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Antiviral defense in salmonids – Mission made possible?

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ABSTRACT

Viral diseases represent one of the major threats for salmonid aquaculture. Survival from viral infections are highly dependent on host innate antiviral immune defense, where interferons are of crucial importance. Neutralizing antibodies and T cell effector mechanisms mediate long-term antiviral protection. Despite an immune cell repertoire comparable to higher vertebrates, farmed fish often fail to mount optimal antiviral protection. In the quest to multiply and spread, viruses utilize a variety of strategies to evade or escape the host immune system. Understanding the specific interplay between viruses and host immunity at depth is crucial for developing successful vaccination and treatment strategies in mammals. However, this knowledge base is still limited for pathogenic fish viruses. Here, we have focused on five RNA viruses with major impact on salmonid aquaculture: Salmonid alphavirus, Infectious salmon anemia virus, Infectious pancreatic necrosis virus, Piscine orthoreovirus and Piscine myocarditis virus. This review explore the protective immune responses that salmonids mount to these viruses and the existing knowledge on how the viruses counteract and/or bypass the immune response, including their IFN antagonizing effects and their mechanisms to establish persisting infections.

1. Several paths to antiviral defense

Viruses outnumber other microbes in the aquatic environment, and fish are constantly bombarded with them [1]. With that in mind, it is not surprising that some of the most common health threats for seawater aquaculture are viral diseases. Moving aquaculture from the sea to closed recirculating aquaculture system (RAS) facilities may solve the direct contact between aquaculture cages and the pathogens of the ocean. However, viruses can adapt to and establish themselves in closed facilities, and new viruses can emerge in the new environment. It is likely that viral infections in aquaculture will continue to be a significant future challenge.

With the constant exposure of farmed fish to this viral burden, their antiviral immune system needs to be alert and functioning. Effective vaccines are obvious solutions to the viral threat, but vaccines fail if the pathogenic viruses mutate, if new disease-causing viruses emerge, or if the immune system is suppressed. By use of various tactics, viruses are experts in hiding from immune detection and dampening host defense. Emerging pathogenic viruses possess the most successful immune evasion strategies, and the fish need to counteract the viral strategies in order to survive. To ensure that farmed fish are genetically equipped, adequately vaccinated and optimally prepared for antiviral defense, we need to understand the specific interplay between virus and host in depth.

Fish are covered with mucosal tissue, which is where the first encounter between virus and host occur. Mechanisms like mucus shedding and reproduction, antiviral peptides and enzymes, but also mucosal antibodies, act to fight pathogens in the mucus layer [2,3]. Details of the mucosal immune system have been thoroughly reviewed elsewhere [2–4], and will only be touched upon for certain viruses here.

In fish, like in mammals, the center of the antiviral innate immune mechanism in internal organs is focused around type I interferon (IFN) production and its actions as a secreted “early warning” signal from the infected cell to cells in the tissue environment [5–7]. IFN production is triggered by binding of viral nucleic acids to either Toll-like receptors (TLRs) in the endosomes or plasma membrane, or to cytoplasmic receptors, mainly within the RIG-like (RLR) receptor family [8]. Viral glycoproteins can also be recognized by pattern recognition receptors (PRRs), and it has been speculated that the G protein of rhabdoviruses binds to a glycoprotein recognizing PRR [9,10]. All these receptors trigger activation of IFN response factors (IRFs) to induce IFN promoters. IFN type I variants in fish differ between species, and details of salmonid IFNs are well covered elsewhere [6,7,11]. The IFNs are secreted to act on IFN receptors resulting in activation of a downstream

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signaling cascade that induces a large number of interferon-stimulated genes (ISGs) that confer direct antiviral activities to counteract further virus dissemination in the host. Most pathogenic viruses have evolved to develop specific mechanisms that bypass or block IFN production, and/or interfere with the IFN receptor-mediated activation of ISGs or the ISGs themselves. In salmonids, and most likely in other fish species, the numerous nucleated red blood cells population also play an important systemic role in mediating the antiviral innate immune response [12,13].

Whereas the general mechanisms for IFN-mediated antiviral innate immunity are well conserved in vertebrates [11], the execution of adaptive immune responses differ more between fish species and other vertebrate groups [14]. As the first vertebrate group that possesses all elements of adaptive immunity, the teleosts lack lymph nodes, follicular structures and do not form germinal centers, which are the specialized lymphoid structures in higher vertebrates [14]. Instead, recruitment of immune cells to the site of infection is important for initiating the adaptive response.

Except from some very primitive examples, vertebrate fish have IgM as their main systemic antibody, IgD at low expression levels, and IgT as a dominating antibody at mucosal surfaces [15], but not exclusively so [16]. Salmonids develop specific, often neutralizing, antibody responses to viral infections [17]. The B cells in salmonids have maintained some innate functions, like phagocytosis [18] and expression of PRRs including TLRs [19,20], suggesting that fish B cells resemble mammalian B-1 cells more than B-2 cells [21,22]. In line with this, IgM + B-cells from teleost fish express the B-1 marker CD5 [23]. For T cells, differences between fish species are more evident. While cod completely lack MHCII and CD4 T cells [24], salmonids have T cell immune functions resembling their mammalian counterparts, including both cytotoxic ($CD8^+$) and helper ($CD4^+$) T cells, and corresponding MHCI and MHCII [25,26]. Typical cytokines linked to Th polarization are also present, exemplified by the Th2 cytokines IL4/IL13 [27]. A pending question is still how Th cells are related to B cell activation in different fish species.

The innate and adaptive immune equipment of teleost fish should lay the ground for producing effective vaccines that trigger both humoral and cellular immune defense. Despite this, the long-term effects of several virus vaccines have not been optimal, and the answers may lie in the characteristics of the viruses that have emerged in salmonid aquaculture, and/or their ability to avoid immune surveillance.

2. Five RNA-viruses and their strategies to circumvent antiviral defense

Five RNA viruses have had major impact on Atlantic salmon farming by causing frequent or high mortality disease: Salmonid alphavirus (SAV) and infectious salmon anemia virus (ISAV) are lipid membrane enveloped, single stranded (ss) RNA viruses, whereas Infectious pancreatic necrosis virus (IPNV), Piscine orthoreovirus (PRV) and Piscine myocarditis virus (PMCV) are naked, double stranded (ds) RNA viruses [28–31]. All five viruses belong to different virus families (Table 1). Through studies on infection strategies and host interplay for these viruses, important aspects of teleost antiviral defense have been unraveled. Here, we describe the status of knowledge on the virus-host interaction pattern for each of them. This includes existing knowledge on virus receptors and sensors, their target cells and tissues, the IFN response and downstream activation of antiviral effector proteins. Next, we have looked into timing and localization of the humoral and cellular arm of the adaptive immune response elicited by viral infections. Large –omics studies have to some extend helped us understand virus strategies, immune responses and development of disease (Table 2). As our knowledge of the teleost immune system expands, a more detailed understanding of fish-virus interactions will develop. In this review, we point out some research gaps that, if answered, would enable a more elaborative understanding of immunity to fish viruses.

2.1. Salmonid alphavirus (SAV)

Salmonid alphavirus, also referred to as Salmon Pancreas Disease Virus (SPDV), is a significant pathogen for Atlantic salmon and rainbow trout in Europe. SAV is the causative agent of pancreas disease (PD) affecting Atlantic salmon or sleeping disease (SD) affecting rainbow trout. The latter was initially reported for fresh-water reared trout in France [32], but SD in trout has since then been described in several continental European countries [33], and more recently Poland and Switzerland [34,35]. PD is found throughout Europe including Norway, UK, Ireland, France, Italy, and Spain [27]. SAV causes lethargy and anorexia of the infected fish, leading to slow growth and runting [36]. Significant mortality rates (up to 60%) have been observed in PD outbreaks [37], and the disease is a major economical and welfare problem for farmed salmon in Europe. Despite vaccination programs, SAV is becoming increasingly predominant in the field and the eradication of this pathogen is a challenge for most fish farms.

2.1.1. Subtypes and genome

Based on differences in the E2 and nsP3 genes, SAV strains are grouped into 6 different subtypes, SAV1-6 [38]. SAV1, first isolated from Atlantic salmon, is the causative agent of PD in Ireland and Scotland [39]. SAV2 comprises two subgroups, a freshwater variant (SAV2-FW) causing SD in trout, and a marine variant (SAV2-MW) responsible for PD in seawater reared salmon and rainbow trout [33]. SAV3 is a Norwegian subtype and affects both Atlantic salmon and rainbow trout [40,41]. The subtypes 4–6 are found in Atlantic salmon from overlapping areas of the coast of Ireland, Northern Ireland and Scotland [36]. The focus of this review will mainly be on SAV1, SAV3 and PD.

SAV belongs to the genus *Alphavirus* in the family of *Togaviridae* [39,42]. Alphaviruses have ssRNA genomes with positive polarity enclosed in an enveloped icosahedral nucleocapsid [43] (Table 1). The 5′ two-thirds of the nearly 12 kb genome encode the non-structural proteins nsP1-4, which comprise the viral components required for replication of the viral genome and for the transcription of subgenomic RNA. The 3' one-third of the genome contains subgenomic RNA, which translates into the structural capsid proteins (E3, E2, 6K, TF, and E1) that form the virus particle. Knowledge of the determinants for SAV virulence is limited. A reverse genetics system for SAV2 was developed by INRA France [44] and has enabled identification of mutations within the E2, 6K and E1 proteins associated with virulence in rainbow trout. Later a reverse genetic system was reported for SAV3 [45], which allows functional studies into the virulence determinants of SAV3 as well [46].

