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IPNV modulation of pro and anti-inflammatory cytokine expression in Atlantic salmon might help the establishment of infection and persistence

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ABSTRACT

IPNV is the agent of a well-characterized acute disease that produces a systemic infection and high mortality in farmed fish species and persistent infection in surviving fish after outbreaks. Because modulation of the host expression of pro and anti-inflammatory cytokines can help establish persistence, in this study, we examined the expression of IL-1β, IL-8, IFNα1 and IL-10 during acute and persistent IPNV infection of Atlantic salmon. Results showed that IPNV infection induces an increase of the IFNα1 and IL-10 mRNA levels in the spleen and head kidney (HK) of fish after acute experimental infection. Levels of the pro-inflammatory cytokines IL-1β and IL-8 did not rise in the spleen although an increase of IL-1β, but not of IL-8, was observed in head kidney. In carrier asymptomatic salmon, cytokine gene expression of IFNα1 in the spleen and IL-10 in head kidney were also significantly higher than expression in non-carrier fish. Interestingly, a decrease of IL-8 expression was also observed. IPNV infection of SHK-1, which is a macrophage-like cell line of salmon, also induced an increase of expression of the anti-inflammatory cytokine IL-10 with no effects on the expression of IL-1β and IL-8. The effects are induced by an unknown mechanism during viral infection because poly I:C and the viral genomic dsRNA showed the opposite effects on cytokine expression in SHK-1 cells. In summary, IPNV always induces up-regulation of the anti-inflammatory cytokine IL-10 in Atlantic salmon. As this is accompanied by a lack of induction of the pro-inflammatory cytokines IL-1β and IL-8, the anti-inflammatory milieu may explain the high frequency, prevalence and persistence of IPNV in salmon. Effects might be part of the viral mechanisms of immune evasion.

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1. Introduction

The infectious pancreatic necrosis virus is the aetiological agent of a well-characterized disease (Infectious pancreatic necrosis, IPN), which produces systemic infection and mortality in farmed salmonid species causing negative economic impact [1,2]. In young salmonids, IPNV produces an acute disease episode with high mortalities, followed by life-long persistent infection in the survivors. The IPNV carrier fish are asymptomatic and have virus in many visceral organs and in leucocytes of blood and head kidney at extremely low multiplicity of infection [3–6]. Fish can periodically shed infectious IPNV in their feces and reproductive products and transmit the virus to their progeny and other susceptible species [7,8].

Viral persistence has been characterized in mammals where it has been observed that a susceptible host may have an infection associated with cytokine activity inhibition [9–12]. Modulation of the host expression of anti-inflammatory cytokines can help the establishment of chronic infection [13–15]. The nature of the inflammatory mediators induced following IPNV infections in fish is poorly characterized. Studies of Atlantic cod have demonstrated that IPNV induced up-regulation of IL-1β and IL-8 expression in head kidney cells after 1–2 days post-infection [16]. None of these cytokines increased in head kidney of Atlantic cod injected i.p with IPNV between 7 and 42 days [17].

In brown trout, IL-1β and IL-8 expression increased in the spleen by days 1 and 2 after i.p. inoculation of IPNV [18] and in Atlantic salmon (*Salmon salar*), IL-1β expression was not induced in kidney and gills between days 16 and 34 after challenge by cohabitation [19], although in gills, constitutive expression was observed. The induction of other pro-inflammatory cytokines after IPNV infection has not been explored, although IL-1β, IL-6 IL-8, IL-11,

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IL-12 expression were detected in six cDNA libraries from liver, kidney, spleen, peripheral blood and thymus of infected Atlantic halibut [20]. On the other hand, there is little information regarding anti-inflammatory cytokines. The balance of pro-inflammatory and anti-inflammatory cytokines is a factor that determines the characteristics of infection; therefore it seems important to establish the role of cytokines such as IL-10 during acute and chronic IPNV infection. Up-regulation of IL-10 may be related to the immunosuppressive effects described in IPNV infection [21–23] and to the establishment of IPNV persistence. In fact, IL-10 in mammals is able to inhibit monocyte/macrophage antigen presentation, macrophage activation, Th1 proliferation and the development and synthesis of Th1 cytokines [24], and there is growing evidence indicating that this cytokine has a role in regulating persistent viral infections [25]. In regard to the anti-viral cytokines, it has been demonstrated that IPNV induces IFN and interferon stimulated genes (ISGs) in salmon and trout [19,26–28], which may play a role in protection when the cytokine is induced prior to infection [29].

Thus, in order to determine whether modulation of the host expression of pro and anti-inflammatory cytokines is associated with IPNV persistence, in this work, we have evaluated the gene expression of IL-1β and IL-8, the anti-viral cytokine IFNα1 and the anti-inflammatory cytokine IL-10 in Atlantic salmon that have been intraperitoneally challenged with IPNV and in asymptomatic carrier fish surviving a natural outbreak. We also analyzed *in vitro* expression of these cytokines in a macrophage-like cell line (SHK-1) derived from head kidney of salmon in order to understand the role of macrophages in the cytokine response. Results showed that anti-inflammatory cytokine IL-10 is always induced during IPNV infection, which may be associated with mechanisms of immune evasion.

