Faculty of Biosciences, Fisheries and Economics

Norwegian College of Fishery Science

Atlantic salmon type I interferons: Protection against virus infection *in vivo* and function as adjuvants in a virus DNA vaccine.

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Chia Jung Chang (Sami)
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Contents

Acknowledgements

Abbreviations

List of Papers

Abbreviations

APC Antigen presenting cells

ADCC Antibody-dependent cellular cytotoxicity

CDCC Antibody-directed complement-dependent cell lysis

CHSE-214 Chinook salmon embryo cells

CISH Cytokine-inducible SH2- containing protein

CpG Cytosine-phosphate-guanine

CMS Cardiomyopathy syndrome disease

cGAS Cytosolic GAMP synthase

CRFB Cytokine receptor family member

DBD DNA-binding domain

DCs Dendritic cells

DNA Deoxyribonucleic acid

DAI DNA-dependent activator of ifn-regulatory factors

HK Head kidney

HSMI Heart and skeletal muscle inflammation

HA Haemagglutinin

HEF Hamagglutinin-esterase-fusion

ISG15 IFN-stimulated protein of 15 kDa

IFN Interferon

IFN-I Type I interferon

IFNAR Interferon α/β receptor

IFNGR Interferon γ receptor

IPNV Infectious pancreatic necrosis virus

IHNV infectious hematopoietic necrosis virus

IRF Interferon regulatory factor

ISAV Infectious salmon anemia virus

ISG Interferon stimulated gene

ISGF3 Interferon stimulated gene factor 3

ISRE Interferon stimulated response element

IFIT IFN-induced proteins with tetratricopeptide repeats

IFITM IFN-induced transmembrane proteins

JAK Janus kinase

LGP2 Laboratory of genetics and physiology 2

mRNA messenger RNA

MHC Major histocompatibility complex

MDA5 Melanoma differentiation-associated gene

Mx Myxovirus resistance

MyD88 Myeloid differentiation primary response gene 88

PD Pancreas disease

PAMP Pathogen-associated molecular pattern

PCR Polymerase chain reaction

PKR Protein kinase R

pDC Plasmacytoid dendrittic cell

Poly I:C Polyinosinic polycytidylic acid

PRRs Pattern recognition receptors

qPCR quantitative PCR

PTP protein tyrosine phosphatases

RIG-I Retinoic acid inducible gene I

RLR RIG-I- like receptors

RNA Ribonucleic acid

PMCV Piscine myocarditis virus

SAV Salmonid alphavirus

ssRNA Single-stranded RNA

STAT Signal transducers and activators of transcription

TRIF TIR-domain-containing adapter-inducing interferon

TLR Toll-like receptor

TO Atlantic salmon head kidney cell-lin

Tyk2 Tyrosine kinase2

Viperin virus inhibitory protein, endoplasmic reticulum-associated, interferon-inducible

Paper I-IV

List of papers

Paper I

Protection of Atlantic salmon against virus infection by intramuscular injection of IFNc expression plasmid. Chia-Jung Chang, Camilla Robertsen, Baojian Sun, Børre Robertsen. Vaccine. Volume 32, Issue 36, 6 August 2014, Pages 4695–4702.

Paper II

Protection of Atlantic salmon against salmonid alphavirus infection by type I interferons IFNa, IFNb and IFNc. Chia-Jung Chang, Iris Jenssen, Børre Robertsen. Submitted on May 2016.

Paper III

Adjuvant activity of fish type I interferon shown in a virus DNA vaccination model. Chia-Jung Chang, Baojian Sun, Børre Robertsen. Vaccine. Volume 33, Issue 21, 15 May 2015, Pages 2442–2448.

Paper IV

IFN-adjuvanted DNA vaccine against infectious salmon anemia virus: Antibody kinetics and longevity of IFN expression. Børre Robertsen, Chia-Jung Chang and Lisa Bratland. Fish Shellfish Immunol. Volume 54, July 2016, Pages 328–332.

1. Introduction

Fish farming have been considered the importance resource for supplying food for global requirement (1). Norway by far is the country that produce the most salmon in the world, and virus disease is one of the major problems that causes the economic loss in fish farming. For combating the diseases, vaccines have been developed.

In Norway, traditional vaccines based on inactivated virus are available against IPN, PD and ISA, but do not appear to give adequate protection. IPN and vaccine based on recombinant VP2 is the only commercial available subunit vaccine in Norway that provide high and long-lasting protection (2). There is thus a clear need to develop effective vaccines against these and other virus caused diseases of Atlantic salmon. To develop improved vaccines and other prophylactic methods, it is important to increase the understanding of the interaction of viruses with the immune system of Atlantic salmon.

This doctoral thesis encompasses studies include the function of salmon type I interferon (IFN-I) in vivo, the use of IFN-I in vaccine study. How IFN-I triggering both innate and adaptive immune response gives protection against virus infection.

In the introduction, I will first give a brief over view of the virus disease from salmon farming in Norway and then introduce two important viruses ISAV and SAV3 that were used in this thesis. IFN-I activated in innate and adaptive immune system are separated and described in two major sections. In the sub-sections, antiviral response and proteins expression triggered by IFN-I are reviewed and described. The effect of IFN-I in immune cells and the use of IFN-I as vaccine adjuvant have also been discussed. In last few sections I will focus on how IFN-I contributed as vaccine adjuvants and the effect of IFN-I in immune cells.

1.1 Virus diseases in Atlantic salmon farming and the demand for effective virus vaccines

Atlantic salmon is an important aquaculture species globally. Farmed salmon is, however, attacked by several viruses, which represent a continuous threat to the aquaculture industry and cause large economic losses.

Infectious salmon anemia (ISA) is caused by ISA virus (ISAV), which belong to the *Orthomyxoviridae* family (3). Fish infected by this disease show anaemia in blood system and the virulence varies depends on the strain of infection. Outbreaks of ISA have declined from 2008 in 17 cases to 1 cases in 2011. However, the numbers of outbreaks were increased from 2012 in 2 cases to 15 cases in 2015. Most outbreaks have occurred in specific areas in Northern Norway in recent years and have start with low mortality. ISA has been combatted by separation of generations and stamping out of fish in affected areas (4).

Pancreas disease (PD) is caused by salmonid alphavirus (SAV) which belong to *Togaviridae* family (3). Fish infected by this disease show reduced food consumption and growth. PD currently is the most important virus disease in salmon farming in Norway with 137 new cases registered in 2015. SAV subtype 3 (SAV3) causes the epidemic western Norway that usually cause low to moderate mortality while SAV2 causes the epidemic in mid Norway that often cause low mortality (4). This leads to serious economic losses in salmon farming industry (4).

Infectious pancreatic necrosis (IPN) is caused by IPNV which belong to *Birnaviridae* family(3). IPNV can infect a wide range of fish species in the worldwide and cause significant problems in Atlantic salmon farming. IPNV-infected salmon post-smolts show reduced food consumption and increased mortality. The outbreaks of IPN cases in salmon have shown a reduction in cases from 223 cases in 2009 to 30 cases in 2015. The selection of fish that genetic associated to infectious disease resistance using DNA marker identification have been developed, using IPNV resistance Atlantic salmon in the farming was considered the reason for the reduction of outbreaks (4, 5).

The increased cardiomyopathy syndrome disease (CMS) caused by PMCV which belong to *Totiviridae* family (3) and the Piscine myocarditis virus was first described in 2010 and considered as the main agent causing cardiomyopathy syndrome disease. Fish infected with

this virus usually develop circulatory disorder and large inflammatory changes at heart. The CMS disease mainly occurs to a large fish before harvesting, thus resulting in a substantial economic impact (4). Heart and skeletal muscle inflammation (HSMI) caused by PRV which belong to *Reoviridae* family (3). Increased numbers of HSMI cases have been recorded in past few years, 135 new cases outbreaks were register in Norway in 2015 and mortality is highly associated with other factors such as stress caused by transportation, routine management or the control of sea lice. Piscine orthoreovirus (PRV) was first identified from HSMI inflicted tissues and considered to be significantly associated with this disease that causes clinical sign of circulatory disturbance. Heart is the organ that shows the most changes after virus infection, and inflammation with pathological changes are usually displayed in the skeletal muscle, and may also discover in liver and other tissues as well.

Infectious hematopoietic necrosis disease (IHN) caused by IHNV (Rhabdoviridae) (3). IHNV is commonly found in salmonid species in Canada and the USA, and causes a significant economic problems but has never been detected in Norway. IHNV caused a serious epidemic in Atlantic salmon farming in British Columbia in 2001-2003 (6), which has been defeated by the use of a DNA vaccine (7).
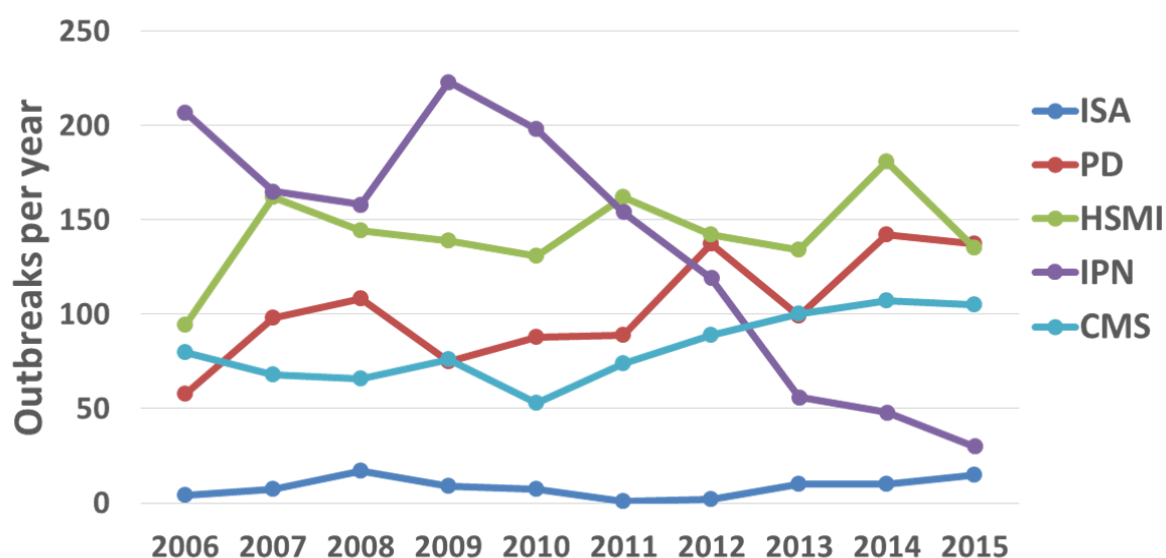
Figure 1. The outbreaks of ISA, PD, HSMI, IPN and CMS from 2006 to 2015 in Norwegian salmonid farming (4).