2.1.2. Tissue tropism and pathogenesis

SAV has a wide tissue tropism in Atlantic salmon [47], however, heart, skeletal muscle, and pancreas are the organs mainly affected by PD and SD and descriptions of the pathology associated with the disease are described in detail elsewhere [37,41]. The histopathology for these diseases are sequential where the pancreatic lesions appear first after infection, followed by heart muscle lesions and finally extensive lesions of skeletal muscle fibers. By using both in vivo and in vitro models, Biacchesi et al. [48] reported that muscle satellite cells are targets for SAV2 infection in rainbow trout. Virus replication was detected in *ex vivo* cultivated satellite cells [48]. Together with the loss of exocrine pancreas leading to defective uptake of nutrients, this may explain the reduced muscle growth observed in surviving SAV-infected fish [37].

2.1.3. Innate immunity

For alphavirus species in higher vertebrates, type I IFN-dependent innate immune response is essential for protection [49]. IFN receptor deficient mice are highly susceptible to alphavirus infections signifying the importance of IFN-mediated defence [50]. The ability of IFNs to restrict viral replication in vertebrate cells is largely mediated trough

Table 1
Description of five salmonid viruses, their structure, genome and associated diseases.

Virus	Family	Structure	Genome (segments)	Variants, diseases and target species
Salmonid alphavirus SAV	*Togaviridae*	Enveloped Spherical, icosahedral single capsid ~70 nm	+ssRNA (1)	SAV1,2,3: Pancreas Disease (PD) in Atlantic salmon SAV2: Sleeping disease (SD) in rainbow trout
Infectious salmon anemia virus ISAV	*Orthomyxo-viridae*	Enveloped ellipsoidal single capsid ~100 nm	-ssRNA (8)	ISAV HPRdel: Infectious salmon Anemia (ISA) in Atlantic salmon
Infectious pancreatic necrosis virus IPNV	*Birnaviridae*	Non-enveloped Icosahedral single capsid ~70 nm	dsRNA (2)	Infectious pancreatic necrosis (IPN) in Atlantic salmon
Piscine orthoreovirus PRV	*Reoviridae*	Non-enveloped icosahedral double capsid ~80 nm	dsRNA (10)	PRV-1: Heart and skeletal muscle inflammation (HSMI)-Atlantic salmon PRV-2: Erythrocyte inclusion body syndrome (EIBS) in Coho salmon PRV-3: HSMI-like disease w/anemia in rainbow trout
Piscine myocarditis virus PMCV	*Totiviridae*	Non-enveloped icosahedralS single capsid ~40 nm	dsRNA (1)	Cardiomyopathy syndrome (CMS) Atlantic salmon

the induction of ISGs and a subset of ISGs have been shown to impede alphavirus replication in mammals [50]. In line with this, up-regulation of type I IFNs and ISGs during SAV challenges in salmonids are reported [51–56]. Two microarray studies are performed for SAV1 challenged trout [57] and SAV3 challenged salmon [53] (Table 2), using head kidney and heart, respectively. Despite different target organs, profound induction of ISGs including Mx, Viperin, ISG15, and several IFIT and GIG family member genes were demonstrated for both SAV subtypes. By using SAV3-infected TO cells, increased TLR3/8, MDA5, RIG-I, and LPG2 transcription were shown [58,59] suggesting that TLR and RLR pathways are involved in SAV recognition and signaling. In both studies, LPG2 and RIG-I in the SAV3 infected cells were more highly upregulated than MDA5. The RLRs are RNA helicases that signal through the adaptor molecule MAVS/IPS1 to activate the transcription factors IRF3, IRF7 and NF-kB, driving transcription of proinflammatory cytokine genes including type I IFN encoding genes [60]. The mammalian alphavirus chikugunya (CHIKV) is RLR restricted as MAVS deficient mice have increased viremia and show reduced IFN levels in serum [50]. Studies of mice deficient for TLR7 or TLR3, as well as their adaptor molecules MyD88 and TRIF, suggest that TLR signaling also contribute to control CHIKV infection, as these mice show increased viremia and enhanced dissemination compared with wild type [50].

Several in vivo studies have demonstrated the induction of TLRs and the adaptor molecule MyD88 [52,61] as well as the RLRs MDA5 and LGP2a [55] in SAV-infected salmonids. In the study by Moore et al. [55], these PRRs showed an earlier peak response in the head kidney for SAV3 intramuscular injected fish compared to bath challenged fish. Grove et al. [52] reported induced levels of TLR8 mRNA in salmon head kidney, heart and gills derived from SAV3 fish challenged by cohabitation, while in another study of SAV3 ip injected fish, TLR8 mRNA levels in spleen were unaffected [61]. In the latter study, MyD88 transcripts remained unchanged at day 3, while at day seven and 28 post SAV injection their levels were four-fold upregulated compared to untreated controls. In recent studies, up-regulation of both TLR7 and TLR8 in SAV3 bath-challenged salmon were reported, where TLR7 was more highly induced than TLR8, while TLR8 showed higher constitutive levels [55]. Collectively, the findings illustrate that the challenge route is of importance for the onset and duration of innate responses, and that bath immersion challenges induce lower and more sustained responses compared to injection challenges [55]. Reduced PD pathology and SAV levels in salmon injected with the TLR-ligands poly I:C or CpG, correlating with up-regulated levels of both type I IFN, IFNγ and Mx are found [62]. The study points to importance of the IFNs for host defence against SAV. This is supported by data showing a direct antiviral effect of salmon IFNa1 against SAV3 in cell lines [63,64]. However, while the study by Xu et al. [63] did not detect antiviral activity upon IFNγ treatment of TO cells, Sun et al. [64] showed potent antiviral activity against SAV3 by IFNγ in the same cells. In the presence of anti-IFNa1 antibodies, the antiviral activity of IFNγ was reduced, proposing that the antiviral activity is partly dependent on type I IFN-induction [64].

2.1.4. Adaptive immunity

Atlantic salmon or rainbow trout surviving PD outbreaks are less susceptible to further infection, as observed experimentally or in the field [65,66]. This shows that salmonids develop an efficient adaptive immune response against SAV following a primary exposure. The detection of neutralizing antibodies associated with protective responses against PD suggests involvement of antibody mediated immunity [67–70]. Passive immunization of SAV in live fish is also reported, providing evidence that these antibodies are protective [71]. For mammalian alphaviruses, antibodies directed against the glycoprotein E2 are often more neutralizing than antibodies reactive to E1 [41]. Recently, Jenberi et al. documented strong anti-E2 responses in SAV3 infected salmon, confirming the reactivity directed against this viral protein for salmon B cells as well [72]. By using a SAV3 replicon, Hikke et al. [46] showed the importance of E1 and low temperature, for virion formation and proper E2 cell surface expression.

The complement system has key roles for the protection against alphavirus infections by stimulating adaptive responses through antibody mediated neutralization of the virus, as well as causing opsonisation of viral particles and complement-dependent lysis of viral infected cells [43]. In general, the role of complement in the protection against PD is scarcely described, although a few reports suggest its importance [73,74]. In a study revealing the serum proteome of SAV3-infected salmon (Table 2), the complement components C3, C9, complement factor B and H and the complement inhibitor C1 were identified [73]. C9 is an important component of the complement-membrane-attack-complex (MAC) known to lyse enveloped viruses. In another study, increased levels of C4, an important factor for classical component activation, were shown in sera from SAV-infected salmon [74]. In the same study, C4 levels were depleted in SAV-vaccinated groups, which showed protection against PD. The consumption of C4 is a sensitive measure of classical activation, suggesting a role of antibody mediated complement activation in protection against piscine alphaviruses.

T cell associated genes including CD8α, CD3z/b, CD4-2 and CD40 are shown to be up-regulated following SAV3 infection in heart [51–53], head kidney [51,52], spleen [51], pancreas [51], gills [52], and also by SAV whole virus vaccination [68–70]. In general, the expression levels of these genes were moderately affected in the immunological organs; gills, spleen and head kidney, and they were shown to decline over time. In contrast, CD4, CD8, CD3ε, MHCII and IL-12 levels were, in general, higher in the primary target organs heart and pancreas at later time points (6–8 weeks) [51,52], which also correlated with the highest IgM mRNA and antibody levels. The results suggest

Table 2
Transcriptomic and proteomic studies describing innate and adaptive immune responses in Atlantic salmon infected with salmonid alphavirus (SAV), infectious salmon anemia virus (ISAV), infectious pancreas necrosis virus (IPNV), Piscine orthoreovirus (PRV) and Piscine myocarditis virus (PMCV). wpc; weeks post challenge (for cohabitation), wpi; weeks post infection, dpi; days post infection, I.p.; intraperitoneal, I.m.; intramuscular.

