-R	CCTCTGGCCGTACCACC
	Actin-probe	CCGGAGTCCATGACGATACC

Fig. 1

Fig. 2**A. CK9** **B. CK10** **C. CK11** **D. CK12**

Fig. 3

A. CCR7

B. CCR9

C. CCR9b

Fig. 4**A. MHC-II** **B. TNF- α**

Fig. 5

Figure
Fig. 6

Figure 7
Fig. 7

Figure
Fig. 8

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