1.2 Characteristics of ISAV and SAV3

Infectious salmon anaemia virus (ISAV)

Infectious salmon anaemia virus (ISAV) is the only aquatic orthomyxovirus described and belongs to the genus of *Isavirus* (8). This enveloped virus consists of eight negative sense single–stranded RNA (ssRNA) segments (9), which encode at least ten proteins (10, 11) as shown in Fig. 2. The virus segment 1 encodes viral RNA polymerase (PB2) (12), segment 2 encodes RNA-dependent RNA polymerase core motif (PB1) (13), segment 3 encodes nucleoprotein (NP) (12, 14, 15), segment 4 encodes RNA polymerase (PA) (15), segment 5 encodes fusion protein (F) (10, 16), and segment 6 encodes the haemagglutinin-esterase (HE), which is a glycoprotein containing both receptor-binding and receptor-destroying enzyme domains (17-20). Segment 7 encodes two small ORF (ORF1, ORF2); s7 ORF1 has IFN-I antagonist activity and s7 ORF2 appear to be a nuclear export protein (NEP) (21-24). Like s7, segment 8 encodes two ORFs; ORF1 encodes surface protein Matrix (M) while ORF2 has IFN-I antagonist activity that modulates s7 ORF1 (21, 25). Recently, s7ORF1 and s8ORF2 have been shown to have IFN-I antagonist activities whereby they inhibit induction of IFNa mediated by interferon regulatory factors (IRFs) (26).
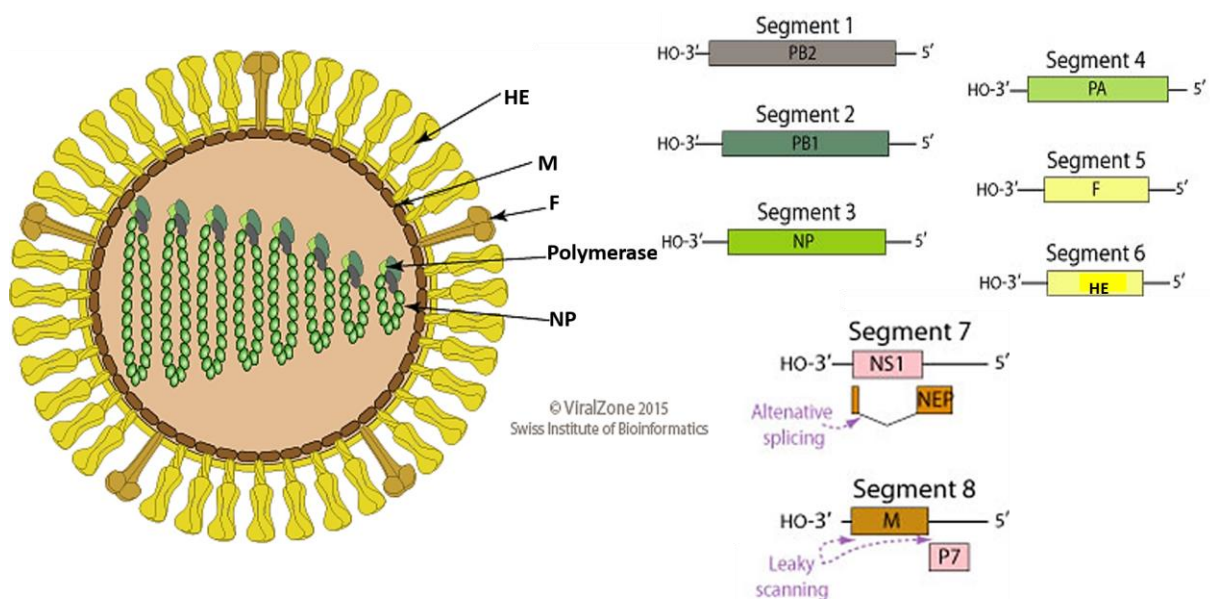
Figure 2. ISAV and Genome derived from http://viralzone.expasy.org/all_by_species/95.html

Source: ViralZone:www.expasy.org/viralzone,SIB Swiss Institute of Bioinformatics and used under a Creative Commons Attribution-Non Commercial 4.0 International License. (adapted and simplified by Chia Jung Chang)

Two major groups of ISAV have been described, one from Europe and one from North America (27). The genotype of European ISAV is spread more widely and shows greater variation in genetic and virulence than North American genotype (28). However, the whole genome in ISAV is considerably conserved. The region with the highest variation called the high polymorphism region (HPR) was found near the transmembrane domain of HE protein (29, 30). The full length segment 6 of ISAV HPR0 was suggested to be the precursor for other HPR-deleted ISAV (ISAV-HPRΔ) strain. However, due to lack of direct evidence, the association between HPR0 and other virulent ISAV remains unclear (29, 31, 32). In addition to the deletion of HPR region in segment 6, the insertion of a sequence in the Fusion protein of segment 5 has been considered necessary in determining the pathogenicity of the virus (33, 34).

Gills was considered as a major entry port for ISAV (35). The infection of ISAV through other entry ports of the mucosal systems like eye, pectoral fin, skin and GI tract have also been identified (36). ISAV initiatially infects the epithelial cells at mucosal barriers and later infection of endothelial cells occurs systemically following the circulatory infection (36, 37). ISA symptoms include severe anemia, hemophagocytosis, ascites, petechial and hemorrhagic necrosis in the liver and congestion in multiple organs (38-41). The function of Haemagglutinin (HA) is important for host specificity and tissue tropism for influenza virus. Haemagglutinin-esterase (HE) from ISAV consist of receptor-binding and receptor-destroying enzyme (RDE) activities (17, 42, 43), which is similar to the hemagglutinin-esterase-fusion (HEF) protein from influenza C virus that has functions in binding to the host cell receptor glycoprotein, virus and cell membrane fusion and receptor destruction (44-47). The binding of ISAV HE and cell receptor has also been identified as important for the infection in Atlantic salmon (42, 43). In addition, the surface HA protein from influenza A virus or the HEF protein from influenza C virus are commonly use for vaccine study (48), Surface protein HE and F protein from ISAV have also been studied that shows some protection in fish against ISAV infection with HE encoded DNA vaccine, or yeast expressed HE and F protein embodied oral vaccine (49, 50).

Salmonid alphavirus (SAV)

Salmonid alphavirus (SAV) is an enveloped virus with a genome consisting of one 12 kb positive-sense ssRNA molecule (51, 52). The genome has two ORF; one which encodes five structural glycoproteins included E1, 6K, E2, E3 with capsid protein, and one ORF, which encodes the four non-structural proteins nsp1-nsp4 as shown in Fig. 3 (53). Like alphavirus from other species, glycoprotein E2 from SAV is suggested to interact with cellular receptors and seems to contain a virus neutralizing site for antibodies (53). The relatively conserved E1 protein is required for trafficking and cell surface expression of E2 protein (54).
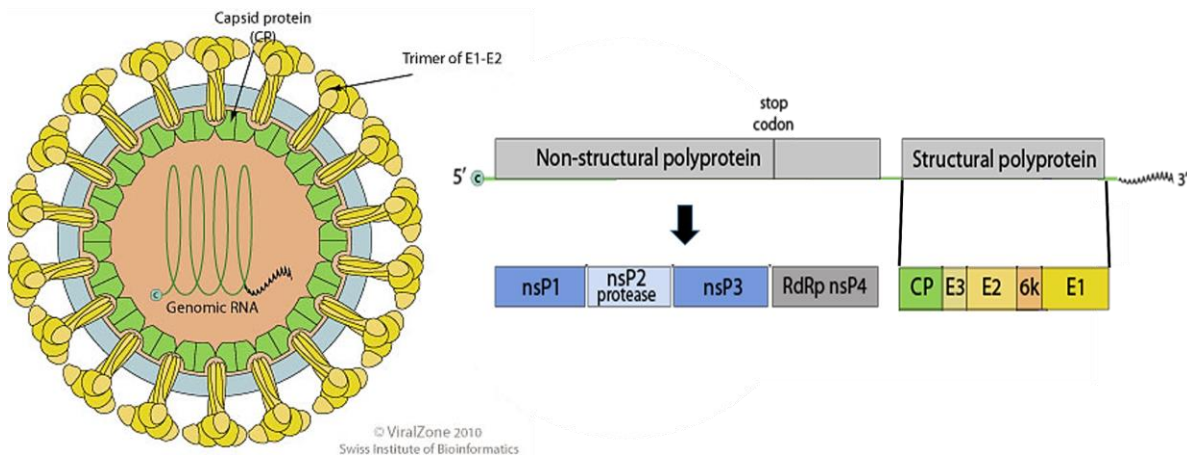
Figure 3. Alphavirus and Genome derived from http://viralzone.expasy.org/all_by_species/625.html
Source: ViralZone:www.expasy.org/viralzone,SIB Swiss Institute of Bioinformatics and used under a Creative Commons Attribution-Non Commercial 4.0 International License. (adapted and simplified by Chia Jung Chang)

Phylogenetic analysis based on sequence analysis of E2 and nsp3 has led to the identification of six subtypes of SAV (SAV1-SAV6) (55, 56). SAV3 was first identified at 2005 in Norway and share 91.6% and 92.9% similarity in nucleotide sequence compared to SAV1 and SAV2, respectively (57). Different geographic locations of the PD epidemics are associated with different strains of SAV. SAV1 is the main problem in Ireland and Scotland while SAV3 is the cause of the PD epidemic in western Norway and a marine SAV2 is causing the epidemic in mid-Norway (58).

Histology of SAV infected fish shows severe degeneration, necrosis and inflammation in pancreas, heart and skeletal muscle (59-62). The virus can be detected in brain, gill,

pseudobranch, heart, pancreas, kidney, skeletal muscle and also in mucus and faeces of infected fish. At early virus infection, the histopathological changes appears only as focal necrosis in exocrine pancreatic tissues and variable inflammation in peripancreatic fat tissue. Myocyte degeneration and focal necrosis were developed at the same stage. Soon after, the infection causes multifocal and severe necrosis with total loss of exocrine pancreas and appearance of myocardiocyte necrosis and increased inflammation. Ordinarily, the development of skeletal muscle degeneration, inflammation and fibrosis appear 3-4 weeks after virus infection (53, 63). Organs and tissues like gill, heart and mid-kidney are commonly used for analysis of samples from the disease outbreak while serum is used for detecting the virus with Real-time PCR in early infection at the initiatal phase of viraemia (64).

1.3   The importance of type IFNs in innate and adaptive immunity against virus infection

The immune response against virus infection consists of both innate and adaptive immune defense mechanisms. The innate immunity against virus infection is activated when virus related particles are recognized by receptors, this trigger immediate and short defense system. Type I interferon (IFN-I) is one of the major cytokines and is secreted from virus infected cells. The secreted IFN enters the blood stream and activates antiviral signaling pathway (JAK/STAT signaling pathway) in other cells. If an early antiviral immune response is not able to eliminate virus replication, then the second line of defense, the adaptive immune response, is activated. Activity of adaptive immunity include activation of many adaptive immune related cell types and secretion of antibody (Ab) against the virus.

Ordinary, the engagement of innate and adaptive immunity require communication from various immune cells and cytokines. The immune cells such as dendritic cells (DCs), B and T lymphocytes are important in this process. Upon infection, virus components such as RNA or DNA are recognized by pattern recognition receptor (PRRs) from cells. Activated Dendritic cells after infection secrete IFN-I and different cytokines which regulate lymphocytes via MHC molecules and co-stimulators. B and T lymphocytes are then activated and turn into effector cells such as antibody producing cells or cytotoxic cells. Modulation of IFN-I in DCs, B and T lymphocytes will be further discuss in later sections.

Interferons

Interferons (IFNs) are cytokines that interfere with virus infection by inducing an anti-viral state in cells. Three families of IFNs (type I, type II and type III) can be distinguished by sequence, gene structure and synteny, and receptor specificity (65). Type I IFN (IFN-I) and type III IFN (IFN-III) play a pivotal role in innate immune responses against virus infections, whereby host cells produce and secrete IFNs upon recognition of viral nucleic acids. These IFNs protect other cells from further viral infection by binding to cell surface receptors, which result with induction of hundreds of IFN stimulated genes (ISGs) (66-69) . The ISGs, several of which encode antiviral proteins such as Mx, ISG15, and viperin. Importantly, IFN-I also stimulates adaptive immune responses, which is described in later sections. Mammals possess multiple subtypes of type I interferon which bind to the same heterodimeric receptor composed of the polypeptide chains IFNAR1 and IFNAR2. Receptors for type I interferon are present in most cell types (70, 71). IFN-II is IFN-γ and is produced by natural killer cells (NK cells) and T lymphocytes and binds to the receptor composed of IFNGR1 and IFNGR2, which is present in a broad range of cell types (72). IFN-γ is important in T-cell mediated adaptive immunity and its function is different from IFN-I and IFN-III. All three IFN types have identified in mammals, birds, and amphibians, while only IFN-I and IFN-II have been found in fish. The thesis thus focuses on the function of type I interferon.

