Effect of viral RNA mimics and ISA virus infection on expression of key genes of the Atlantic salmon interferon system

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Abbreviations

AP-1	Activated protein-1
APC	Antigen presenting cells
ATF-2	Activated transcription factor-2
CCV	Channel catfish herpesvirus
CD	Clusters of differentiation
CPSF	Cleavage and poly-adenylation specificity factor
CpG	Cytosine-phosphate-guanine
CTL	Cytotoxic T lymphocyte
DAI	DNA-dependent activator of IFN regulatory factors
DC	Dendritic cell
DRAF	dsRNA activated factor 1
ds	double stranded
ELF	Elongation initiation factor
HIV	Human immunodeficiency virus
IFN	Interferon
IFNAR	IFN-α/β receptor
IHNV	Infectious haematopoietic necrosis virus
IκB	Inhibitor of NF-κB
IKK	IκB kinase
IL	Interleukin
IPNV	Infectious pancreatic necrosis virus
IRAK	IL-1 receptor associated kinase
IRF	IFN regulatory factor
ISAV	Infectious salmon anemia virus
ISG	IFN-stimulated gene
ISGF3	IFN-stimulated gene factor-3
ISRE	IFN-stimulated response element
LPS	Lipopolysaccharide
MDA5	Melanoma-differentiation-associated gene

mDC	myeloid DC
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation factor 88
NEMO	NF-κB essential modifier
NF-κB	Nuclear factor-κB
NK	Natural killer cells
NS	Non-structural
OAS	Oligoadenylate synthetase
ORF	Open reading frame
pDC	Plasmacytoid DC
PKR	Protein kinase R
PKZ	Z-DNA binding protein kinase
Poly I:C	Polyinosinic polycytidylic acid
RIG-I	Retinoic-acid-inducible gene I
Rip1	Receptor interacting protein-1
RNA	Ribonucleic acid
rSasa IFN–α1	Recombinant salmon IFN–α1
ss	Single stranded
STAT	Signal transducers and activators of transcription
TBK	Tank-binding kinase
THOV	Thogoto virus
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF-related-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein inducing IFN-β
WNV	West Nile virus
VHSV	Viral hemorrhagic septicemia virus

Summary

Atlantic salmon is the predominant species in Norwegian aquaculture industry, and viral outbreaks cause heavy economical losses every year. Although farmed salmon appears to be very sensitive to viral infections, this species and all bony fishes in general possess an immune system fully capable of handling these pathogens. In this work we have studied the type I interferon (IFN) system of salmon, which is critically important in the innate immune defences against viruses in all vertebrates. IFN is produced by virus infected cells and is secreted as an alarm protein that induces an antiviral state in neighbouring cells. This antiviral state is mediated by a variety of antiviral proteins encoded by IFN-stimulated genes (ISG).

The first stage in activation of the IFN system is induction of IFN transcription upon recognition of pathogens. Viruses are detected by a variety of cellular receptors which recognize conserved features associated with viral nucleic acids. These receptors are thoroughly studied in humans and mouse and even though the data in fish is scarce, genetic studies indicate that fish also possess similar receptors. We have investigated the stimulatory effect of two synthetic ligands that mimic single stranded (ss) and double stranded (ds) viral RNA and compared the immune response against the orthomyxovirus infectious salmon anemia virus (ISAV), which possess a segmented ssRNA genome. Polyinosinic polycytidylic acid (poly I:C) mimics viral dsRNA that is recognized by receptors in all nucleated cells. Our studies show that poly I:C induces IFN transcription both in vivo and in vitro.

Imidazoquinolines, in this work represented by S-27609, are nucleotide analogues that mimic ssRNA and is recognized by receptors in immune cells that respond to viral infection by producing high levels of IFN. S-27609 proved to be a potent inducer of IFN in salmon, but showed no stimulatory effect in salmon TO cells grown in culture. ISAV proved to be a powerful inducer of IFN both *in vivo* and *in vitro*. However, ISAV infection of TO cells

induced IFN transcription at considerably later time points compared to the kinetics observed after poly I:C stimulation.