Infection	Challenge model	Target tissue	Immune system	Analysis	Reference	Main discovery
SAV	I.p. injection SAV1 2–5 dpi	Head Kidney	Innate	Microarray	Herath et al. 2012 [57]	• Up-regulated IFN mediated/antiviral responses • Up-regulated MHC class I and II pathway genes
	Cohabitation SAV3 0–12 wpc	Serum	Humoral	Proteomics	Braceland et al. 2013 [242]	• Complement factors dominate early, then decline when antibodies sharply rise at 5 wpc • Correlation of tissue injury with creatine kinase, enolase and malate dehydrogenase during peak PD • Hemopexin, transferrin, and apolipoprotein sustain during later stages
	Cohabitation SAV-3 2,5-5 wpc	Heart	Innate Adaptive	Microarray	Johansen et al. 2015 [53]	• Up-regulated IFN-mediated responses • MHCII gene expression increases in concert with PD development • Lowly adaptive immune gene expression within the PD trial period
ISAV	Cohabitation HPR7b ISAV 3–14 dpc	Head kidney Liver Gills	Innate, adaptive	RNASeq	Valenzuela-Miranda et al. 2015 [270]	• Up-regulated IFN-mediated responses in all tissues • Up-regulated B− and T-cell gene transcript levels, primarily in head kidney • Immune cell migration genes in gills and liver
	Cohabitation HPR7b ISAV 30–47 dpc	Spleen	Adaptive	RNASeq	Dettlef et al. 2017 [98]	• Viral susceptibility associates with differences in immune gene expression, including lectins-, perforin- and the transcription factor hivep2-transcripts
IPNV	Immersion IPNV (NFH−Ar/NFH-El) 6–29 dpi	Head Kidney	Innate adaptive	Microarray	Skjesol et al. 2011 [246]	• Early innate antiviral response and IFNγ expression differ between infections with the virus genotypes
	Immersion IPNV V0512-1 (Sp) 1–5 dpi	Head kidney	Innate, adaptive	Microarray	Reyes-Lopes et al. 2015 [271]	• Susceptible individuals are characterized by a short-lasting, inflammatory innate response • Protected individuals have lower and more sustained innate antiviral immune responses • Resistance is associated with TGFβ-expression
	Immersion IPNV V0512-1 (Sp) 1–20 dpi	Whole fry	Innate, adaptive	Microarray	Robledo et al. 2016 [272]	• Susceptible individuals are characterized by high early innate immune response • Resistant individuals possess a moderate, putative macrophage-mediated inflammatory response
PRV	Cohabitation PRV-1-Nor 5–10 wpc	Erythrocytes	Innate	Microarray	Dahle et al. 2015 [13]	• Long-lasting innate immune response parallels induction of genes related to antigen presentation and antibody production
	I.p injection PRV-1-Nor 5–11 wpi	Heart	Innate Adaptive	Microarray	Johansen et al. 2015 [53]	• Long-lasting IFN-regulated antiviral response up to 11 wpc • Adaptive immune responses, dominated by B− and cytotoxic T-cell responses, triggered from 6 wpc
	Cohabitation PRV-1-Nor 4–8 wpc	Spleen Head Kidney	Innate Adaptive	Microarray	Johansen et al. 2016 [205]	• Infected parr show earlier induction of genes involved in innate antiviral immunity compared to smolt • Also B− and T-cell response genes appear earlier in infected parr compared to smolt
PMCV	I.p injection 2–12 wpi	Heart	Innate, Adaptive	Microarray	Timmerhaus et al. 2011 [13]	• Systemic induction of antiviral- and IFN-dependent genes from 2 wpi • Cardiac activation of complement genes prior to cardiac-specific upregulation of T cell response genes
	I.p. injection 2–10 wpi	Heart	Innate Adaptive	Microarray	Timmerhaus et al. 2012 [273]	• Earlier activation of NK cell-mediated cytotoxicity and NOD-like receptor signaling pathways in CMS low responder hearts • Induced T cell response genes in high responder hearts reflect increased CMS pathology
	I.m injection 6–14 wpi	Heart	Innate, adaptive	Microarray	Martinez-Rubio et al. 2014 [274]	• Functional feeds reduce CMS pathology, associated with lower expression of genes associated with cytotoxic T-cells

that a combined cytotoxic and Th1 mediated response takes part in the heart at late phases of the infection. Immunohistochemistry on hearts from salmon from a natural PD outbreak has verified an influx of immune cells, dominated by $CD3^+$ T lymphocytes [75]. The presence of MHCII expressing cells with macrophage or lymphocyte morphology in the infected hearts suggest trafficking of immune cells with antigen presenting capacity i.e. macrophages, DC cells or B cells. While a Th1/cytotoxic response associated with inflammation/pathology appeared late (8 weeks) in the SAV ip injected salmon, the reaction in the pancreas was greatly downscaled suggesting a contraction phase [51]. Together, the published studies suggest that both humoral and cellular adaptive responses contribute to the protection against these viruses. Presently, there is limited understanding of the role T cells play during alphavirus infection with most work focusing on gene expression and a lack of studies focusing on cell-mediated cytotoxicity.

2.2. Infectious salmon anemia virus (ISAV)

ISAV, first described as the virus that causes Atlantic salmon anemia (ISA) in 1995 [76], causes an acute high mortality disease in Atlantic salmon and has been responsible for high loss epidemics with devastating economic consequences for aquaculture, primarily in Chile in 2007 when ISA outbreaks hampered the planned aquacultural expansion [77,78]. The disease ISA is characterized by severe anemia and organ necrosis linked to collapsed circulation and gas exchange, in part induced by red blood cell agglutination [31,77,79,80]. Many wild salmonids are also susceptible to ISAV infection, but no disease have been found in the wild [81].

2.2.1. Subtypes and genome

ISAV belong to the family of *Orthomyxoviridae*, and is related to the mammalian influenza virus [82]. ISAV is an enveloped virus with a segmented ssRNA genome [79] (Table 1). Both the influenza virus and the ISAV genome can mutate, recombine and reassort, giving it numerous possibilities to change [82,83]. ISAV exists as an initially harmless wild-type strain, the so-called highly-polymorphic region (HPR) 0 strain, which has not been associated with mortality or disease [84]. The presence of ISAV HPR0 is a potential risk factor for disease, since mutation into pathogenic strains can cause ISA [85–88]. Two glycoproteins in the virus envelope, the hemagglutinin esterase protein (HE) and the fusion protein (F) are essential for virus uptake [89]. ISAV pathogenicity is related to a diverse set of deletions in the HPR-region of the HE protein (known as HPRdel), combined with mutation in the F-protein [87,89–91]. The HPRdel variants, commonly numbered HPR1-x, show different levels of pathogenicity.

2.2.2. Tissue tropism and pathogenesis

The pathogenesis of ISAV have been previously reviewed [31]. ISAV uses 4-*O*-acetylated sialic acids as attachment sites or receptors [92–97]. A transcriptome study on Atlantic salmon families with differential resistance to ISAV revealed that the resistant family (> 35% less mortality and lower virus levels) showed upregulated expression of sialic acid-binding Ig-like lectin (*siglec14*) (Table 2), which may aid virus binding to 4-*O*-acetylated sialic acids [98]. Siglec14 is also shown to be an immune cell stimulator [99,100]. Increased expression was also observed for epithelial catherin (cdh1), which has been linked to resistance against IPNV as well [101].

The HPR0 strain primarily infects the gill and skin epithelial cells and replicates at low levels with no pathological consequences [102]. In contrast, virulent ISAV establishes a systemic infection, infects endothelial cells, and bud virus into the blood stream [92].

Atlantic salmon experimentally infected through ip injection or bath/immersion of pathogenic ISAV variants, like the Chilean HPR7b strain, develop ISA in approximately 10–15 days causing 70–100% mortality within the following month [98].

2.2.3. Innate immunity

The high mortality from ISAV infections indicates that the immune system is unable to control and limit the virus replication. There is an intricate interplay between the IFN system and ISAV replication. Whereas type I IFNs, more specifically IFNa1 and IFNc, inhibit ISAV replication [103], at least two ISAV-encoded proteins exhibit type I IFN antagonist properties [104,105] (Fig. 1). The segment 7-encoded s7ORF1 is a cytoplasmic protein that inhibits activation of both IFN and Mx transcription [104,105]. The segment 8-encoded s8ORF2 is located in the nucleus, binds dsRNA and polyadenylated ssRNA, act as an RNA silencing suppressor [106], and inhibit poly (I:C)-mediated IFN promoter activation [104].

A comparison between low and high virulent ISAV strains using mucosal bath challenge revealed that the low virulent ISAV initiated an earlier IFN-mediated response in the gills, heart and kidney, compared to the high virulent variant which had a slower response pattern [107]. This gives the immune system a head start in developing a specific immune response against the low virulent virus. In line with this, the most virulent ISAV variant also inhibited Mx production more efficiently in cell culture compared to low virulent strains [103].

The related influenza virus mechanisms of interaction with IFN production and signaling have been thoroughly studied. The multifunctional NS1 protein interacts with several intracellular proteins essential for initiating the antiviral immune responses [108], and thereby interfering with the activation of RIG-I, the NF-κB signaling pathway [17], the stress granule-mediated antiviral activity [18], cell survival pathways and cellular transcription-translation [27–30]. Several of the other influenza proteins interact with antiviral proteins as well, but not to the same extent [108]. Whether ISAV possess a similar entity to inhibit IFN and ISG production, and if the ISAV proteins are equally multifunctional are presently a pending question.