The following sections will first describe IFN-I triggered innate immune system and the function of antiviral proteins mainly in mammals but also in fish. Second, the thesis will describe the role of adaptive immunity in combating viruses and the role of type I interferon in connecting both innate and adaptive immune systems. Third, the thesis will describe the use of type I interferon in vaccine study and derived outcomes.

1.4 **Type I IFNs in mammals and fish**

In mammals, IFN-I is a multigene family with at least 9 subclasses including IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω, which has been found in most species that have been studied. Besides, IFNδ, IFN-ζ, IFN-τ and IFN-ω are more distinct than others and have been found in pig, mice, cattle and horse (73). IFN-I are encoded by intron-less genes and bind to the same

heterodimeric receptor containing the IFNAR-1 and IFNAR-2 subunits in mammals. Fish type I IFNs were first discovered in 2003 and have been cloned from several different fish species, among those zebrafish, pufferfish, channel catfish and Atlantic salmon. Unlike mammalian IFN-I, fish IFN-I genes are interrupted by introns and signal through at least two heterodimeric receptors (74-76). The largest IFN-I family has been found in salmonids and comprise at least six subtypes including IFNa, IFNb, IFNc, IFNd, IFNe and IFNf, several of which are encoded by multiple genes (77). IFN-I differ in the number of disulfide bridges formed by cysteine residues. In mammals, IFNα possesses four cysteines while two cysteines were present in IFNß. These cysteines residues have been found to be essential for protein conformation and biological activity (78-81). Likewise, conserved region of cysteines residues have been found in fish and have been used for classifying fish IFN-I into two groups. Group I IFN-I contain two cysteine residues with one disulfide bridge while group II IFN-I contain four cysteine residues with two disulfide bridge as shown in Table 1 (77, 82-84). However, a recent IFN-I found in turbot reveals that the group system based on cysteines residues may not suitable for all the fish species (85).

Table I : Classification of type I interferon in teleost

Species	Group I IFN-I (2 cysteines)	Group II IFN-I (4 cysteines)
Atlantic salmon (*Salmo salar*)	IFNa, IFNd	IFNb, IFNc
Rainbow trout (*Oncorhynchus mykiss*)	IFNa, IFNd, IFNe	IFNb, IFNc, IFNf
Zebrafish (*Danio rerio*)	IFN1, IFN4	IFN2, IFN3
Species that IFN-I found not belong to any groups		
Turbot (*Scophthalmus maximus*), IFN2 **(5 cysteins),** IFN1 **(6 cysteins)**		

The table was made mainly according to a previous phylogenetic tree analysis (77) and IFN-I from Turbot (86).

In salmonids, IFNa and IFNd contain two cysteines, IFNb and IFNc contains four cysteines and form one and two disulfide bridges respectively (87, 88). Antiviral activity has so far only been proved for the IFNa, IFNb and IFNc subtypes (89). Therefore, our work focuses on these three salmon IFN-I subtypes, (IFNa, IFNb and IFNc) especially the antiviral immune response in salmon pre-smolt.

1.5 Induction of IFNs with virus infection

The central role of IFN-I is induction of an antiviral state in cells through interaction of IFN with interferon receptors, resulting in induction of IFN-stimulated genes (ISGs) some of which inhibit virus replication (90, 91). In order to combat virus infection, virus must be detected through several virus sensors in the cell. The sensors for virus RNA includes RIG-I Like receptors (RLRs), Toll like receptors (TLRs) and virus DNA-receptors as described below.

RIG-I like receptors (RLR) and Toll-like receptors (TLR) for RNA viruses

In mammals, receptor responsible for virus-RNA recognition is localized in the cytoplasm and belongs to the RIG-I-like family (RLR) (92). These cytosolic receptors; RNA helicases retinoic acid-inducible gene I (RIG-I), Melanoma differentiation-associated gene (MDA5) identify and bind to viral RNA (93). Signaling through the adaptor protein IPS-1 (also named MAVS) leads to activation of Interferon Regulatory Factor 3 (IRF3), Interferon Regulatory Factor 7 (IRF7) and Nuclear factor-κB (NF-κB) and transcription of IFN-I genes (94). Laboratory of genetics and physiology 2 (LGP2) is another cytosolic receptor and was found play a role as a downstream regulator for RIG-I and MDA5 (93). In some cell types, virus dsRNA is recognized by Toll-like receptor 3 (TLR3). TLR3 is embedded in the membrane on the cell surface and/or in endosomes and signals through the TIR-domain-containing adapter-inducing interferon-β (TRIF) protein. Signaling through TRIF results in activation of TANK-binding kinase 1 (TBK1), which activate IRFs and NF-kB as shown in Fig. 4 (95). In mammals, plasmacytoid dendritic cells (pDCs) are super-producers of IFN-I. pDCs recognize viral ssRNA through TLR7 in endosomes and signals through the adaptor protein MYD88 (96). This process activates IFN-I transcription through IRF7 (96).

Although less studies have been done in fish, fish appear to possess similar IFN induction pathways as mammals. Similar signaling members have been found in fish as in mammals (8, 23-29). The RLRs RIG-I, MDA-5, LGP2 have been found in salmonid fish (82, 97, 98). The Toll-like receptors TLR3, TLR22, TLR7, TLR8 and TLR9, TLR21, which all recognize virus RNA, have also been identified in salmonids (99-104). TLR22 has only been found in fish, it is expressed on the cell surface and recognizes long dsRNA molecules (105). In Atlantic salmon, specialized leucocytes have been detected in head kidney and spleen that produce

high levels of IFNb and IFNc in response to a TLR7 ligand (89). Homologs of MAVS which is an adaptor protein in the RLR-pathway and MyD88 which is an adaptor protein in the TLR7/8 and TLR9 pathways have been identified in salmonids (98, 101, 106, 107). The adaptor TRIF (TICAM-I) protein was found to be associated with TLR3 and activate NF-kB response in zebrafish (108, 109).
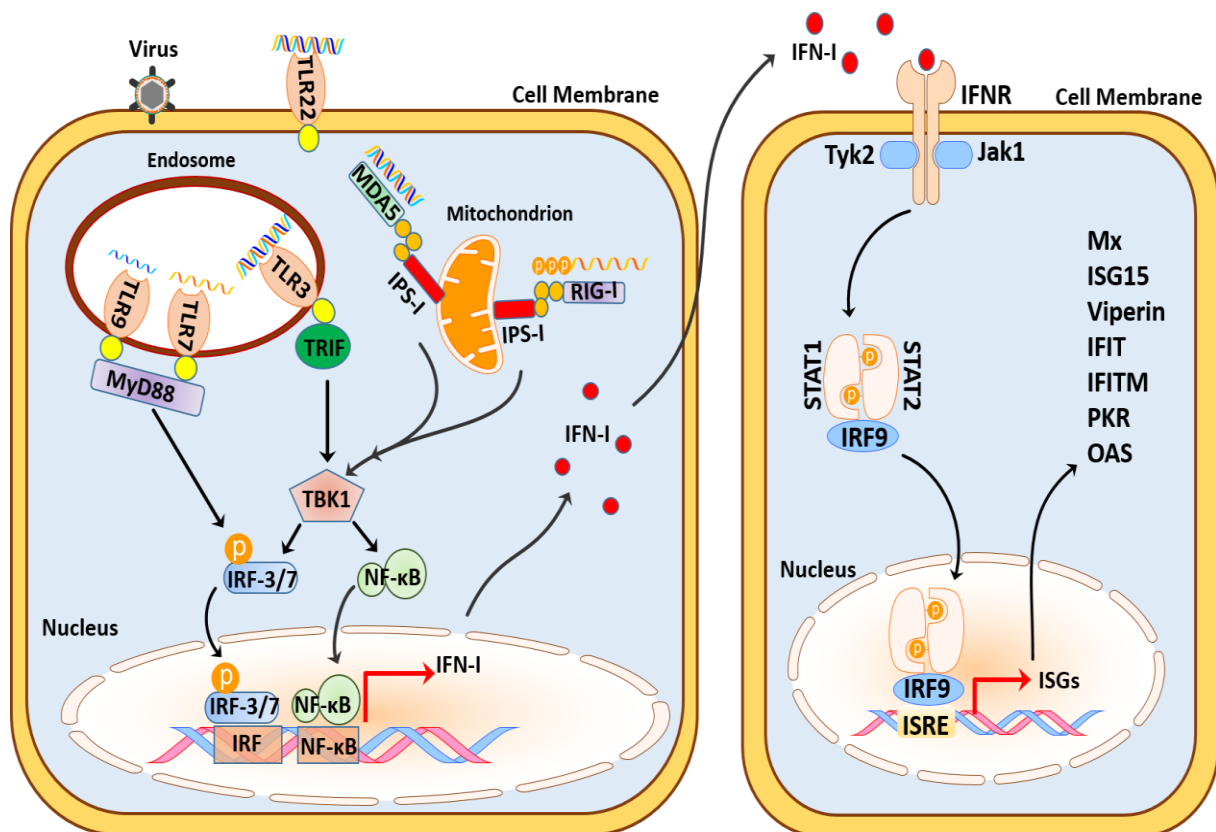
Figure 4. Signaling of virus RNA mediated type I interferon and antiviral response. RIG-I and MDA5 recognize 5' triphosphate uncapped single stranded RNA and double-stranded RNA, they signal via IPS-I (MAVS) and TBK1 leading to activation of IRF-3/7 or NF-kB and IFN-I production. TLR3 recognizes short dsRNA, fish TLR22 recognizes long dsRNA, TLR7/8 recognize ssRNA and TLR9 recognizes CpG containing dsDNA. These TLRs signal through the adaptor proteins MyD88 or TRIF for further downstream signaling. IFN-I is released and bind to IFN-receptors on the surface of cells resulting in triggering of the JAK-STAT pathway leading to antiviral protein production. Some elements in this drawing was derived from Biomedical-PPT-Toolkit-Suite of Motifolio Inc., USA.

In addition to the sensors that recognize virus RNA, IFN-I synthesis may also be induced by recognition of viral DNA. In mammals, several DNA sensors from host cells have been discovered recently. Cytosolic DNA such as DNA-dependent activator of ifn-regulatory

factors (DAI), DEXD/H box helicases, and cytosolic GAMP synthase (cGAS) recognize B-DNA and TLR9 recognizes DNA containing unmethylated CpG motifs and their activation leads to IFN-I production (110-112). So far DDX41 in olive flounder is the only fish homolog for cytosolic DNA sensor identified (113).

1.6 IFN-I receptors and induction of interferon stimulated genes (ISGs)

IFN-I bind to IFN-I receptors that are composed a heterodimeric receptor of class II cytokine receptor family member (CRFB), which results in signaling through the JAK/STAT pathway and activate downstream antiviral protein production (75). In mammals, IFN-I receptor is comprised of the IFNAR1 and IFNAR2 chains (114). Upon binding to IFN-I, IFNAR1 and IFNAR2 receptors are associated with the Janus family tyrosine kinases Tyk2 and Jak1 respectively (114). The transcription factors STAT1 and STAT2 are phosphorelated by IFNAR1 and IFNAR2 associated tyrosine kinases and then form homo or heterodimers. The dimer of phosphorylated STAT1 and STAT2 associates with IRF9 and form the transcriptional activator protein complex3 (ISGF3). ISGF3 then translocates into the nucleus and activates transcription of hundreds ISGs by binding to the interferon-stimulated signaling element (ISRE) in their promoters (114, 115).