Viral recognition by cellular receptors activates signalling pathways that regulate transcription of IFN and ISGs. The major regulator of IFN transcription in mammals is IFN regulatory factor (IRF) – 7, and we have studied the structure and function of a salmon IRF-7 like protein. Phylogenetic studies indicate close evolutionary relationship between salmon and other fish IRF-7, and a more distant to IRF-7 of higher vertebrates. At the structural level we identified several domains of the protein important for its function as an IFN regulatory transcription factor that is activated by virus infections. Functional studies revealed that IRF-7 expression is inducible by poly I:C, recombinant salmon IFN and ISAV infection, and that the protein is a positive regulator of the IFN promoter.

Activation of IRF-7 and other transcription factors results in transcription and synthesis of IFN. The secreted IFN binds to surface receptors present on all cells, and activates a signalling cascade that induces transcription of ISGs. Two of the best described ISGs are Mx and ISG15. Mx possesses antiviral properties against many viruses including infectious pancreatic necrosis virus (IPNV) that also infect salmon. Salmon responds to poly I:C and S-27609 stimulation by expressing high levels of Mx and ISG15 transcripts. Similar responses are seen after ISAV infection. However, TO cells respond almost immediately to ISAV infection by inducing Mx and ISG15 transcription even though IFN transcription is impaired. This suggest not only that ISAV antagonize IFN at the transcriptional level, but also that TO cells are able to synthesize important ISGs independent of IFN stimulation. Even though the IFN system of salmon is activated during ISAV infection, it does not protect the host against infection. This is best seen when stimulating TO cells with recombinant salmon

IFN prior to infection. IFN treatment makes TO cells resistant against IPNV infection, but IFN is not able to protect the cells against ISAV.

Orthomyxoviruses produce IFN antagonistic proteins during replication, and we have investigated two candidate genes from the ISAV genome. The two ISAV genes, known as S7ORF1 and S8ORF2, have different functions and are localized to different compartments in the infected cell. Using an IFN-promoter luciferase assay we observed that both genes had the ability to down regulate activation of the IFN promoter. The negative impact on the IFN promoter, the impaired IFN transcript kinetics in TO cells and the inability of recombinant salmon IFN to protect TO cells against ISAV infection indicate that the virus have adopted strategies that circumvent the actions of the IFN system.

List of papers

I. **Kileng, Ø., Brundtland, M. I. and Robertsen, B.;**

Infectious salmon anemia virus is a powerful inducer of key genes of the type I interferon system of Atlantic salmon, but is not inhibited by interferon (2007) Fish & Shellfish immunology 23; 378-389.

II. **Kileng, Ø., Albuquerque, A. and Robertsen, B.;**

Induction of interferon system genes in Atlantic salmon by the imidazoquinoline S-27609, a ligand for Toll-like receptor 7 (2007) Fish & Shellfish immunology 24; 514-522.

III. **García-Rosado, E., Markussen, T., Kileng, Ø., Bækkevold, E. S., Robertsen, B., Mjaaland, S. and Rimstad, E.;**

Molecular and functional characterization of two infectious salmon anaemia virus (ISAV) proteins with type I interferon antagonizing activity (2008) Virus Research 133; 228-238.

IV. **Kileng, Ø., Workenhe, S. T., Bergan, V. and Robertsen, B.;**

An Atlantic salmon IRF-7 gene identified by analysis of sequence and function and its expression compared with a STAT1 gene (2008). Manuscript.

1. Introduction

Farmed Atlantic salmon is highly susceptible to viral infections and viral diseases results in reduced production and economical losses in the Norwegian aquaculture industry every year. Recently the aquaculture industry in Chile was struck hard by outbreaks of infectious salmon anemia virus (ISAV), showing that viral infections cause problems for the industry on a global scale. The growth of the fish farming industry, and its problems with viral infections, has lead to an increased interest in understanding the immune system of fish.