2.2.4. Adaptive immunity

Several vaccines against ISAV have been tested in immunization trials with good results [109–111]. In these studies, the protection has been closely associated with ISAV-specific and neutralizing antibodies indicating an important role of humoral immunity in protection. When focusing on genes associated with ISAV resistance, a role of T cells in immune protection against ISAV was indicated [98]. The transcription factor human immunodeficiency virus type I enhancer binding protein 2 (*hivep2*), which have previously been identified as a potential protective quantitative trail locus (QTL) for ISAV resistance [112], was induced in a transcriptome study on ISAV resistant salmon [98]. Hivep2, also known as Shnappi 2, is a lymphocyte transcription factor shown to be involved in Th2 differentiation [113]. Furthermore, Perforin 1-like protein (*prf1*) which is involved in activity of cytotoxic T cells was also induced in ISAV resistant fish, indicating antiviral cytotoxic immune activity against ISAV [112]. In line with this, a larger $CD8^+$ population at early stages of infection have been reported in tissues from salmon infected with a virus strain with low virulence, compared with the high virulent strain [114]. Taken together, these observations indicate that both T helper and cytotoxic T cell activity are protective towards ISAV infection. However, the T cells have to appear early and act strongly enough to control the virus. Since innate antiviral responses may be inhibited by ISAV segment 7 and segment 8 proteins, and neutralizing antibodies may be induced too late to fight ISAV in the critical phase, T cell-mediated responses may be crucial. However, this warrants further investigation.

2.3. Infectious pancreatic necrosis virus (IPNV)

Infectious pancreatic necrosis (IPN) is caused by IPNV. IPNV belongs to the genus *Aquabirnavirus* within the family *Birnaviridae* (Table 1). Aquatic birnaviruses have a worldwide distribution and are detected in more than 30 different families of fish [115]. Some hosts are subclinically infected, while in other hosts the viruses cause disease, such as IPN in salmonids [115]. Susceptibility of salmonids to IPNV is age dependent [116], and outbreaks are most often observed in fry during the freshwater stage, in post-smolts up to 10 weeks after transfer to sea, and in larger sea-reared salmon [116,117]. IPN has a history as one of the major loss factors in international salmonid aquaculture. Vaccines against IPN were introduced into aquaculture in the mid-90s, but they were not as efficient as the vaccines against bacterial diseases, which made the control of IPN a challenging task for the fish farmers. Breeding of salmon that are resistant to IPN has been a success and is proposed to be the main reason for the dramatic decline of IPNV outbreaks in Norwegian aquaculture after 2009 [118]. Genetic mapping of QTLs affecting disease resistance against IPN has made it possible to produce IPN resistant salmon for the aquaculture industry [54]. The epithelial protein cadherin (CDH1) has been linked to this resistance [101]. This protein strongly binds to IPNV virions, and co-localization between the virus and CDH1 in endosomes indicates that the protein is involved in IPNV internalization [101].

2.3.1. Subtypes and genome

Birnaviruses possess a bi-segmented dsRNA genome, designated segment A and B that is enclosed in a non-enveloped single-shelled icosahedral particle about 60 nm in diameter [119]. VP1, the viral RNA-dependent polymerase encoded by segment B is the largest of the viral proteins (94 kDa) [120]. Segment A has two overlapping open reading frames, of which the smaller encodes VP5 and the larger encodes a 103-kDa polyprotein [121,122]. After its translation, the polyprotein is cleaved and further processed to form the mature outer capsid protein VP2, in addition to VP3 and VP4. VP3 is the suggested key organizer of particle assembly in birnaviruses and is shown to interact with VP1 and

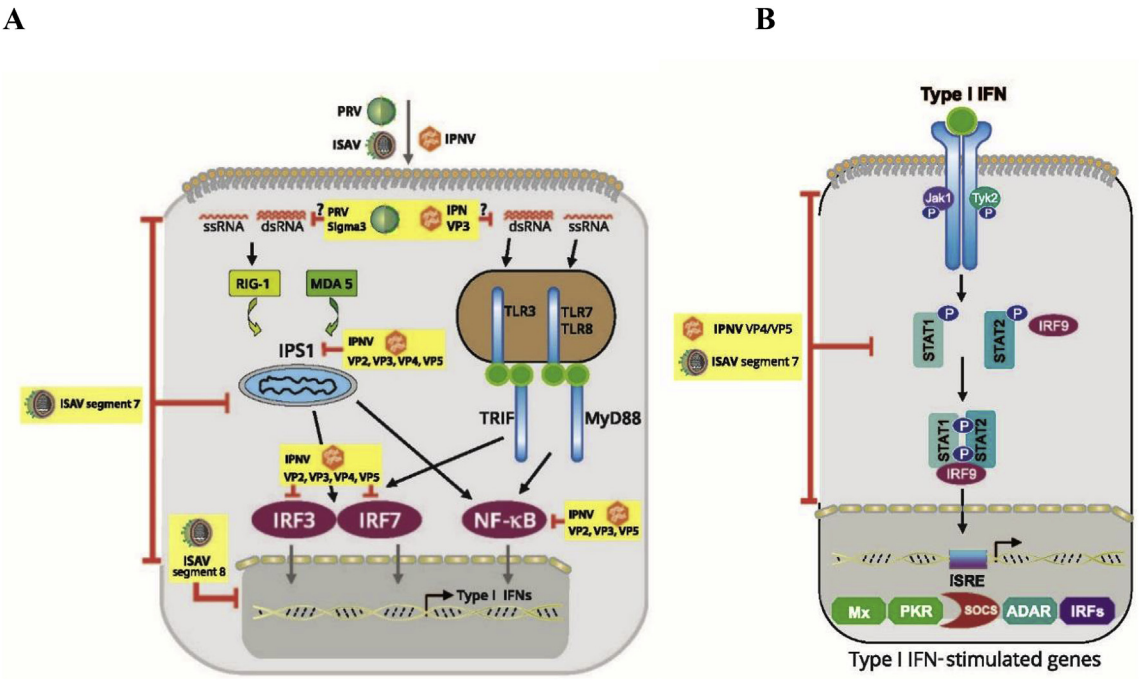

Fig. 1. Type I interferon signal pathway and evasion strategies for some salmon viruses. (A) Cells sense virus dsRNA and ssRNA via pathogen recognition receptors (PRRs), RIG-I, MDA-5, TLR3/7/8/19/22. These receptors activate their adaptor molecules IPS1, TRIF and MyD88, which initiate the signaling cascades that activate IRF3, IRF7 and NF-kB. The evasion mechanisms of IPNV include impairment of IPS1 signaling by VP2, VP3, VP4 and VP5 [153]. All the four IPNV proteins act on IRF3 and IRF7, while only VP2, VP3 and VP5 act on NF-kB. ISAV segment 7 and ISAV segment 8 inhibit salmon IFN promoter activation in the cytoplasm and nucleus, respectively [104,105]. PRV Sigma 3 [228] and IPNV VP3 have RNA-binding capacities [123], and may hinder recognition of the viral RNA by PRRs. (B) Binding of type I IFN to their receptors activates the JAK/STAT signaling pathway. Specifically, Tyk2 and Jak1 kinases activation results in the generation, phosphorylation and assembly of the trimeric ISGF3 complex, which consists of a STAT-STAT2 heterodimer and IRF9. This complex translocate to the nucleus, binds to IFN-stimulated elements (ISRE) and induces ISGs production. IPNV VP4 and VP5 and ISAV Segment 7 are shown to reduce Mx promoter activation [145], suggesting that ISG functions might be impaired. So far, which host molecules in the signaling cascade that are targeted by these virus proteins are unknown.

ds-RNA [123]. Nucleotide sequence analyses of IPNV isolates differing in pathogenicity have revealed that VP2 is a major determinant of virulence for the virus. There is experimental evidence showing that viruses of the Sp strain encoding Thr217-Ala221 are highly virulent, while isolates encoding Pro217-Ala221 are moderately virulent and Pro217-Thr221 isolates are classified as avirulent [124,125]. However, conflicting data exist which describe strains with Pro217-Ala221 to be highly virulent both in experimental conditions and in the field [126,127].

2.3.2. *Tissue tropism and pathogenesis*

Both gills and intestine are suggested as routes of entry for IPNV. As the name implies, IPNV primarily affect the pancreas and the most consistent histopathological finding is necrosis of exocrine pancreatic tissue [128]. Necrosis in other organs, e.g. liver, gut and hematopoietic tissues are also common findings [129].

2.3.3. *Persistence*

Fish that survive IPN outbreaks may become persistently infected, and the virus is likely to persist by subverting the host defence for a long time, even throughout their lives [130]. The virus has been reported to hide and multiply within head kidney derived leucocytes from carrier fish without causing lytic infection, suggesting that these cells could be a site of persistent infection [131–135]. While Sommer & Johansen [133] reported a low IPNV replication rate in head kidney macrophages derived from infected fish, a separate study found it to be non-detectable [136]. In addition to macrophages, IPNV is detected in head kidney derived neutrophils and B cells originating from IPNV challenge survivors, suggesting that these cells are potential targets for IPNV infection [135]. Santi et al. [137] have implied that IPNV strains differ in their ability to establish persistent IPNV infections. The authors suggested that Thr residue 221 is a prerequisite for persistent infections, while strains with Ala at this position did not establish long-term persistent infections as efficiently as the avirulent virus. Later, Julin et al. [138] showed that both virulent and avirulent strains cause persistent infections. In their study, infections with both the strains were followed at freshwater and seawater phases. In freshwater, higher levels of IPNV transcripts were detected in head kidney of fish infected with low virulent compared to high virulent virus, suggesting that the latter is able to limit its own replication to a level where the innate response is not alerted [138]. When *ex vivo* cultivated adherent leukocytes were stimulated with the TLR ligand CpG, known to induce the IFN pathway in these cells [139], significantly higher IFN levels were detected in the low virulent infected group compared to the high virulent group in the fresh water phase [138]. The data imply that high and low virulent IPNV strains modulate innate responses in head kidney differently. However, how these differences between the strains affects the scale and character of host immune responses warrants further studies.