Like mammalian interferon receptors, the conserved sequences are first found between pufferfish (*Tetraodon*) and mammals as CRFB and IFN receptor genes (75). Sequence analysis suggested that pufferfish CRFB1 and CRFB2 were IFNAR2 homologs while CRFB5 is an IFNAR1 homolog. So far, the receptor for two-cysteine IFNs are found to be composed of CRFB1 and CRFB5, while receptor for four-cysteine IFNs is composed of CRFB2 and CRFB5 in zebrafish. Characterization of IFN-I receptors in salmon requires further studies. A hypothetical model for receptors of Atlantic salmon IFNs have been described recently. IFNa is proposed to bind to a receptor composed of CRFB1a combined with CRFB5a, CRFB5B or CRFB5c (116). IFNc is proposed to bind to a receptor composed of CRFB2 coupled with CRFB5a or CRFB5c (116). IFNb is suggested to signal through a receptor composed of CRFB2 and CRFB5X (116). Functional studies of STAT protein have shown association of Tyk2-STAT molecules and the subcellular localization of IRF9 and ISG3 proteins complex in carp and salmon (117-121). The expression of antiviral Mx protein triggered by Tyk2-STAT signaling in salmon (117) and the conserved region of ISRE between fish and higher

vertebrates indicates similarity in IFN-I mediated signaling between fish and mammals (117, 119, 122-126).

1.7 Antiviral proteins induced by IFN-I

IFN-I induce several hundred different ISGs in mammalian cells (66-69). More than 380 of these human ISGs have been shown to be involved in antiviral activity (127), but only a limited number of IFN-I induced proteins have been shown to directly inhibit replication of viruses. The most studied IFN-I induced antiviral proteins include the GTPase Mx (myxovirus resistance gene), ISG15 (IFN-stimulated protein of 15 kDa), protein kinase R (PKR), ribonuclease (RNAase) L and oligoadenylate synthetases (OAS). More recent studies have added some other ISGs to the list, proteins such as virus inhibitory protein, endoplasmic reticulum-associated, interferon-inducible (Viperin, Vig-1 in fish), IFN-induced proteins with tetratricopeptide repeats (IFIT) and IFN-induced transmembrane proteins (IFITM), some of which are described below. Many interferon-inducible genes have been discovered from various fish species such as ISG15 (128-130), IFIT5 (131) and Mx protein (132-136). Additionally, homologs of Vig-1 protein that was first identified in rainbow trout and renamed as Viperin after being identifed as a IFN-I inducible protein in human fibroblasts (137-139). These proteins inhibit different stages of virus replication, which in concert create the antiviral state induced in cells by IFN-I.

Myxovirus resistance protein (Mx)

The first IFN-I induced antiviral protein which was discovered was the Myxovirus resistance protein (Mx1) (140, 141). Mx belongs to the GTPase family and exists in most vertebrates. The main structural domains of the protein are the large N-terminal GTPase domain (G domain), the middle domain (MD), the GTPase effector domain (GED) and the stalk region that is formed between MD and GED domain. G domain is associated with GTP hydrolysis while protein oligomerization is mediated by MD, GED domain and stalk region.

Studies have shown that GTP hydrolysis and oligomerization are crucial for antiviral activity. Experiments with human MxA that targets viral nucleoprotein from La Crosse virus (142) and form MxA-N complex which lead the depletion of the nucleoprotein from viral

replication sites (143). It was suggested that oligomeric rings from MxA surround the viral nucleoprotein, and later GTP binding and hydrolysis lead to conformation changes and the disintegration of the viral N protein (144). In mammals, the antiviral activity of Mx protein has been shown against the orthomyxoviruses influenza A, influenza B, influenza C virus, and Thogotovirus (THOV) in addition to hepatitis B virus and Semliki Forest virus (145). In fish, multiple Mx proteins were also found in rainbow trout (146-148) and in Atlantic salmon (132). Atlantic salmon Mx1 protein constitutively expressed in CHSE-214 cells showed inhibition of IPNV replication (149). Experiments with constitutive expression of Atlantic salmon Mx1 (AsMx1) in chinook salmon embryo (CHSE-214) cells shows reduction of cytopathic effects with ISAV infection (150). Studies of antiviral activity of IFNs shows IFNa, IFNb and IFNc provide transient inhibition of ISAV replication in TO cells, and IFNa induced Mx expression is correlated to reduce virus load at early ISAV infection time point (151).

Interferon-stimulated gene 15 (ISG15)

Another notable antiviral protein induced by IFN-I is the Interferon-stimulated gene 15 (ISG15). ISG15 is an ubiquitin-like protein that possesses a C-terminal LRLRGG motif, which is essential for its antiviral activity. ISG15 is present in the cells either in free form or covalent-bound to substrate proteins during post-translational process (152). The C-terminal glycine (LRLRGG) from ISG15 covalently binds to the lysine residues derived from target proteins (ISGylation) and forms the ISG15-substrates protein conjugations. The conjugations with substrate proteins such as MxA, PKR, and RNaseL extends the function of virus inhibition by increasing their stability, activity, or the interaction with other proteins (153). Additionally, ISG15 has been shown to increase the antiviral response via counteraction of Newcastle disease virus-induced IRF3 degradation (154). ISG15 has been shown to restricts influenza virus replication by targeting the virus NS1 protein directly and to inhibit HIV-I virus release by influencing protein trafficking within the cellular endosomal pathway (155, 156). Homologs of ISG15 is well conserved in many vertebrate species and share the conservative of sequences of mammalian ISG15 (128, 129, 157-160). The consensus LRGG Protein motif at C-terminal and ISGylation have been identified to be associated with the antiviral activities in fish (129, 160).

Virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible (Viperin) is an ER-associated protein, which uses lipid rafts to interfere with viruses such as influenza and hepatitis C virus infection during their entry, budding, and release (161). IFIT protein family (interferon-induced proteins with tetratricopeptide repeats), that IFIT1 (ISG56) recognize viral RNA that contains 5'-triphosphate moiety or lacks 2'-O-methylation thus inhibit RNA translation (162). Experiments show that IFIT5 (ISG58) enhance the innate immune response upon interacting with RIG-I and MAVS and co-mediate with IRF3 and NF-κB in innate immune response (163). In Atlantic salmon IFNa, IFNb and IFNc induce expression of ISG15, Viperin, and ISG58 in salmon cell line that against IPNV. IFNa gives similar antiviral activity as IFNc and greater than IFNb. IFNd shows no antiviral activity with *in vitro* antiviral assay (89). IFNa have also shown antiviral activity against SAV3 replication (164, 165). In the present study we have used Mx, ISG15, Viperin and IFIT5 (ISG58) as markers for antiviral gene induction by IFN-I, and use antibody against Mx and ISG15 for protein induction analysis.

1.8 The use of IFN-I in protection of vertebrates against virus infection

IFN-I (IFN-α and IFN-β) has been used for treatment of humans against hepatitis B virus either alone or in combination with nucleos(t)ide analogues (166). Protective effects of IFN-I against influenza A virus has been shown in guinea pig, ferrets and chicken (167, 168). Protection of live fish with recombinant IFN-I has been tested against infectious spleen and kidney necrosis virus (ISKNV) in zebrafish, but showed only protection in the first week after injection probably due to the short life time of IFN-I *in vivo* (169). The short duration of protection and the high cost of production make IFN-I less interesting for prophylaxis of fish. On the other hand, studies of DNA vaccination of salmonids with the G-protein from the rhabdoviruses VHSV or IHNV have demonstrated systemic up-regulation of Mx and other antiviral proteins accompanied by protection against virus infection in the first weeks after vaccination (170). This has inspired the studies in the present thesis where we tested the *in vivo* antiviral effects of IFN-I expressing plasmids in Atlantic salmon. A recent study in turbot gives similar evidence as our paper I and II that protection against VHSV virus after intramuscular delivered of turbot IFN-I DNA plasmid (85).

1.9. Adaptive immune responses against viruses

The second line of defense against viruses is the adaptive immunity, which in contrast to innate immunity is very specific and long lasting. Activation of innate immune response to virus infection is required for initiation of adaptive immune response (171). Cytokines and ISGs that induced by rapid inflammatory response which eliminate the virus infection, and mediate macrophage activation, and dendritic cells maturation (172). DCs are professional antigen presenting cells that are responsible for initiating adaptive immune response.

Adaptive immunity engages B and T lymphocytes. B cells are involved in humoral immunity, they produce and secrete virus-specific antibodies whereas virus-specific CD4+ and CD8+ T cells mediate cellular immunity. B cells and T cells originate from bone marrow and mature in spleen and thymus respectively in mammals. Dendritic cells express MHC-I and MHC-II molecules coupled with antigen peptides which are presented to naive T-lymphocytes. This process lead to maturation of naïve T-cells and formation of CD4+ and CD8+ effector cells. The encounter of antigen presenting cells with lymphocytes are shown in Fig. 5 (173-176).

While both B and T cells are found in fish and activation of adaptive immunity seems to happen in similar manner, head kidney and thymus are the primary hematopoietic organs. B-cell production and maturation as well as immunoglobulin production are localized in head kidney (177, 178). Spleen is the house organ for lymphocytes, macrophages and plasma cells development, while thymus is the organ that produce and develop T cells (179, 180). Interestingly, fish have a unique T-cell rich organ at the base of the gills (181). Like mammals, a lineage of B cells is also found in fish that produce the mucosal specific antibody IgT/Z, and T cells are also found in mucosal- associated tissues (182-184).

The following sections will describe the role of DCs and B and T lymphocyte mediated humoral and cellular immune response, and information available about these cells in fish.
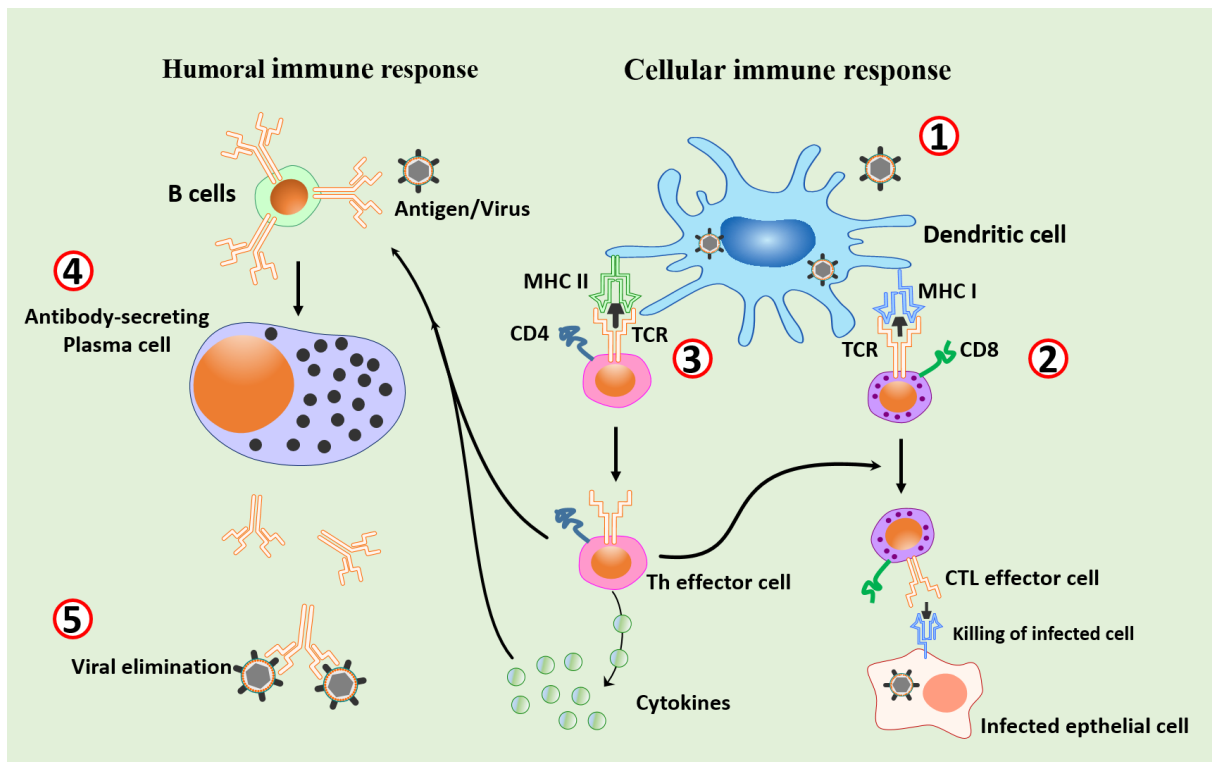
Figure 5. Humoral and Cellular immune response eliminate virus infection. Step 1. Virus recognition through direct infection or phagocytosis of dendritic cells (DCs). Step 2. Antigen peptides that derived from viruses are presenting and activate CD8+ T cells via MHCI molecules/peptides complex. Activated CTL are mediating the cytotoxicity of killing virus infected cells. Step3. The antigen peptides are presenting and activate CD4+ T cells via MHC II molecules/peptides complex. T helper (Th) cells are secreting cytokines and mediating humoral response. Step 4. B cells differentiate into antibody productin plasma cells after antigen recognition and stimulated by T cells-derived cytokines. Step 5. Virus-specific antibody recognize virus epitopes and eliminate virus infection. Some elements in this drawing was derived from Biomedical-PPT-Toolkit-Suite of Motifolio Inc., USA.