The type I interferon (IFN) response is of crucial importance for immunity against viruses. IFN possess a central position in innate immunity and is essential for regulation of adaptive immune responses. Accordingly, viruses have developed mechanisms to antagonize the IFN response. Little is known about IFN induction pathways in fish and virtually nothing is known about how viruses antagonize IFN production in fish host cells. The main objective of this work has been to study functional aspects of the IFN system of salmon in response to viral infections. Firstly, we have compared the induction of IFNs and ISGs upon stimulation of two synthetic compounds that mimic viral RNA and that induce IFNs via different receptors and pathways in mammals. The dsRNA poly I:C induces IFNs via the RIG-I/MDA5 pathway or the TLR3 pathway, which activates IFN-β transcription through the activation of IRF-3, IRF-7 and NF-κB. The imidazoquinoline S-27609 induces IFN-α via the TLR7 pathway, which activates IRF-7. Secondly, because IRF-7 plays an important role in the two pathways, we have cloned and functionally studied an IRF-7 – like gene from salmon. Thirdly, we have chosen infectious salmon anemia virus (ISAV) as a model to study the interactions between the IFN system of salmon and viruses. These studies show that the IFN system of salmon is similar to that of higher vertebrates. However, because our knowledge about fish immunity is still rather limited, the introduction will focus on the mammalian IFN system followed by a summary of the present status in fish.

1.1 The innate immune system

The innate immune system is often referred to as the first line of defence against pathogens. This terminology is quite obvious because it starts with the physical barriers represented by skin and mucus and expands to defence mechanisms inside all nucleated cells. When pathogens infect a host, their presence is recognized by cellular receptors which trigger the cell to initiate countermeasures to limit their spread. These countermeasures includes synthesis of effector proteins that function inside the cell, and cytokines that are secreted as alarm signals to warn neighbouring cells of imminent danger. In addition, the innate immune system have specialised effector cells (e.g. dendritic cells (DCs), natural killer cells (NK) and macrophages) ready to combat pathogens as they arrive. These effector cells constitute a small fraction of the total cells in the blood. Although few in numbers, these cells probe peripheral tissues where they phagocytose foreign particles or infected cells in search of pathogens.

Cytokines are proteins that regulate important cellular functions such as growth, immune activation and defence against invading pathogens. Cytokines also regulate the adaptive immune system by promoting B and T cell differentiation, and thus serve to bridge innate and adaptive immunity [1,2]. Among the cytokines, the interferons (IFN) were discovered based on their capacity to induce cellular resistance to viral infections. Given their ability to interfere with influenza virus infection in chicken cells, these cytokines were named interferons by Isaacs and Lindenmann in 1957 [3].

1.2 Interferons

Since their discovery in 1957, the numbers of IFNs has expanded and are now commonly grouped into type I, type II and type III IFNs. This classification is based on sequence homology and which receptor complex they use for signalling.

The mammalian type I IFNs constitute a multi-member cytokine family which include multiple subtypes of IFN-α and a single member of IFN–β, –ε, –κ, –ω, –δ and –τ [4]. Human type I IFN genes are clustered on chromosome 9 and all lack intron structures. Of these, only IFN-β and the multiple subtypes of IFN-α have immunological functions.

IFN-α/β are produced and secreted as a response to virus infection by all nucleated cells, and they induce an antivirale state by binding to the IFN-α/β receptor complex (IFNAR) that is ubiquitously expressed on the surface of cells [5,6]. In addition, a specific subset of immune cells known as plasmacytoid DCs (pDCs) are identified as the main producers of IFN-α/β upon stimulation [7].