2.3.4. *Innate immunity*

Both type I and type II IFNs contribute to protection against IPNV in permissive cell-lines [140], although in vitro studies have shown that the antiviral activity by IFNγ is not as pronounced as described for IFNa1 [64]. At least some of the antiviral activity ascribed to IFNγ may be due to its ability to increase the transcription of type I IFNs [64]. Prior to the identification of the IFN genes themselves, activation of functional IFN responses was proven indirectly by measuring the transcription of ISGs i.e. the Mx gene [141], Mx protein [142] or Mx promotor activation [143]. Early studies measuring Mx protein levels suggested lack of IFN responses in IPNV infected salmonid cell-lines [142]. However, in later studies, augmented IFNa1 mRNA were detected in different IPNV infected cell-lines [59,144,145] and in

cultivated trout head kidney macrophages [144]. An explanation to these contradicting results is provided by Skjesol et al. [145], showing that IPNV possess strategies to antagonize IFN-induced responses by weakening IFN-signaling and thereby ISG expression.

In vivo, increased levels of PRRs, type I IFNs and several ISGs have been perceived in Atlantic salmon challenged by both virulent and avirulent IPNV strains, during acute and persistent infections [146–149]. In the study by Skjesol et al. [146], coordinated induction of RIG-I, MDA5, TLR8, TLR9, PKR, MyD88, IFNa1 and IFNc, and several ISGs were detected in the head kidney (Table 2), however, at significantly lower levels in the avirulent IPNV infected fish [146]. Compared to the virulent strain, lower virus load and mortality appeared in the avirulent strain, suggesting that the immune gene expression levels are directly related to the rate of virus of replication [146]. A subsequent study has confirmed that the levels of IFNa1 and Mx transcription in different target organs for the virus correlates with virus load and pathology [150].

The process where juvenile salmon undergo parr-smolt transformation is a stressful stage in the production cycle, and for IPNV-infected Atlantic salmon stress can induce a delay in the transcription of immune markers [151,152]. Julin et al. [138] supported this notion, as Mx mRNA levels in the infected fish dropped during the fresh water stage, while transfer to sea provoked reappearance of IPNV for both high and low-virulent IPNV. In seawater, a long-term expression of Mx was detected over a period 19 weeks and correlated with the IPNV levels [138]. However, as the results demonstrate, high levels of Mx does not necessarily correlate with protection.

Since IFN treatment [64] or induction [141] inhibits IPNV replication, the virus depends on strategies to modulate IFN activity to be able to establish an infection in its host. Lauksund et al. [153] identified the proteins preVP2, VP3, VP4 and VP5 as powerful inhibitors of IFNa1 activation through the RIG-I/MDA5 signaling pathways. RIG-I and MDA5 interact with the adaptor protein IPS-1 upon binding viral RNA, which activates the transcription factors IRF3, IRF-7 and NF-κB resulting in initiation of type I IFN transcription [154] (Fig. 1A). Although all four segment B encoded proteins inhibited IPS-1 mediated activation of the IFNa1 promoter [153], the protease VP4 showed the strongest inhibition and abolished the promoter activation. However, VP4 mutated to eliminate the protease activity retained its ability to inhibit IPS-1 activation, proposing that protease activity of VP4 is not mediating this effect. In contrast to the segment B encoded proteins, the viral polymerase VP1 showed IFN inducible properties [153]. Overexpression of VP1 alone potently activated the IFNa1 promoter and increased the activation mediated by IRF1 and IRF3. Furthermore, VP1 combined with the IRFs upregulated IFNa1 mRNA levels and antiviral activity against IPNV in TO cells. Since VP1 utilize both viral and non-viral sources for RNA-synthesis in vitro [155], the IFN inducing properties of VP1 might be due to dsRNA synthesis. In support of this, a VP1 mutant deficient in producing dsRNA abolished VP1 mediated IFNa1 activation [153]. During IPNV infection, RNA synthesized by VP1 might be recognized by PRR and thus trigger IFN activation. This may force the virus to develop multiple IFN antagonizing mechanisms to avoid triggering IFN synthesis. The dsRNA binding of VP3 is also proposed as a mechanism for avoiding antiviral host responses [123] (Fig. 1A).

Besides encoding mechanisms that inhibit the production of IFNs, IPNV have developed strategies to inhibit responses to IFN as well [145]. Experimentally, IFNa1 treatment after IPNV infection results in restrained virus production and in parallel reduced Mx protein levels [145]. In the same study, protein synthesis of housekeeping genes were not affected, implying that the results might be representative for other ISGs as well. Further, when testing the influence of the different IPNV encoded proteins on Mx-promoter activation in response to IFNa1, VP4 and the non-structural protein VP5 inhibited the activation of this promoter [145]. The results suggest that these IPNV proteins are able to circumvent the action of IFN, most likely by targeting JAK/STAT signaling (Fig. 1B). The exact mechanisms by which IPNV encoded proteins impairs signaling pathways leading to type IFNa1 or Mx expression are presently not understood.

In addition to alerting the IFN system at systemic sites, IPNV is shown to upregulate splenic and head kidney mRNA levels of IL-10, an anti-inflammatory cytokine, after acute IPNV infection in salmon and in asymptomatic IPNV carriers [149]. In the same individuals, there was a lack of induction of the inflammatory cytokines IL-1β and IL-8, and for IPNV carriers even IL-8 expression decreased. In accordance with this, other studies have also reported the absence of IL-1β and TNFα induction in tissues from IPNV infected fish [147,156]. IL-10 is a cytokine with many regulatory functions, and among these the cytokine is known to modulate the expression of other cytokines [157]. In mammals, IL-10 has a role in regulating immunity to persistent viral infections [157]. Reyes-Cerpa et al. [149] hypothesized that IPNV-induced IL-10 creates an anti-inflammatory milieu, which could explain the high prevalence and persistence of IPNV in salmon.

2.3.5. Adaptive immunity

Vaccination against IPNV in salmonids has a long story and high survival rates from experimental trials [158] and field [159] have been reported, however the vaccines available on the market have not fully prevented IPNV outbreaks in the field. Studies of the protective responses have demonstrated that IPNV specific antibodies are elicited [148]. The major neutralization epitopes of IPNV are localized within VP2 [160–162], and recombinant VP2 alone, DNA vaccines or multivalent vaccines including this component are shown to elicit specific antibodies against the virus [148,163,164]. More recently, an IPNV vaccination trial including high and low doses of inactivated virus vaccine revealed unique signatures of the antibody response before and after IPNV challenge [148]. Upon immunization, antibody levels in the high antigen group were substantial higher than for the low antigen group suggesting an antigen dose dependent response. However, upon challenge 8 weeks post vaccination, no classical secondary immune response with increased antibody titers were apparent, instead, antibody levels in both the vaccinated groups declined [148]. The study suggest that the antibodies in the vaccinated groups waned due to their usage in virus neutralization. In addition to neutralizing activity, it is likely that specific IPNV antibodies opsonize free virus particles or infected cells and thereby stimulate macrophages, induce cytokine production and activate cellular responses. Cellular immunity is scarcely investigated during fish IPNV infections, although the upregulation of T cells or antigen presenting derived molecules are reported [146]. As knowledge of cellular immunity directed against IPNV is still sparse, basic research is needed to identify cells that contribute to cellular effector functions against this virus and their mode of action in fish.

2.4. Piscine orthoreovirus (PRV)

No virus is more commonly found in farmed Atlantic salmon than PRV, a member of the family of *Orthoreoviruses*. PRV infection can cause heart and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon, erythrocytic inclusion body syndrome (EIBS) in Coho salmon, and HSMI-like disease with anemia in rainbow trout [165–168]. Recently, association with jaundice syndrome in chinook salmon and proliferating darkening syndrome (PDS) in brown trout have been reported as well [169,170]. PRV infection does not necessarily cause mortality, but its effect on the heart and red blood cells may weaken the infected fish and make it less tolerant to stress and hypoxic conditions [171]. Since PRV is near ubiquitous in farmed Atlantic salmon and HSMI outbreaks are very common in Norway, the virus is considered as a significant cause of loss. PRV also infects wild salmon, and may cross-infect between different salmonid species [172].

2.4.1. Subtypes and genome

Orthoreoviruses are non-enveloped viruses with a double protein

capsid and a segmented dsRNA genome encoding at least eleven proteins [173] (Table 1). Three subtypes of PRV have been identified in salmonids so far; PRV-1 in Atlantic salmon and Chinook salmon [165,169], PRV-2 in Coho salmon [167], and PRV-3 in rainbow trout and Brown trout [166,167,170]. A more distantly related orthoreovirus have been found in largemouth bass [97]. Most research have so far been performed on PRV-1 in Atlantic salmon, and this is the subtype referred to here unless otherwise is stated.