Humoral immune response

The role of B cells

Activation of humoral immunity requires the interaction of virus antigens and B cell receptors (BCR) which leads to the maturation of naïve B cells. After maturation and class switching, naïve B cells are turned into memory B cells and produce high-affinity antibodies that recognize virus antigens from blood and infected cells in the plasma thus eliminate virus infection (176).

In placental mammals, five isotypes of immunoglobulins (IgG, IgE, IgA, IgM and IgD) with distinct structures and biological activities have been found (176). The major isotypes of IgG are present in serum and play an important role against infection with virus and bacteria (176). IgA is responsible for mucosal immunity, against for example influenza virus (185). In fish, three Ig isotypes (IgM, IgT, IgD) have been discovered. Secreted IgM is the main immunoglobulin in the blood stream and mucus, and is important in systemic immunity, whereas secreted IgT is more important in mucosal immunity (186). However, there is no evidence of class switch recombination have observed in fish (187, 188). Additionally, the finding of phagocytic B cells in fish, suggests that this cell type may play a similar role as dendritic cells in mammals in connecting innate and adaptive immunity (189).

The role of antibody in elimination of virus

The main function of virus specific antibodies is neutralization of the virus. Neutralization refers to the binding of antibody to viral particles without other assistance, which lead to loss of infectivity by virus (190). Passive immunization is a method, which shows neutralizing activity of antibody by transfer of virus-specific antibody to naïve animals leading to protection against infection by the virus (191). Even though the mechanism remains unclear, it is hypothesized that antibodies neutralize free virus via direct contact (Fig. 6). It has been shown that neutralizing antibodies block conserved epitopes of hemagglutinin of influenza virus or envelope protein of HIV-I and as a result prevent virus from attaching and entering the cells (192, 193).

Alternatively, antibody may also provide protection through antibody-directed complement-dependent cell lysis (CDCC), Fc receptor-mediated phagocytosis and antibody-dependent cellular cytotoxicity (ADCC). Cell lysis is caused by formation of membrane attack complex formation as result of CDCC mediated complement cascades. Fc receptors expressed on macrophage and natural killer cells are important for phagocytosis and release of perforin and granzymes for cytotoxicity. Both the CDCC and ADCC lead to the destruction of cells as shown in the Fig. 6 (190). Antibody can also inhibit virus release and transmission from the cell surface (194). Passive immunization have shown to protect against IHNV, ISAV and SAV3 after naïve fish received the virus-specific antisera (195-197). Serum with neutralizing activity has been shown to inhibit infection of cells by IPNV, SAV3 and ISAV (198-200).

Complement-dependent neutralization of virus with antibody have been demonstrated against VHSV in rainbow trout (201, 202).
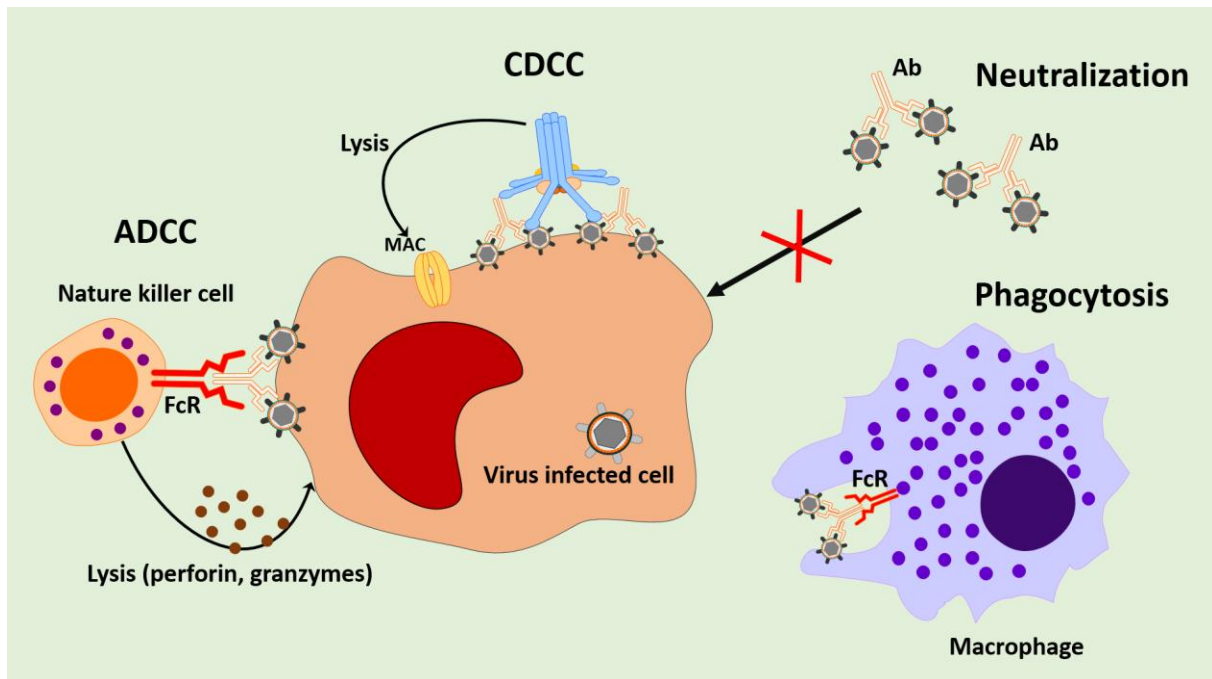
Figure 6. Neutralizing antibodies and antibody-directed complement-mediated cytotoxicity (CDCC), Nature killer cell with Fc receptor-mediated antibody-dependent cytotoxicity (ADCC), Macrophage with Fc receptor-mediated phagocytosis. Some elements in this drawing was derived from Biomedical-PPT-Toolkit-Suite of Motifolio Inc., USA.

Cellular immune response

The role of DCs

In mammals, dendritic cells (DCs) are members of APC family, and are derived from hematopoietic tissue in bone marrow. Two major DC subsets, plasmacytoid DCs (pDCs) and conventional DCs (cDCs) have been identified (203). pDCs are the main IFN-I producing cells (204), while cDCs are divided into several subpopulations including resident DCs (205). The contribution of DCs in virus clearance is as follows. When DCs are infected by influenza virus or take up virus antigens from infected cells, chemokine receptor CCR7 is up-regulated and modulate migration of DCs to lymphoid tissue for maturation (206). The cytosolic virus proteins taken up by DCs are processed and degraded into peptide fragments by the proteasome or endosome/lysosome. These peptides are bound to and translocated by MHC I

or MHC II molecules and presented on the cell surface. The combination of MHC/virus peptide complex on DCs surface is identified by CD8+ and CD4+ T-cells and this leads to their activation (207, 208). Besides, activated pDCs secrete IFN-I which prolong CD4+ and CD8+ T cells survival and differentiation (209).

Dendritic cell like features such as surface MHC II molecules and TLRs have been found in hematopoietic tissues from rainbow trout (210). Similar features like MHCII, CD83 molecules have identified in DC-like cells in Atlantic salmon and the up-regulation of IFN-I can be induced in these cell types by stimulation with TLR ligands and co-stimulatory CD40L-like molecules (211, 212).

The role of T helper cells

CD4+ T cells can differentiate into two helper T cell subsets that Th1 and Th2 cells. As shown in Fig. 4, CD4+ Th1 cells recognize peptide/MHC class II complex presented by APCs. Th1 cells produce IFN-γ and IL-2 cytokines which participate in adaptive immunity by activating cytotoxic T lymphocytes and B cells. Th2 cells produce IL-4, IL-5, IL-13 cytokines that promoting B cell response and also regulate Th1 cells (213).

The role of Cytotoxic T-cells

CD8+ T cells are cytotoxic T lymphocytes (CTL) that eliminate virus replication through secreting IFN-γ or lysis of virus-infected cells through release of cytolytic mediators: perforin and granzymes (214). IFN-γ induces synthesis of interferon-stimulated genes (ISGs) gives direct interfere in virus replication (215). CD8+ T cells secreted IFN-γ also improving CD4+ Th1 cells differentiation (216, 217). The function of perforin is permeabilization of virus-infected cell membrane and induction of cell apoptosis through granzymes. Granzymes further induce pro-inflammatory cytokines production and influence protein synthesis in virus-infected cells (218).

CD4+ T helper cells and CD8+ cytotoxic T cells have been identified in fish, (219, 220). In ginbuna crucian carp transfer of virus sensitized donor cells with CD4+ T cells to naïve fish was shown to induce a more rapid and stronger antibody response (221). Cytotoxicity of

fish T cells has been proved by using clonal fish with cell-mediated cytotoxicity assays in ginbuna crucian carp (222) and rainbow trout (223). In those experiments, cytotoxic T cells recognized intracellular peptides and destroyed infected cells through CD8 T cells receptor and MHC I molecules. In ginbuna carp, a recent study showed that CD8α+ lymphocytes are using perforin and granzyme for eliminating virus-infected cells (224).

1.10 Vaccine against fish virus

Traditional vaccines

Live attenuated viruses have been considered to be the most effective virus vaccines since they trigger both humoral and cellular adaptive immunes in the host. Live vaccine in human such as poliovirus vaccine (225) and yellow fever vaccine provides long lasting protective immunity (226, 227). Similarly, greater efficacy was found in live attenuated influenza vaccine compared to inactivated vaccine (228, 229). However, due to the risk of using a live virus as vaccine, alternatives like inactivated whole virus vaccines and subunit vaccines have been developed. Define subunit vaccines! Additionally, DNA vaccines have been studied intensively in last decades since they are safe and elicit both humoral and cellular immune responses (230, 231).

In fish, several inactivated vaccine and subunit vaccine are used commercially while only one attenuated virus vaccine, and one DNA vaccine have been licensed (232). Inactivated virus vaccine against IPNV, ISAV and SAV3 have been shown to promote humoral response and protection against virus infection in a dose-dependent manner (198, 233, 234). The problem with inactivated virus vaccines for fish is that relatively high doses are needed to obtain sufficient protection and such vaccines may be too expensive to be used for farmed fish. Subunit vaccines, like recombinant VP2 from IPNV, can improve the IPNV-specific antibody response, and recombinant HE and F from ISAV that improve the ISAV-specific antibody response and protection against ISAV (235, 236). Besides, subunit vaccines using E2 and E1 protein from SAV3 give some protection against SAV3 in salmon (237). The problem with subunit vaccines is that it is very difficult to obtain correct folding of recombinant virus proteins.