2. Materials and methods

2.1. Cell culture

SHK-1 cells (*Salmo salar*; ATCC, American Type Culture Collection, Manassas, VA, USA), which were described as macrophage-like cells [30], were grown at 16 °C in Leibovitz 15 medium (L15; Gibco, Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (FCS; Hyclone, Thermo Fischer Scientific, Logan, Utah, USA), 4 mM L-glutamine (Gibco), 2-mercaptoethanol (2-ME; Gibco) and 50 μg/mL Gentamicin (US Biological, Swampscott, MA, USA). Cell cultures of 40–60 passages were used in this study.

2.2. Virus

The IPNV strain Sp (ATCC) was propagated in CHSE-214 cells (*Salmon Chinook*, ATCC) in infectious medium (IM) that contain minimum essential medium (MEM, Gibco) supplemented with 2% FCS, 100 UI/mL/100 μg/mL penicillin/streptomycin (Gibco) and 2 mM L-glutamine (Gibco). The viral inocula were titrated by plaque assay lysis. Briefly, serial dilutions of virus (10^{-1} to 10^{-11}) were prepared in MEM and incubated 1 h in IM. Cells were washed twice with phosphate buffer saline (PBS) and kept for 72 h in semisolid medium that contain MEM supplemented with 2% FCS, 0.5% low-melting-point agarose (Gibco), 100 UI/mL/100 μg/mL penicillin/streptomycin (Gibco) and 2 mM L-glutamine (Gibco). After this, 1 mL of 37% formaldehyde (Winkler, Santiago, Chile) was added on semisolid medium for 30 min to fix the cell monolayer. Finally, the agarose was removed and crystal violet was added on each well and maintained for 30 min. Excess of crystal violet was removed and the lysis plaques formed were counted. The genomic double-stranded RNA (dsRNA) of IPNV (gIPNV) was prepared from 300 μL of a supernatant of CHSE-214 cells with IPNV-cytopathic effects. 75 μL of digestion solution (200 mM Tris, 10 mM EDTA, 50 mM SDS and 1% 2-ME) and 4 μL of 200 μg/mL Proteinase K (Promega) were added to the supernatant and incubated for 1 h at 50 °C. Then, nucleic acid extraction was performed with 1 volume of phenol:-chloroform (1:1), stirred for 1 min and centrifuged for 10 min at 10,000*g*. The RNA present in the aqueous phase was precipitated with absolute ethanol at −20 °C for 15 h. Finally, a pellet was obtained by centrifugation at 4 °C, 10,000*g* for 30 min at and the dsRNA was resuspended in 30 μL of nuclease free water.

Table 1
Sequence of primers used for PCR analysis.

Gene	Primer sequence	EMBL accesion	Amplicon size (pb)
β-Actin	FW: 5′- ATGGAAGGTGAAATCGCC - 3′ RW: 5′- TGCCAGATCTTCTCCATG - 3′	AF157514	260
EF1a	FW: 5′- CTGAGAGGGAGCGTGGTATC - 3′ RW: 5′- GGGGGCTCAGTAGAGTCCAT - 3′	BT043569	289
IL-1β	FW: 5′- AGGGAGGCAGCGGCTACCACAA - 3′ RW: 5′- GGGGGCTGCCTTCTGACACAT - 3′	AY617117	353
IL-8	FW: 5′- AGCGGCAGATTCAAACTCAC - 3′ RW: 5′- GTTGTTGGCCAGCATCTTCT - 3′	BT046706	445
IL-10	FW: 5′- GAACTCCGCACATCCTTCTC - 3′ RW: 5′- CGTTGATGTCAAACGGTTTCT - 3′	EF165029	301
IFNα1	FW: 5′- ATCACAAAACAGAATGCCCC - 3′ RW: 5′- AGACTGACAGGGTCCCACAT - 3′	AY216594	463
VP2	FW: 5′- TACGAGATCGACCTCCCATC - 3′ RW: 5′- CCACCAGTGTGATTGGTCTG - 3′	FN257531	451

2.3. Fish and treatment

Atlantic salmon (*S. salar*) of 20–30 g were obtained from a local salmon farm and maintained in tanks with a freshwater system at a biomass of 10–12 kg/m^3, 14 °C and with continuous support of oxygen. Fish were fed with commercial trout pellets twice a day and acclimated for 2 weeks prior to treatment. The presence of IPNV was discarded by PCR detection of VP2 in a randomly selected sample of the fish before experiments. Three groups of eight fish were challenged with intraperitoneal injection of 1.5×10^{-5} PFU/g of IPNV [7] for 24, 48 and 96 h. As control, groups of five fish were intraperitoneally injected with IM and treated for the same periods of time. After treatment, all fish were tested for transcripts of the viral major capsid protein (VP2) by PCR [31]. For comparison, a group of five fish was challenged with intraperitoneal (ip) injection of lipopolysaccharide (LPS) of *Escherichia coli* (Sigma, St Louis, MO, USA) (6 mg/kg) [32] and another group injected with Poly I:C (Sigma) (500 μg) [32,33] and treated for 24 h. As vehicle control, groups of five fish were intraperitoneally injected with PBS and treated for the same period of time. At the indicated times, all fish were sacrificed with 100 mg/mL of benzocaine [20] and spleen, gills and head kidney were extracted and stored at −80 °C.

2.4. Naturally infected fish

Fifteen fish were obtained after a natural outbreak of IPNV at a local farm. No fish showed any clinical signs. Fish were sacrificed

Table 2
Cycling conditions for standard PCR.

Step	Temperature (°C)	Minutes	Cycles
1	94	3:00	1
2	94	0:45	5
	55	0:45	
	72	0:45	
3	94	0:45	30
	60	0:45	
	72	0:45	
4	72	5:00	1

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