2.4.2. Tissue tropism and pathogenesis

Orthoreoviruses enter target cells either through a receptor-mediated mechanism, or through a combined process of extracellular outer capsid proteolysis and receptor-independent uptake [174]. The replication cycle is located to the cytoplasm [173]. PRV, like the mammalian and avian reoviruses, infect through an intestinal route [175]. Reported target cells for PRV in Atlantic salmon are erythrocytes, myocytes and macrophages [176–178]. The PRV infection cycle can be divided into three main phases, where each phase is described in details in the following paragraphs.

In the acute phase of infection, up to 50% of the red blood cells contain dark inclusions visible in a light microscope [177,179], and electron microscopy shows inclusions filled with virus progeny [177]. PRV is also released to high levels in plasma in this phase [168,177]. If virus release occurs through cell-lysis is unclear. Although hemolysis of blood cells have been observed in the spleen and head kidney [169,180], anemia is usually limited in PRV-1 infected Atlantic salmon [168,171]. In contrast, rainbow trout and Coho salmon develop anemia upon infection with PRV-2 and PRV-3 [166,167]. In erythrocytes, increased expression of ISGs is associated with decreased PRV protein production, while PRV RNA remains unaffected [181], indicating blocking of translation and virus release. The second phase of a PRV infection occurs from two to eight weeks after infection and the phase is characterized by heart inflammation and pathology [168,177,182,183]. The heart tissue seems visually unaffected by PRV infection until an influx of immune cells starts in the epicardium and continues into the compact layers [182–184], forming the characteristic pathology of HSMI. The heart can then regenerate, and in experimental studies this process is usually without mortality, but is a vulnerable phase for fish exposed to environmental stress [171]. In the *Onchorhynchus* species, rainbow trout, Coho, Sockeye and Chinook salmon, PRV infections have been associated with liver necrosis and jaundice syndrome/yellowish liver [166,169,185]. In PRV infected Chinook salmon, the liver and head kidney were more heavily infected by PRV, compared to Atlantic salmon [169]. The authors suggested that toxicity from excess hemolysis caused the liver injury.

2.4.3. Persistence

The persistent phase of PRV appears to be life-long in farmed salmon and have been linked to black spot formation in the salmon filet [178], an economically important quality problem for salmon producers. The prevalence of PRV in farmed Atlantic salmon reaches 90–100% in many countries [186,187], whereas wild Atlantic salmon have a prevalence of 10–20% [172]. It has been proposed, although not proven, that wild Atlantic salmon have the ability to completely eradicate a PRV infection as opposed to farmed salmon. It also appears from experimental infection studies that rainbow trout are capable of getting rid of an infection by PRV-3 [188]. Orthoreoviruses are also persistent in mammals, primarily in the intestine where they have been associated with inflammation and onset of celiac disease [189]. Assemblies of PRV-infected macrophages have been found in the muscle tissue, associated with red or melanized (black) spots [178], indicating that PRV infection of macrophages induce or worsen the formation of such spots. Macrophages may be where PRV primarily hide from the immune system during persistence.

2.4.4. Innate immunity

So far, not much is known about the initial mucosal immune response to PRV infection upon entering the host. It is shown that PRV translocates over the intestinal epithelial wall, but is sensitive to enzymatic degradation and/or low pH in the digestive system [175].

The erythrocytes have an important role in the initial dissemination phase of PRV [177,179]. Studies on the erythrocyte transcriptome response to PRV infection revealed upregulation of a wide range of innate antiviral effector genes, and points towards an essential immunological role of the red blood cells in the initial pathogen sensing and response [13,179,181], which is in line with findings of others [12,190]. The erythrocytes respond with an antiviral immune response characteristic for RNA viruses, and genes encoding proteins involved in direct sensing of dsRNA are upregulated in correlation with the level of virus, including TLRs 3 and 22, RIG-I, and dsRNA-activated protein kinase (PKR) [13]. For the related grass carp reovirus (GCRV), TLR19, TLR22, RIG-I and MDA5 have been reported to play roles during infection [191–193]. The type I IFN pathway is also strong in the erythrocytes. While the red blood cells isolated from PRV infected salmon produce IFNa1 for several weeks [13], the IFNa1-production in cultured erythrocytes lasts only for days [179]. Furthermore, the expression of the IRFs 1, 7 and 9 and mediators of the JAK-STAT signaling pathway genes are also induced in correlation with expanding PRV levels. Upon GCRV infection, IRF7 was shown to be phosphorylated and dimerize with IRF3, indicating that upregulated IRF7 may potentiate the response to PRV infection [193]. PKR, ISG15 and Viperin were all strongly induced in concert with levels of infection [13,181]. These ISGs are previously shown to control production of mammalian orthoreoviruses at the translational and posttranslational level [194,195]. When this blockage occurs, the first phase of virus progeny production ends [181]. The mammalian orthoreoviruses counteract the antiviral response by binding and inactivating IRF3 and thereby blocking IFN production and ISG expression [196]. The long lasting transcription of IFN-regulated genes in PRV-infected salmon does not indicate a blockage of IFN signaling. MRV is also reported to block host translation through interaction with PKR and inhibition of stress granule formation [197]. This is in line with the translational inhibition observed in PRV-infected erythrocytes. However, MRV is shown to bypass the host translational block and continue its own virus protein production [197], which does not seem to be the case for PRV.

Coho and Sockeye salmon mount a limited innate immune response to PRV-1 when compared to the Atlantic salmon [198,199]. Similarly, Atlantic salmon respond less to PRV-3 compared to rainbow trout [188], indicating that the antiviral response is stronger in the host that suffer the most severe pathological outcome. This implies PRV-mediated pathology could be coupled to its ability to induce antiviral immune responses in the host. According to transcriptome data, the innate immune response in erythrocytes continues through the second phase of infection [13]. This long-lasting innate immune response may explain the observed cross protection against unrelated secondary viral infections (SAV and IHNV) that last (for) many weeks after infection with PRV [200–202].

2.4.5. Adaptive immunity

According to detection of PRV RNA in different organs after infection, PRV accumulate in the spleen after the peak phase in blood [177]. Fish erythrocytes were recently reported to go into a shape-shifted phase where they express both MHCI and MHCII, indicating that they may present both intra- and extracellular antigens [203]. This shape-shifting may drive accumulation of infected erythrocytes in the spleen following PRV infection. However, macrophages may also engulf the PRV-infected erythrocytes (hemophagocytosis) and serve as PRV antigen presenting cells.

Using bead-based multiplex immunoassays, PRV specific IgM targeting both outer capsid proteins and intracellular virus factory proteins of PRV have been detected in plasma [204]. This specific humoral

response against PRV is induced at the end of the HSMI phase. Small differences in antibody kinetics are observed for the different PRV proteins [204].

The HSMI hearts primarily recruit CD8$^+$ T cells [183], but transcriptomic data indicate that also helper T cell, macrophage and B cell levels increase after disease onset [53,183]. The increase in CD8$^+$ cells is associated with elevated granzyme A mRNA levels, which indicates a cytotoxic T cell attack on PRV infected heart cells [53,183]. The immune cell recruitment parallels decreased virus levels in the heart, suggesting an immunological attack specifically directed against virus-infected myocytes [177,182].

After PRV infection, there is an increase in soluble and membrane-bound IgM transcripts in the head kidney, while CD8 and granzyme mRNAs are induced in the spleen [205]. This may represent proliferating B− and cytotoxic T-cells in head kidney and spleen, respectively. No obvious differences in cytokine/chemokine patters have been reported in spleen and head kidney, apart from an increased upregulation of the chemokine CCL19/MIP-3β in spleen [205]. CCL19 is a chemokine reported to attract dendritic cells, and T- and B cells that express the chemokine receptor CCR7 [19,206]. Presently, the role of CCL19 is not defined in salmonids.

The specific adaptive immune response elicited in PRV infected fish implies that effective vaccines can be developed, and recently a vaccine based on inactivated PRV particles was reported to protect from HSMI [207].

2.5. Piscine myocarditis virus (PMCV)

PMCV is associated with cardiomyopathy syndrome (CMS), a disease that kills large farmed Atlantic salmon in seawater. CMS is characterized by inflammation and necrotic damage in the atrium and ventricle, which can lead to by sudden death and heart rupture [208].

2.5.1. Subtypes and genome

PMCV is a simple, non-enveloped dsRNA virus belonging to the family of *Totiviridae* (Table 1). The PMCV dsRNA genome encodes three proteins, in contrast to most other *totiviruses* that encode only two [30]. A related virus that also encode a third protein, have been found in Golden shiner [209], and along with PMCV, these are the only variants shown to infect vertebrates. This third protein (ORF3) may therefore play a role in virus dissemination within higher organisms.

2.5.2. Tissue tropism and pathogenesis

The atrium is where pathology initially occurs [210]. The amount of PMCV in the heart is directly associated with the pathological changes, and the peak in PMCV-levels corresponds with the pathology both in time, and in the localization within the specific microscopic sites of necrosis and inflammation [30,210–212]. Existing data indicate that the heart is the primary target organ for PMCV replication, and accordingly, high virus levels are present in the heart at the peak of infection and disease [30,211]. PMCV origin and transmission pathways are not well understood [213].