DNA vaccines for fish

The advantages of DNA vaccines include that they are safe, give less side effects and are easier and less expensive to produce compared to traditional vaccines (238). In addition, they may be produced against viruses that cannot be cultured. DNA vaccines are plasmids that…. Synthesis of the virus protein antigen by the host cell provides correct folding and elicits both cellular and humoral response similar to a virus infection (239). After DCs have taken up the DNA vaccine plasmid via intramuscular injection, peptide fragments of the virus protein antigen are presented to immune cells by DCs via both MHC I and II molecules (240). Plasmid encoded protein may also be secreted by transfected cells or released from apoptotic transfected cells and taken up by DCs for presentation to T cells as describe before of DCs maturation (240, 241).

DNA vaccines against the rhabdoviruses VHSV and IHNV consist of a plasmid encoding the virus G protein and were shown to provide higher neutralizing antibody response than inactivated and subunit vaccine in fish (242, 243). The upregulation of MHC II molecules at muscle injection site indicated the involvement of DCs after vaccination (244). Cellular responses triggered by rhabdovirus DNA vaccine have been described that VHS virus infected cells were killed by MHC I coordinated cytotoxicity (245, 246). Protection derived from cellular response was illustrated in a long term study after two years IHNV DNA vaccination study (247). A DNA vaccine against IHNV has been licensed for use in Canada and provides remarkable protection against virus infection (7). A DNA vaccine encoding the polyprotein from IPNV has shown moderate protection. that protection was suggested derived from vaccine induced neutralizing antibody and cellular response (248). Interestingly, the same IPNV polyprotein DNA vaccine also shows upregulation of IFN-I, Mx, MHC molecules and T cells at muscle, head kidney and spleen after vaccination (248). Besides, moderate protection against ISAV was obtained by vaccination with a plasmid expressing HE, and the protection was suggested to be mediated by cellular immune responses (49, 249). In addition, challenge experiments of alphavirus replicon-based DNA vaccine encoded with SAV3 E2 virus protein illustrated that the protection was derived from the correct folding and expression of E2 protein on the cell surface (54).

1.11 Type I IFN as adjuvant

In general, subunit vaccines show less immunogenicity than inactivated whole virus, which is due to the absence of intrinsic components that act as pathogen-associated molecular patterns (PAMPs). PAMPs interact with pattern recognition receptors (PRRs) such as TLRs and RLRs for activating not only innate immune responses, but also for activating adaptive immune responses (250, 251). Adjuvants are used for solving the problems by enhancing the immune response in vaccination. Many adjuvants contain TLR or RLR ligands, which stimulate maturation of APCs and next activate B- and T-cells (252).

IFN as adjuvant

Several lines of evidence demonstrate that IFN-I function as adjuvants. The TLR9 ligand CpG and TLR7/8 ligand R848, have been shown to act as adjuvants through induction of IFN-I and show adjuvant activity with hepatitis B virus surface antigen in mice by enhancing the antibody response (253). Experiments using chicken gammaglobulin as antigen showed a clear increase in specific antibody response when using the synthetic double-stranded RNA polyinosinic:polycytidylic acid (poly IC) as adjuvant (254). The antibody enhancement by poly IC was shown to be due to IFN-I which using IFN-I receptor knockout mice (254). Potent adjuvant effect of IFN-I was also shown in influenza vaccine experiments in mice with enhanced antibody response and protection against virus infection (255). Similarly, experiments using IFN-I receptor knockout mice have shown that endogenous IFN-I is the main mediator in the Th1-type immune response derived from wide range adjuvants (255).The adjuvant effect of fish IFN-I has apparently not been demonstrated until our work in this thesis.

Mechanism of action of IFN-I as adjuvant

Effect of IFN-I on DC

IFN-I have been shown to stimulate maturation and activation of DCs. Peripheral blood monocytes were turned into antigen presenting cells after co-incubation of IFN-α and granulocyte-macrophage colony-stimulating factor (256). The monocytes developed dendritic cells like morphology and expressed high levels of the MHC I and II molecules, B7 co-stimulatory molecules, adhesion proteins and CD40, which are considered as DCs features (256). Moreover, low level IFN-α treatment of peripheral blood mononuclear cells has been shown to up-regulate the co-stimulator CD86 (B7.2) and accelerate the maturation of dendritic cells (257). In human, IFNα/ß promotes dendritic cells trafficking and maturation (258).Experiments using TLR7 and TLR9 ligands illustrated that pDCs activation depends on the induction of IFN-I (258). After activation via TLR ligands, IFN-I also induces the expression of CXCR3 ligands in pDCs in response to CCR7 ligands and improve the migration of pDCs (258).

Effect of IFN-I on B-cells

Type I IFN produced by pDCs have been shown to enhance B-cell differentiation into plasma cells (259). As described above, IFN-I was shown to influence DCs maturation, which lead the enhancement of B cells isotype switching and antibody levels (254). Experiments with co-administration of chicken gamma globulin (CGG) and IFN-α were conducted using IFN-αβR-deficient B and T cells (260). IFN-α was unable to increase the CGG-specific antibody response in IFN-αβR-deficient B cells which illustrated the requirement of IFN-α for antibody production in B cells (260). Moreover, the experiment also showed that CGG-specific antibody response also required the direct influence of IFN-α on T cells (260). A possible explanation for the positive affect of IFN-I in humoral response might be due to its role in improvement of B cells differentiation and proliferation, and the protection of B cells and T cells from apoptosis (260).

Effect of IFN-I on cytotoxic T cells

The activated DCs are the powerful APCs thus initiate T cells response (173). Naïve CD8+ T cells require the third signal for fully activation (261). Experiments using IFN receptor deficiency CD8+ T cells that illustrated the direct effect of IFN-α (261). IFN-α that provide a third signal for CD8+ T cells responding to antigen and costimulatory B7-1 complex and stimulate T cell clonal expansion, differentiation and the development of cytolytic function (261). Likewise, IFN-I have shown that improve the cross-priming of CD8+ T cells by providing the third signal to T cells without involving signal 2 interaction (CD40 ligand /CD40) provided from DCs (262).

Effect of IFN-I on CD4+ T cells

IFN-I can directly improve the lifespan and clonal expansion of CD4+ T cells and CD8+ T cells, prolong the survival of memory T cells and promote the differentiation of CD4+ T cells (263-266). An indirect effect of IFN-I was observed in IL-15 secreting cells such as antigen presenting cells or inflammatory monocytes, which upon stimulation with IFN-I prolong the survival of memory T cells or increase the number of Th1 cells (267, 268).

1.12 The role of IFN-I in DNA vaccines

One weakness of DNA vaccines is poor immunogenicity due to low level of protein expression caused by the low amount of plasmid injected, codon usage from target protein and choice of promoter (239). Therefore, like other vaccines, DNA vaccines also require adjuvants for improving the immune response. However, DNA vaccines have a "build-in" adjuvant activity triggered by the plasmid DNA, which in part is due to unmethylated CpG motifs (269). It has been shown that CpG motifs in the DNA backbone is important for promoting T cells response (269). This motif was considered to be a TLR-9 ligand and trigger the corresponding immune response (270). However, experiments with TLR9-deficient mice showed that TLR9 ligand is not the only mediator for triggering the plasmid adjuvant activity (271-273). Experiments, by intracellular delivery of double-strand B-form DNA plasmid

illustrated that the TLR-independent response was dependent on TBK-1 and IRF3 (274-276). The TLR9-independent response was further investigated by IFNαβR receptor-deficient mice, this confirmed the importance of IFN-I which is essential for DNA vaccine-induced immunogenicity (277).

Some examples exists where IFN-I has been used as DNA vaccine adjuvant in mammals and chicken. Co-administration of bovine IFN-α as adjuvant with Foot-and-mouth disease virus (FMDV) in pcDNA3.1 backbone plasmid shows IFN-α elicited higher antigen-specific antibody titer, virus specific T cells proliferation and protection in guinea pigs (278, 279). Similar experiment has been done in mice, use of IFN-alpha as an adjuvant for adenovirus-vectored FMDV subunit vaccine showed upregulation of IgG antibodies and generation of T follicular helper cells (280). Likewise, recombinant IFN-I protein or DNA plasmid encoded with IFN-I from chicken used as adjuvant with Infectious bursal disease virus antigen increased both primary and secondary antibody response in chicken (281).

Altogether this inspired us to investigate the adjuvant effect of IFN-I in vaccines against virus in Atlantic salmon. We decided to study the adjuvant effect of IFN-I in DNA-vaccines because recombinant IFN-Is are rapidly degraded *in vivo*. We decided to use ISAV as a model because the DNA vaccine against ISAV previously was shown to give low protection and because ISAV gives high mortality in challenge exeriments.

2.1 Aim of the study

Function of type I interferon has been studied and described previously in salmonid cell lines. The goal of this project was to investigate the antiviral activity of IFNa, IFNb, and IFNc, against virus infection in vivo. To achieve this, we injected salmon pre-smolts with expression plasmids for each of the three IFNs and measured induction of antiviral genes and protection against infection with ISAV and SAV3. We also investigated the adjuvant activity of IFNa, IFNb, and IFNc co-injected with hemagglutinin-esterase from ISAV as DNA vaccine against ISAV in vivo. To achieve this, we injected salmon pre-smolts with plasmids encoded hemagglutinin-esterase from ISAV and IFNa, IFNb, IFNc, and measure the induction of antibody response and protection against infection of ISAV.

Primary goal I:

To study the antiviral effects of IFN expression plasmids injected i.m. into Atlantic salmon.

Subgoals:

1) Measure the antiviral protein response mediated by IFN expression plasmids at liver, heart and pancreas, and the kinetic response of IFNc plasmid injection.

2) Measure the protection against ISAV and SAV3 after IFN plasmid injection.

Primary goal II:

To study the adjuvant effects of IFN expression plasmids injected i.m. together with a plasmid expressing the Haemagglutinin-esterase (HE) ISAV protein into Atlantic salmon.

Subgoals:

1) Measure the antibody response modulated by co-injection of IFN expression plasmids and ISAV HE plasmid and the protection against ISAV.

2) Determine the immune cells at muscle injection site attracted by IFN-I after IFN plasmids injection.

3) Measure the long term antibody and antiviral protein response after co-injection of IFN plasmids with or without ISAV HE.

3 Summary of papers

Paper I

Protection of Atlantic salmon against virus infection by intramuscular injection of IFNc expression plasmid. Chia-Jung Chang, Camilla Robertsen, Baojian Sun, Børre Robertsen. Vaccine. Volume 32, Issue 36, 6 August 2014, Pages 4695–4702.

In this work we have tested the in vivo antiviral activity of type I interferons (IFNs) in Atlantic salmon by injecting presmolts intramuscularly (i.m.) with plasmids encoding IFNa1, IFNb or IFNc under the control of a CMV promoter, and measured expression of antiviral genes in organs and protection against infection with infectious salmon anemia virus (ISAV) infection. All three IFN plasmids induced expression of antiviral genes (Mx, Viperin, ISG15 and IFIT5) at the muscle injection site while the control plasmid had little effect. Only IFNb and IFNc plasmids induced expression of antiviral genes in head kidney, liver and heart. This suggests that IFNb and IFNc are distributed systemically while IFNa1 is active only at the injection site. Injection of IFNc plasmid was found to induce expression of antiviral genes and receptors for virus RNA (RIG-I, TLR3 and TLR7) in head kidney from 1 to at least 8 weeks. Immunoblotting showed increased expression of ISG15 and Mx protein in liver with time during this time period. Challenge of presmolts with ISAV 8 weeks after injection of IFN plasmids, showed strong protection of the IFNc plasmid injected fish, low protection of the IFNb plasmid injected fish and no protection of the IFNa1 plasmid injected fish. Clues to the difference in protection obtained with IFNb and IFNc plasmids were found by immunohistochemical and immunoblot studies of Mx protein, which indicated that IFNc plasmid stimulated stronger Mx protein expression in heart tissues and liver endothelial cells than IFNb plasmid. Taken together, these data suggest that i.m. injection of the IFNc expression plasmid may be a new method for protecting Atlantic salmon against virus infection.

Paper II

Protection of Atlantic salmon against salmonid alphavirus infection by type I interferons IFNa, IFNb and IFNc. Chia-Jung Chang, Iris Jenssen, Børre Robertsen. Submitted on May. 2016

Salmonid alphavirus 3 (SAV3) causes pancreas disease (PD), which is a major problem in Norwegian aquaculture of Atlantic salmon. In this work we studied antiviral activities of salmon type I interferons IFNa, IFNb and IFNc against SAV3 infection in cell culture and in live fish to increase the understanding of the innate immunity of salmon against this virus. Recombinant IFNa, IFNb and IFNc all induced antiviral activity against SAV3 in ASK cells. For in vivo studies, we injected salmon presmolts intramuscularly with plasmids encoding salmon IFNa, IFNb and IFNc or a control plasmid and measured expression of the antiviral protein Mx in pancreas after 2 and 10 weeks and protection against SAV3 infection after 10 weeks. IFNb and IFNc plasmids, but not IFNa plasmid induced Mx expression in pancreas as shown by RT-qPCR and immunohistochemistry. A high level of protection against SAV3 infection by IFNc plasmid was observed by a strong reduction of virus load in serum and by a marked reduction in pathology of pancreas and heart compared to control fish. Lesser but significant protection was observed with IFNb plasmid while no protection was observed after treatment with IFNa plasmid. Taken together, this work suggests that IFNa provides protection of salmon against SAV3 locally in an infected area while IFNb and IFNc provides systemic protection against the virus.

Paper III

Adjuvant activity of fish type I interferon shown in a virus DNA vaccination model.
Chia-Jung Chang, Baojian Sun, Børre Robertsen. Vaccine.Volume 33, Issue 21, 15 May 2015, Pages 2442–2448.

There is a need for more efficient vaccines to combat viral diseases of Atlantic salmon and other farmed fish. DNA vaccines are highly effective against salmonid rhabdoviruses, but have shown less effect against other viruses. In the present work we have studied if type I IFNs might be used as adjuvants in fish DNA vaccines. For this purpose we chose a DNA vaccine model based on the hemagglutinin-esterase (HE) gene of infectious salmon anemia virus (ISAV) as antigen. Salmon presmolts were injected with a plasmid encoding HE alone or together with a plasmid encoding Atlantic salmon type I IFN (IFNa1, IFNb or IFNc). Sera were harvested after 7-10 weeks for measurements of antibody against ISAV and the fish were challenged with ISAV to measure protective effects of the vaccines. The results showed that all three IFN plasmids delivered together with HE plasmid potently enhanced protection of salmon against ISAV mediated mortality and stimulated an increase in IgM antibodies against the virus. In contrast, HE plasmid alone gave low antibody titers and a minor protection against ISAV. This demonstrates that type I IFNs stimulate adaptive immune responses in fish, which may be a benefit also in other fish DNA vaccines. Quantitative RT-PCR studies showed that the salmon IFNs caused an increased influx of B-cells and cytotoxic T-cells at the muscle injection site, which may in part explain the adjuvant effect of the IFNs.

Paper IV

IFN-adjuvanted DNA vaccine against infectious salmon anemia virus: Antibody kinetics and longevity of IFN expression. Børre Robertsen, Chia-Jung Chang and Lisa Bratland. Fish Shellfish Immunol. 2016 Apr 20. pii: S1050-4648(16)30178-4. doi: 10.1016/j.fsi.2016.04.027.

Plasmids expressing interferon (IFN) have recently been shown to function as adjuvants in Atlantic salmon when co-injected with a DNA vaccine encoding hemagglutinin-esterase (HE) from infectious salmon anemia virus (ISAV). In this work we have compared the antibody kinetics and the systemic Mx/ISG15 response of fish vaccinated with HE-plasmid using either IFNa plasmid (pIFNa) or pIFNc as adjuvants over a longer time period, i.e. 22 weeks post vaccination (pv). The results showed that the antibody response against ISAV with pIFNa as adjuvant arose earlier (7 weeks pv) than with pIFNc as adjuvant (10 weeks pv), peaked at week 10 and declined at week 22. The antibody response with pIFNc as adjuvant peaked at 16 weeks and kept at this level 22 weeks pv. Fish injected with pIFNc alone expressed high levels of Mx and ISG15 in liver throughout the 22 week period. In contrast, fish injected with pIFNc together with HE-plasmid expressed high levels of Mx and ISG15 in liver for the first 10 weeks, but at week 16 this response was absent in two of three fish at week 16 and was absent in all tested fish at week 22 pv. This suggests that cells expressing HE and IFNc are intact at week 10 pv, but are eliminated by adaptive immune responses after week 10 due to recognition of HE. The longevity of the Mx/ISG15 response in pIFNc treated fish is likely due to the fact that IFNc is a self-antigen of salmon and is not attacked by the adaptive immune system.

4. Discussion

Induction of ISGs and antiviral proteins Mx and ISG15 by IFN-I plasmids at the muscle injection site and in internal organs.

Previous work has shown that recombinant IFNa1, IFNb and IFNc protect salmon cells against IPNV and ISAV infection *in vitro*, IFNa1 and IFNc having similar and stronger antiviral activity than IFNb (282, 283). In paper II we tested the antiviral activity of these three IFNs against SAV3 in ASK cell and showed all three IFNa, IFNb and IFNc induce similar protection against SAV3 in salmon cell line.

In paper I and paper II we studied the *in vivo* antiviral activity of these IFNs against ISAV and SAV3 when delivered as genes in expression plasmids by intramuscular injection. The results demonstrated unexpected differences between IFNa1, IFNb and IFNc with the ability to induce systemic up-regulation of antiviral genes in Atlantic salmon. Paper I revealed that all three IFN expression plasmids induced similar levels of ISG transcripts at the muscle injection site, which suggests that similar amounts of IFNa1, IFNc and IFNb were produced by the transfected muscle cells. In contrast, only IFNb and IFNc plasmids induced antiviral genes in head kidney, liver, heart (paper I) and pancreas (paper II). Similar levels of ISG transcripts were, however, induced in head kidney leucocytes treated with recombinant protein IFNa1 and IFNc. Taken together, this suggests that IFNc and IFNb travel with the blood stream after being expressed at muscle injection site and induce antiviral genes systemically in fish, while IFNa in only active locally at the production site. Thus, during virus infection, IFNa is probably mainly important at the infection site, while IFNc and IFNb are distributed that throughout the fish body and trigger synthesis of antiviral proteins in cells systemically. Previous work showed that IFNc is produced by a variety of cell types in live Atlantic salmon, is induced by both viral dsRNA and ssRNA analogs and has equally strong antiviral activity as IFNa1 (282). Altogether this suggests that IFNc has a crucial role in innate immunity against virus infection in Atlantic salmon. IFNb is also distributed systemically, but has less antiviral activity than IFNa and IFNc, and is produced mainly by specialized leukocytes in response to an ssRNA analog (282).

The difference in distribution properties of IFNa compared to IFNb and IFNc may be due to that IFNa is a 2C-IFN, which contains one disulphide bridge, while IFNb and IFNc are 4C-IFNs, which contain two disulphide bridges (284). As mentioned above, the number of

disulphide bridges might influence the half-life of the IFNs. Besides, the isoelectric points of IFNa1 (pI 9.2) and IFNb/IFNc (pI 6.9/pI 5.1) are also quite different and might influence their distribution and degradation properties.

Kinetics of ISG expression induced by the IFNc expression plasmid at the muscle injection site and internal organs.

The time course study from the paper I showed that IFNc plasmid induced up-regulation of not only antiviral genes (Mx, ISG15, Viperin, IFIT5), but also genes for receptors of virus RNA (RIG-I, TLR3 and TLR7, TLR8) in head kidney throughout the 8 week experimental period. The expression of antiviral genes in head kidney was highest 5-7 days after injection of IFNc plasmid, dropped at week 2, but slowly increased during the following weeks. Increased expression of Mx and ISG15 protein was confirmed in liver, head kidney and heart of IFNc plasmid injected fish 8 weeks after injection (paper I). In paper II we showed that pIFNb and pIFNc induced similar levels of Mx transcripts in pancreas after 10 weeks interferon DNA plasmid injection. As expected from paper I, pIFNa had no effect on Mx expression in pancreas. This was confirmed by immunohistochemistry of Mx in pancreas. Injection of pIFNc resulted in a long-lasting expression of Mx and ISG15 in liver, which lasted at least 22 weeks (paper IV). It is thus highly likely that injected pIFNc may continue to provide systemic expression of antiviral genes beyond 22 weeks.

Protective effects of IFN-I expression plasmids against ISAV and SAV3 *in vivo*

The long-lasting induction of antiviral genes by pIFNc injection in salmon, inspired us to study whether this treatment might provide protection of the fish against virus infection.

In paper I, we chose an ISAV infection model since ISAV causes high mortality in Atlantic salmon pre-smolts. In paper II, we chose SAV3 as the model since the virus cause significant economic loss in salmon farming and the challenge model has been established and studied (199). The results of the challenge experiments showed that pIFNa and control plasmid provided no protection as expected from the ISG expression studies. In contrast, pIFNc provided a high level of protection of salmon against ISAV infection 8 weeks after

pIFNc injection and against SAV3 infection 10 weeks after pIFNc injection. Although pIFNc injected fish showed a high level of protection against both ISAV and SAV3 infection, pIFNc did not completely eliminate ISAV in head kidney or eliminate SAV3 in serum or SAV3 mediated pathological changes in pancreas and heart. Whether the viruses would be cleared or would replicate beyond the sampling time points is thus uncertain.

Differences in antiviral activity between IFNb and IFNc

Interestingly, pIFNb and IFNc plasmid induced comparable amount of Mx and ISG15 protein in liver (Paper I) and pancreas (Paper II) while IFNb only gave a low level of protection against both ISAV and SAV3 infection. This may be due to that IFNb and IFNc use different receptors and consequently induce antiviral proteins in different cell types. IFNb may thus not induce antiviral proteins in cells that are crucial for infection by ISAV and SAV3. This hypothesis is supported by the fact that IFNb showed less antiviral activity than IFNc in TO cells (Svingerud et al, 2012), by the finding in paper I which showed that Mx in endothelial cells in liver was more strongly stained in IFNc treated fish compared to IFNb treated fish and by the IFN receptor study done by Sun et al (2014). Moreover, heart tissue showed stronger Mx staining throughout in fish treated with IFNc plasmid compared to IFNb plasmid and this was confirmed by immunoblotting of Mx (paper I). This suggests that IFNc induces antiviral proteins more strongly than IFNb in several different cell types in heart, which is a target organ for both ISAV and SAV3.