2.5.3. Innate immunity

The immune response to PMCV have been explored using microarrays [211] (Table 1), and is characterized by a typical antiviral, IFN-mediated immune response in the first two weeks after infection, which dominate in blood cells, spleen and kidney. The antiviral response resembles that of PRV, and includes expression of dsRNA sensors like RIG-I and TLR3, and antiviral effectors like Mx and Viperin together with IFN signaling mediators like STAT1 [211]. However, in contrast to PRV, the response to PMCV levels off after a couple of weeks, whereas the virus levels keep increasing until pathology develop in the heart several weeks later. IgM transcripts was observed in the heart already at 4 wpc, which could potentially represent an “innate” B cell response. The innate-like B− and myeloid-cell expressed protein CD9, also named tetraspanin, was strongly induced in heart at 4 wpc, which coincided with a decreased antiviral response. CD9 is known to be involved in intracellular mobilization of viruses, including their mechanisms of entry and exit [214]. The levels of CD9 expression in this study corresponded with the increase in PMCV levels [211]. Likewise, when the virus levels plateaued from 6 wpc, CD9 transcript levels decreased as well. The putative role of CD9 in PMCV infection and dissemination warrants further exploration. In PMCV infected salmon elevated IFN responses was observed in the red blood cells and appeared to last longer in the blood compared to other tissues [211]. However, the red blood cells were not found to be infected by PMCV.

2.5.4. Adaptive immunity

The pathology of the heart is associated with a corresponding strong immune cell recruitment and upregulation of adaptive immune gene expression [211]. Immunoglobulin transcript levels in heart, indicating B cell recruitment, increased in line with the progression of CMS pathology. A microdissection study on the CMS lesions revealed higher levels of PMCV, MHCII-, B− and T-cells transcripts in the lesions compared to the surrounding unaffected tissue [212]. This indicates that the immune cell response in the heart targeting the virus occur at late time points, and include both cellular and humoral elements.

The complement components C1, C3, C5 and C8 were also expressed in the heart prior to the immune cell influx, and the authors suggested that complement mediated responses initiate inflammation and pathology [211]. A similar complement expression was observed in the spleen.

3. Towards better understanding of virus-host interactions and antiviral immunity?

3.1. Type I IFN responses and antiviral ISGs

Of the known piscine ISGs, not so many have been characterized in vitro or in vivo for their contribution to antiviral immune responses. For example, only a few of them have been tested for their ability to inhibit virus replication [215–217]. Clearly, more work needs to be done to uncover additional bony fish ISGs and their properties including their antiviral mechanisms. For viruses infecting mammalian hosts and in particular viruses infecting humans and mice, a variety of approaches have been employed to identify ISGs with antiviral activities and how their functions are directed against the virus [218–220]. A novel method for identifying large numbers of antiviral genes has been described based on virus infection of cells, which over-express fluorescent labelled ISGs their ability to inhibit virus replication [221]. Antiviral activity is measured by analysis of fluorescent-labelled viruses and ISGs by flow-cytometry. A similar approach could be used to identify genes with antiviral activity against fish viruses. For SAV and infectious hematopoietic necrosis virus (IHNV), GFP-expressing strains exist that can be directly used for this purpose [44,222]. In addition, in vitro studies using gene knockouts in mice have been useful to detect their phenotype [223]. However, deleting a single ISG from a large pool of IFN-induced genes carries an inherent risk of not detecting an antiviral phenotype due to the redundancy of the IFN system. Via the recent CRISPR/Cas9 technologies, B. Collet and collaborators have disrupted proteins taking part in IFN signaling to uncover their effects on ISG expression [224]. CRISPR/Cas9 or other gene knock-down strategies should be further implemented for studies on ISG functions in fish. As described for IPNV, type I IFNs and ISGs may affect virus persistence [138,225], additionally, they may have roles in virus pathogenesis [226]. Further insight into the effects IFNs and ISGs have on teleost fish could extend our understanding of how these molecules contributes to viral disease outcomes in vivo. Moving forward, new approaches to understand this broad functionality of the IFN system will be needed.

3.2. ISG levels do not necessarily correlate with protection

It is a paradox that the highest ISG levels in viral infected fish often are detected in parallel with the highest virus levels [51,53,146], which implicates that these effectors have not been able prevent virus replication and the establishment of the infection. Virus countermeasures, where the viruses hamper host ISG responses, might partly explain this. It is also possible that these transcripts are not efficient translated and as a result, their mRNA levels do not reflect the amount of protein produced. While ISG levels are frequently measured in the systemic immunological organs and/or target organs for the virus, the host-virus dynamics at virus entry points (i.e. gills and fins) are neglected. However, these initial meeting points might be key arenas to help limit the internal spread of viral infections through alerting IFNs and other cytokines. In addition, other constitutive or innate barriers that do not rely on new protein synthesis are of importance for the disease resistance. These topics warrant further investigations.

3.3. Interferon evasion strategies; the viruses take the stage

One of the first obstacles a virus meet when infecting a host is the IFN system and therefore successful viruses have evolved multiple strategies to antagonize the system [227]. Some of the effects mediated by fish viruses are summarized in Fig. 1, and as shown, there are studies demonstrating that salmonid viruses may inhibit the synthesis of IFNs by targeting the IRFs or block IFN activated signaling mediated by JAK-STAT signaling pathways. Since type I IFNs are produced directly as a response to dsRNA molecules that are sensed by PRRs of infected cells, many viruses produce dsRNA binding proteins to sequester these signals [227]. IPNV VP3 and PRVSigma 3 are examples of viral encoded dsRNA binding molecules [123,228], however, their direct effects on IFN induction remain to be clarified. In general, for most of the studies performed in fish, the molecular mechanisms that these viruses use to target host proteins are not examined in very much details and should be further investigated. A family of host proteins named suppressors of cytokine signaling (SOCS) have also been shown to be a target for viral exploitation [229]. The SOCSs, in particular SOCS1 and SOCS 3 that are ISGs themselves, negatively regulate JAK/STAT signaling. Atlantic salmon SOCS1 has been demonstrated to be a negative regulator of both type I and type II IFN signaling in salmonid cell-lines [230]. Both SAV3 and IPNV are able to modulate SOCS expression in TO cells, and SAV3, especially, induce an early robust SOCS1 expression, which may represent a putative strategy to promote viral survival [231].

3.4. In fish, red blood cells are also immune cells

The role of salmonid red blood cells in the immune response to viral infections is only in its first stage of being explored. Nevertheless, several immune functions have been assigned to red blood cells; they express nucleic acid sensing PRRs [12], components of the IFN signaling system and a large array of ISGs [12,13]. They are themselves primary target cells for PRV infection and replication [13,177,188], and can take up ISAV by endocytosis [232]. Red blood cells also respond to viruses when they are not infected, including PMCV in Atlantic salmon [211], and IPNV and viral hemorrhagic septicaemia virus (VHSV) in trout [203,233]. Expression of MHCI and −II on red blood cells, indicate their ability to present antigen [190]. The red blood cells have been shown to differentiate and change shape when responding to infections and heat stress [179,203], and produce pro-inflammatory cytokines like IL-1β, IL-8 and IFNγ in response to IHNV [203]. Surprisingly, red blood cells significantly induce the expression of IgM in this differentiated state [203]. These findings indicate that the red blood cells in fish have the potential to acquire features that resemble both monocytes and B cells. Typical red blood cell characteristics, like the level of haemoglobin, are reduced in this state [203], and in PRV infected Atlantic salmon the haemoglobin loss is associated with increased sensitivity to hypoxic stress [171]. The role salmonid red blood cell differentiation play in a virus-infected fish is a fascinating new research field.

3.5. Humoral and cellular immunity to salmonid viruses – how do they take the stage?

Secreted IgM specific for viral proteins along with virus-neutralizing activities in serum have been shown to develop in salmon after infections with IPNV, SAV, PRV and ISAV [71,204,234,235]. In line with this, vaccine effects are strongly associated with specific antibodies for SAV and ISAV [109,111,236,237], while for IPNV a clear correlation between virus clearance and the levels of antibodies is questionable [164]. Virus outer capsid proteins represent the most relevant targets for neutralizing and protective effects. SAV E2 [237,238], ISAV HE [239] and IPNV VP2 [160], are considered the primary neutralizing and protective antibody targets. For PRV and PMCV, neutralization assays have not been established due to the lack of susceptible cell cultures to test the effects on. However, multiplex bead-based immunoassays have been used to identify PRVμ1 as an outer capsid target for specific IgM [204]. The bead-based immunoassays also revealed that the non-structural PRV protein μNS, only expressed in virus replicating cell, is an additional antibody target [204]. Likewise, humans infected by the ssRNA-viruses Dengue virus and Zika virus produce antibodies targeting the nonstructural protein NS1 [240], which was identified a vaccine component that promoted protection against Dengue fever [241]. Further exploration of non-structural virus proteins as antibody targets could be of great value for fish vaccine design.

Complement proteins are likely to be involved in the IgM-mediated immunity in fish, and a coordinated expression of IgM and complement factors have been shown for SAV and PMCV infection [211,242]. The role and importance of complement in antibody-mediated protection in fish should be better established, clarifying if complement-mediated cytotoxicity and cell lysis directed by IgM are central mechanisms.