Adjuvant activity of IFNs in DNA vaccination against ISAV

In Paper III, we provide evidence that fish IFN-I also has a major role in kick-starting the adaptive immune responses against the virus. This was shown by demonstration of adjuvant effect of the salmon IFN-I plasmids in DNA vaccination against ISAV. Previous research had shown that DNA vaccines are highly effective against salmonid rhabdoviruses, but are less effective against other viruses. In this work we decided to study if IFN-I plasmids might be used as adjuvants in DNA vaccines against virus in fish since it is known that IFN-I enhance

the adaptive immune response in mammals as described above. Previous work had shown that a DNA vaccine against ISAV based on the virus hemagglutininesterase (HE) only gave a modest increase in antibody response and protection against infection (49). We therefore decided to test if IFNa, IFNb or IFNc delivered as expression plasmids together with the HE plasmid might increase the protective effect of the vaccine and thus function as adjuvants in the vaccine. Salmon presmolts were injected with a plasmid encoding HE alone or together with a plasmid encoding Atlantic salmon IFNa1, IFNb or IFNc. Sera were harvested after 7-10 weeks for measurements of antibody against ISAV and the fish were challenged with ISAV to measure protective effects of the vaccines. The results showed that all three IFN plasmids delivered together with HE plasmid potently stimulated an increase in IgM antibodies against the virus and strongly enhanced protection of salmon against ISAV mediated mortality. In contrast, HE plasmid alone gave low antibody titres and a minor protection against ISAV. Besides, we have also observed adjuvant effect of recombinant IFNc when delivered i.p. together with inactivated ISAV as vaccine. Recombinant IFNc enhanced the IgM antibody response ISAV. Taken together, the results from Paper III demonstrated that the IFN-I function stimulate adaptive immune responses in fish and thus function as adjuvants. The present work thus shows that the link between type I IFNs and the adaptive immune system was established in fish several hundred million years ago. This finding may hopefully be beneficial for developing more potent vaccines against other fish viruses as well.

The present studies lend support to the hypothesis that the potency of DNA vaccine based on VHSV and IHNV G proteins may in part be due to their ability to induce IFN-I (170, 285).

The mechanism of action of IFN-I as adjuvants

The fact that IFNa, IFNb and IFNc all showed similar adjuvant effects was surprising since they have quite different properties in Atlantic salmon. The three IFNs are induced through different signaling pathways and show different expression in cells and tissues (282). Recent work has shown that salmon IFNa, IFNb and IFNc utilize different receptors (116). The immune cells that contribute to the adjuvant effect of IFN-I in Atlantic salmon must thus have receptors for all three IFN subtypes since they all show similar adjuvant properties. The IFNa1 plasmid was shown to induce antiviral genes only at the muscle injection site, while the IFNb and IFNc plasmids induce antiviral genes systemically in the fish (paper I). This

means that the adjuvant effect of the IFNa plasmid is caused by stimulation of immune cells at the muscle injection site since IFNa does not induce antiviral genes systemically in salmon. Accordingly, the adjuvant activity of IFNb and IFNc is also likely to occur at the muscle injection site. As described in the Introduction, mammalian studies suggest that adjuvant activity of IFN-I is pleiotropic and due to direct stimulatory effects on T cells, B cells as well as dendritic cells, which are the main antigen presenting cells (254, 286-288). IFN-I have been shown to be important for clonal expansion of CD4 and CD8 T-cells and for initiation of cross-priming of CD8 T cells (261-263). In addition, IFN-I have been shown to promote survival of B-cells by inhibition of apoptosis (289). The effect of IFN-I on fish immune cells is unknown, but the RT-qPCR studies showed that injection of all three IFN plasmids caused an increase in transcripts for IgM and IgT, which suggest an increased influx of B-cells, and an increase of CD8, perforin and granzyme A transcripts, which suggests an increased influx of cytotoxic T-cells. This might be explained by the fact that the IFNs induce several chemokine genes in the muscle tissue. We did not observe increased transcript levels of CD4, MHCII or CD86, which suggests no increased attraction of CD4 T cells or professional antigen presenting cells at the muscle injection site. It is possible, however, that antigen presenting cells are still involved in the adjuvant activity of the IFNs since they may be resident or attracted by the wounding caused by injection or by the vector.

The mechanism of protection of the IFN adjuvanted DNA vaccine against ISAV is unknown

The mechanism of protection obtained by injection of IFN-plasmid together with the HE-plasmid is uncertain since the antiserum from the vaccinated fish showed low neutralizing activity against the virus, which was not significantly different from serum of fish injected with control plasmids. Poor ISAV-neutralizing activity was also observed by antiserum from Atlantic salmon immunized with a high dose of inactivated ISAV (198). Non-neutralizing antibodies may contribute to protection by increased phagocytosis and destruction of virus with antibody-dependent or complement-mediated cytotoxicity (ADCC, CDCC), but this has yet to be shown for fish antibodies. The role of cytotoxic T-cells in the protective immune response has to be examined in future studies.

Kinetics of the antibody response against ISAV upon vaccination with DNA vaccine containing IFNa and IFNc plasmids as adjuvants

In paper IV, we compared the kinetics of the antibody response against ISAV in DNA vaccines using pIFNa or pIFNc as adjuvants over a period of 22 weeks after plasmid injection. The results showed that pIFNc caused a delayed increase in antibody response compared to pIFNa as adjuvant.

Similar antibody kinetic studies have not yet been performed in DNA vaccinated salmonids earlier. However, high titres of neutralizing antibodies were found in rainbow trout after multiple DNA vaccinations against VHSV and IHNV 38 and 45 days after vaccination (244). In another study, neutralizing antibodies were observed in trout 3 months after DNA vaccination against IHNV, but not at later time points although protection was observed 3 to 25 months after vaccination (247). In carp, DNA vaccination with a β-galactosidase construct showed appearance of antibodies against β-gal at day 7 pv, which increased until day 14 pv and kept at this level throughout the 70 day trial period (290). The earlier antibody response in carp is likely to be due to the higher temperature (22° C) used for this fish species.

Longevity of the injected IFN plasmids in the fish muscle.

The innate immune response of pIFNc triggered Mx protein in liver shows strong and slightly increase after fish injected with pIFNc during the whole 22 week trial period. This suggests that cells transfected with pIFNc may express IFNc for an even longer time period. Longevity of IFNc expression is probably due to the fact that IFNc is a self-antigen in salmon. Cells transfected with pIFNc are thus not likely to be attacked by the adaptive immune system of salmon. The long-lasting expression of a transgene after DNA vaccination has also been shown for luciferase in Atlantic salmon, glass catfish and mice (291-293). In addition, the luciferase activity in salmon muscle was highest at day 10 after injection then declined, but was still detectable 535 days after injection (291). Taken together, the longevity of luciferase in plasmid-injected fish and mice suggests that cells expressing luciferase are not attacked by the immune system the due to the poor immunogenicity of luciferase or its intracellular localization(294).

Evidence for elimination of cells expressing the HE construct by the adaptive immune system

In contrast to the long-lived systemic expression of Mx and ISG15 in fish injected with IFNc plasmid alone, fish co-injected with pIFNc and pHE showed disappearance of Mx and ISG15 expression in liver at 16 and 22 weeks after injection. This observation suggests that transfected cells at the muscle injection site have taken up both pIFNc and pHE plasmids and that the HE-expressing cells were destroyed by HE-specific adaptive immune response after 16 weeks immunization. Another possible explanation for destruction of HE-expressing cells could be that IFNc mediated attraction of natural killer cells (NK cells) , which attacked HE-expressing cells. If so, however, the disappearance of Mx/ISG15 should have happened before week 7 post plasmid injection since the HE is expressed in fish at week 1 post vaccination (295).

Information from other DNA vaccination studies may give some explanation for the mechanisms involved in immune mediated destruction of HE-expressing cells. In rainbow trout, the decrease of luciferase activity was observed when plasmid coding for luciferase was co-injection with G-protein encoded plasmid (296). It was suggested that plasmid transfected host cells were killed by the raised cellular immune response (296). Moreover, myocytes expressing G-protein declined rapidly at day 31 to 38 post vaccination (297). Taken together, this suggested that cells expressing antigen were destroyed by adaptive immune mechanisms (296, 297). Cytotoxic T-cells might be involved in this, but antibody dependent mechanisms could not be excluded. The macrophages, B-cells and activated T-cells with the complement system might also have been attracted to the injection site and eliminated the expression of G-protein (297).

In mice DNA vaccinated with luciferase- and HBsAg-expressing vectors, the destruction of HBsAG-expressing myocytes was found to be dependent on MHC II restricted CD4+ T cell activation, but was not mediated by MHC I restricted or perforin-mediated lysis (294, 298). Destruction of HBsAg -expressing myocytes was suggested to be antibody dependent since HBsAg was expressed on the cell surface and since myocyte destruction was associated with an HBsAg specific antibody response (298). Therefore, the mechanism of antibody-directed complement-mediated cytotoxicity and Fc receptor mediated antibody-dependent cytotoxicity (ADCC, CDCC) as shown in Fig. 6 that were suggested for the destruction of cells expressing HBsAg protein (299, 300). This leads to the hypothesis that DNA vaccination

of Atlantic salmon with ISAV HE is associated with an antibody dependent process since HE is expressed on the surface of muscle cells and the disappearance of Mx-expression in fish vaccinated with pIFNc + pHE occurred concomitant with occurrence of peak antibody levels.

The role of T-cells has to await development of adequate assays for Atlantic salmon. At present little is known about induction of cytotoxic T-cell responses upon vaccination of salmonids and other fish species. However, cell-mediated cytotoxicity has been detected in rainbow trout after vaccination with the VHSV G-protein plasmid (301). The disappearance of the HE/IFNc construct occurred much later than the reported disappearance of G-protein in DNA vaccinated rainbow trout is uncertain whether this difference is due to the antigen itself or the way expression has been measured.

5. Future studies

There are a lots more to study of IFN-I in innate and adaptive immunity. Functional study of the role of disulfide bridge that compare to mammalian IFN-I could be important for understanding the stabilization of IFN-I protein. The finding of paring of IFN-I and receptors in different organs could possibly explain the regulatory effect of IFN-I in response to virus infection. As described above, interferon receptor knockout cells have been used as a powerful tool for investigating the function of IFN-I in mammals, thus if we could establish a similar IFN-I receptor deficiency models which would be beneficial in study both innate and adaptive immunity. In mammals, the IFNR deficient cell could be used for study the correlation of IFN-I triggered antiviral activity (302). Besides, lymphocytes with IFNR deficiency could be used for study the effect of IFN-I in antibody production and T cells activation (303).

For the understanding of what mechanism are involved and of which cells are play the major role in triggering humoral or cellular immune response. It would be interesting to know which immune cells are attracted by IFN-I expressed plasmid at muscle injection site, this could be done by microarray or by RNA-Seq analysis. Immunohistochemistry would be a necessary tool for characterization of cells that are involved. Use of antibodies against markers of different immune cells such as B and T lymphocyte or antigen presenting cells would be important tools for analysis of cell population after interferon treatment. Clonal

salmon could be beneficial for cytotoxicity study in T cells, that was considered the major response triggered by DNA vaccine.

In addition, regional B or T cells have been found in the mucosal system, thus study of IFN-I in regional lymphocytes could be important for investigating of the early response after virus infection and the link of innate immunity with antibody response.

6. Prospects of applications

The results from paper I and paper II suggest that i.m. injection of IFNc plasmid might possibly be used as a new method to combat virus infections in farmed salmon since it induces antiviral genes in the fish over a relatively long time period that would at least cover the release of smolts into the sea, which is a critical period in the production.

The benefit of using IFNs in prophylaxis against virus infections is that they induce genes with a broad spectrum of antiviral properties while conventional DNA vaccines are directed towards specific pathogens. Interestingly, DNA vaccines against the G-protein of salmonid rhabodviruses also gives non-specific protection against virus-infection, which was suggested to be caused by IFN-induction (304). However, the non-specific protection obtained in these G-protein vaccines only lasts a few weeks and then disappears.

The present demonstration of adjuvant effects of IFN expression plasmids provides a novel method for improving DNA vaccination of fish. This is important since only DNA vaccines against fish rhabdovirus based on the G-protein have until now shown satisfactory protection against virus infection. A benefit of DNA vaccines is that they induce both humoral and cell mediated adaptive immune responses because the protein antigens are produced within the host cells (305). Moreover, they are easy to accommodate to various virulent virus strains and can be prepared even against viruses that cannot be grown in culture. DNA vaccines are also safe to use and show less side effects than traditional fish vaccines, which have to be delivered in oil adjuvants to give a protective effect. Besides, our results of using IFN-I as adjuvant reveal the possibility of using IFN-I in both traditional and DNA vaccination.

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