Long-lived plasma-cells in head kidney are proposed to be responsible for the specific antibody production after infection [243]. Furthermore, enrichment of certain clonotypes of both IgM and IgT have been shown to develop in spleen from rainbow trout after infection with VHSV, indicating general proliferative B cell responses in spleen in the early phase [16]. Activation-induced cytidine deaminase, an enzyme linked to Ig hypermutation, was found in head kidney melanomacrophage centers of channel catfish, indicating that antibody specificity may develop there [244]. In addition, the B cells in teleost possess properties of innate-like B-1 cells like phagocytosis and CD5 expression [18,21–23,245]. In salmonids, B cells express a range of TLRs [19,20], and respond to the TLR-ligand CpG by differentiating into antibody-secreting cells, up-regulate co-stimulatory molecules and IFNa1 transcripts [19]. Whether all B cells have remained both typical innate and adaptive functions, or the properties belong to separate subpopulations, need to be determined. A tissue-specific B cell response, primarily linked to expression of soluble and membrane bound IgM, are demonstrated in hearts infected with SAV, PRV and PCMV [52,53,211], even at early time points post infection. Interesting questions that arise are how these local B cell are activated and recruited and how they mediate their protective responses. Activation of the B cells directly by viral PAMPs or early innate signals including type I IFNs and other inflammatory cytokines may modulate B cell responses, both by acting directly on the B cells or indirectly via signaling to DC and other cells. Future work should aim at further characterize how the innate-like and adaptive qualities of teleost B cells play together in mediating protection against viruses. T cell responses, their functions and how they co-ordinate their activities with other leukocytes during viral infections are not completely understood in teleosts. As described in earlier sections, transcriptome analyses indicate that $CD4^+$ and $CD8^+$ T cells together with some of their signature cytokines and effector molecules are evoked by the viruses covered here

[53,98,211,246]. Taken together, the data indicate that T cells multiply primarily in the spleen and in the target organs for the virus. For SAV, PRV and PMCV infections, $CD8^+$ T-cells and cytotoxicity genes increase in association with heart inflammation and pathology [52,53,183,211]. This also leads to a reduction in virus levels in heart, which suggest that cytotoxic T cells responses are implicated in protection. Laser-capture microdissection of CMS heart lesions led to findings indicating a direct co-localization between pathological sites, virus-infected cells and cytotoxic T cells, which points to very specific interactions between target cells and T cells that result in cell death [212]. This is in line with other studies that identify cytotoxic T cells in teleost fish as mediators of adaptive immunity [26]. The dual role of T cell cytotoxicity observed in heart infections with SAV, PRV and PMCV where $CD8^+$ T cells are associated with antiviral-, but at the same time pathological effects, needs further study.

Even though progress has been made in recent years regarding the description of bony fish T cells, most of these findings are inferred on the basis of gene expression. For rainbow trout, mAbs against different CD4-1, CD4-2, and CD8a and CD8b are available, and have been used to identify different T cell subtypes [25,247]. It is noteworthy that CD4 and CD8 molecules are not only expressed on T cells, but also on other cell types, e.g. CD4-2 is also expressed in trout monocyte-like cells [247]. Therefore, multiple markers should be used for true identification of T cells. Although signature cytokines and transcriptions factors for different Th subsets are identified in salmonids, their functions are modestly explored. The salmon Th2 cytokines IL-4 and IL-13 were found to increase the number of IgM secreting B cells in vivo [27].

For further development in this field, development of new specific markers and tools is a requisite to pave the way for identifying which T cell subsets that contribute to cellular antiviral immunity in fish. Also identification and separation of lymphoid innate T cells and NK cells are essential to understand the major cell types involved in antiviral cell-mediated immunity.

3.6. Persistent virus infections - a game of hide and seek

Viruses have developed many different strategies to survive and to propagate in a susceptible host. One of them is to remain in a latent phase in the cell with very slow replication and with very little or no virus release, which represents the characteristics of persisting viruses [248]. Understanding the virus-host interplay that leads to a persistent infection requires detailed knowledge of their collective rendezvous. For three of the viruses described in this review, namely IPNV, PRV and SAV, there are reports suggesting that they may persist in their host [47,249], even throughout their lifespan [130,178].

As described in an earlier section, IPNV multiply in head kidney adherent leucocytes, most likely macrophages [133]. Johansen et al. [133] demonstrated that about 1% of the total head kidney leukocytes were infected by IPNV, while higher numbers were reported by Munro et al. [250]. PRV infections in Atlantic salmon also develop into lifelong persistence, and macrophage-like cells are proposed to be the main cell type for this persistence [178]. In contrast to the PRV-1 persistence in farmed Atlantic salmon, the PRV-3 variant in rainbow trout appears to be cleared from the hosts, at least in experimental studies [188]. For the related mammalian orthoreovirus, persistence have been linked to mutations in outer capsid proteins of the virion, allowing the virus to bypass steps in the uncoating process needed for cytoplasmic transfer upon infection [251]. However, whereas MRV is sensitive to IFN type I, is PRV produced at high levels despite IFN signaling [13].

In mammals, therapeutic vaccines against persisting viral infections have been developed with varying success [252]. Muang'andu et al. [253] reported that vaccine generated antibodies were not sufficient to clear persistent IPNV infections and that vaccination did not prevent the fish from becoming viral carriers. Many viruses possess strategies for cytokine evasion to facilitate persistence by interfering with normal cytokine signaling and leucocyte trafficking [248]. In mammals, a suite of cytokines are targets for viral subversion including type I IFNs and IL-10 [157]. As discussed in an earlier section, these cytokines are suggested to be important for the establishment and maintenance of IPNV persistence [149]. In line with this, Saint-Jean et al. [225] found that IFN responses might be involved in retaining IPNV persistence. Similarly, the IL-10 receptor was upregulated in PRV infected erythrocytes along with SOCS1 [13].

Programmed cell death (apoptosis), is a host mechanism to get rid of virus-infected cells. On contact with cytotoxic T cells and NK cells, cells can be killed by granzyme granules, but also by TNF and Fas ligands. These effectors engage proteolytic caspase cascades that leads to apoptotic cell death. Viruses may encode both pro- or anti-apoptotic proteins, and blocking the apoptotic system may represent a positive strategy for virus survival. For IPNV, virus induced apoptosis in infected cell-lines [254,255] and infected fish [254] has been reported. IPNV VP5 shares homology with members of the anti-apoptosis Bcl-2 family [256], which functions is to block caspase activity and enhance viral progeny production. When overexpressed in cell lines VP5 show an anti-apoptotic potential [256]. In contradiction to this, Santi et al. [254] by the use of recombinant IPNV Sp strains demonstrated that the VP5 protein had little influence on apoptosis both in vitro and in vivo. The study concluded that VP5 was not essential for the establishment of a persistent IPNV infection. However, it should be noted that in tissues of IPNV-infected fish there is only a small number of cells that are apoptotic [156]. Interestingly, in IPNV-positive peripheral blood, head kidney and spleen leukocytes from infected rainbow trout no apoptotic nuclei were found [257]. Assuming that IPNV-positive macrophages are present in these cells, a possible explanation for the lack of apoptotic morphology in these cells is that differentiated macrophages, as long-lived cells, are resistant to various death stimuli. It is also possible that persistent infections of macrophages are accompanied with specific inhibition of host-induced apoptosis, which may delay cell death and facilitate viral persistence in these cells.

Both in field trials [249,258] and in experimental SAV challenge experiments [47] the clearance of the virus is not absolute, suggesting that surviving fish may become life-long asymptomatic carriers. In the field trial, viral RNA were detected over a period of nine months and the highest levels, at the latest time points, were found in gill, followed by heart and pancreas [249]. However, attempts of virus isolation from tissue pools in support of carriers were unsuccessful beyond day 71. To our best knowledge, there are no reports of productive in vitro infections of subsets of salmonid leukocytes with SAV.

While progress has been made, there are still remaining questions surrounding viral persistence in fish. For IPNV and PRV, macrophage-like cells are suggested as the main reservoir for persistent virus [133,178]. Macrophages are professional phagocytes specialized in pathogen elimination and antigen presentation, functions the virus may have to suppress to be able to persist. By modulating cytokine secretion, reducing antigen presentation and interfering with cell-death, viruses may functionally impair immune cells for their own benefit.

3.7. Preventing viral disease – success so far?

A range of vaccination strategies have been tested against the viral diseases covered here [69,110,207,259–264], and vaccines targeting IPNV, ISAV and SAV have reached the market. Even if several of the vaccination trials performed have been reported as successful, the vaccines have not given complete protection against viral disease in the field. There could be several reasons for this, like suboptimal antigen doses or adjuvant effects, immunosuppressed hosts, the inability to induce mucosal protection, and/or lack of T-cell mediated responses to the vaccine. New strategies like the IFN adjuvanted DNA vaccines tested against ISA [109,265], or the oral vaccines with viral antigen carried by commensal bacteria tested against IPN [266], could provide more optimal solutions in the future. Another approach to prevent viral diseases in aquaculture is breeding for resistance, which have

successfully reduced IPN in Atlantic salmon [101]. Gene editing will most likely put this strategy on fast forward. However, there might be risks related to a rush to put moderately protective vaccines and partly resistant genetic families to the market. For example, if the virus still has the ability to infect and replicate in the fish, there is a possibility that the virus may adapt, circumvent the resistance, and revert to virulence. An additional approach is to counteract immune suppression and optimize antiviral immune activity using immune-stimulating and immune-optimizing feed [267–269].

4. Concluding remarks

Studies of important virus infections in salmonid aquaculture reveal the need to expand the understanding of the timing and tissue localization of immune activation, the role of viral persistence mechanisms, how erythrocytes contribute to innate immunity, and the requirements for T cell based long-term protection. However, studies on fish immune cell populations and their activity are still hampered by the shortage in available cell population marker antibodies and other immunological tools. A joint effort to develop the critical tools will help advance the understanding of the dynamics of fish antiviral responses.

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