The mammalian type II IFN consists of one member and is synonymous with IFN-γ [8]. The human IFN-γ gene is located to chromosome 12, and the gene contains three introns [9]. IFN-γ is produced exclusively by T- and NK-cells and has important functions later in the immune response [10]. In the innate immune response, production of IFN-γ is mediated by NK-cells stimulated by interleukin 12 (IL-12) and IL-18, and in the adaptive responses by CD4 T helper 1 (Th1) lymphocytes and CD8 cytotoxic T lymphocytes (CTL) as a response to antigens presented on major histocompatibility complexes (MHC) [11,12]. IFN-γ utilize a different receptor than the IFN-α/β receptor and induces a variety of genes including several with antiviral properties [6,13].

The latest IFNs to be discovered where the type III IFNs, termed IFN-λ1, –λ2 and –λ3 (also known as IL-28A, IL-28B and IL-29) [14,15]. This family of IFN is also produced by virus infected cells and they stimulate cells to produce the same antiviral proteins as type I IFNs do. Even though type III IFNs share functional characteristics with the type I, they are different in many aspects. Above all the type III IFNs use a different receptor complex and the gene structure are different from the type I IFNs [16].

As the main focus of this work is antiviral activity exerted by type I IFNs, they will henceforth be referred to as IFN.

1.3 Receptors involved in pathogen recognition

The vertebrate cell has numerous receptors involved in detection of pathogens. In contrast to the receptors involved in adaptive immunity, the innate receptors are germ line encoded and not generated by rearrangements and hyper mutations of the receptor genes. The innate receptors detect conserved structural features associated to pathogens such as DNA, RNA, lipids, polysaccharides and peptides.

There are several receptors that detect viral DNA and RNA. The most thoroughly studied are RIG-I/MDA5 and Toll-like receptors (TLR). RIG-I and MDA5 are located to the cytoplasm of most cells, and they recognize ssRNA and dsRNA respectively. These two receptors are now considered to be the main inducers of IFNs and ISGs in non-immune cells. The different TLRs are predominantly present in immune cells even though some are found in non-immune cells. These are either found in endosomal compartments or as extracellular receptors. The endosomal TLRs detect RNA or DNA of bacterial and viral origin, and the extracellular TLRs detect protein or lipid structures from pathogens.

The different receptors will be addressed in the following sections.

1.3.1 Cytoplasmic receptors

In the cytoplasm of most cells several receptors linked to the IFN system have been described. These receptors have a ubiquitous distribution and they detect DNA or RNA from a variety of pathogens.

Retinoic-acid-inducible gene I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA5) are Dex (D/H) box RNA helicases located to the cytoplasm that applies synthetic and viral RNA as ligands [17,18]. RIG-I detects *in vitro* transcribed double stranded (ds) RNA and 5´-triphosphate single stranded (ss) RNA generated by viral polymerases during replication of viruses, and binding of the ligands to the receptor induces synthesis of IFNs in response to infections of paramyxoviruses, influenza viruses and Japanese encephalitis virus [19-21]. MDA5 is reported to detect the synthetic dsRNA polyinosinic-polycytidylic acid (poly I:C) and picornaviruses, and is able to activate the IFN system through similar mechanisms as RIG-I [21,22]. RIG-I and MDA5 are now considered to be the main inducers of IFN in non-immune cells upon virus infections, and signalling through these receptors is an essential feature of host immunity to infections by RNA viruses [23].

Located to the cytoplasm are several other receptors that detect different forms of RNA and DNA from pathogens. The protein kinase R (PKR) is an IFN induced dsRNA-activated kinase with antiviral and antiproliferative activity [24]. Recognition of dsRNA results in activation of PKR, and the main function of PKR is to inhibit protein synthesis in the cell through phosphorylation of a factor called eIFα2 [25]. Upon stimulation of dsRNA, PKR is also able to activate NF-κB which participates in the transcription of IFNα/β [26,27]. In addition, fish possesses a PKR-like eIFα2 kinase which is activated by dsDNA, the Z-DNA binding protein kinase (PKZ)[28-30]. PKZ have Zα binding motifs which specifically bind dsDNA with the left-handed Z-conformation, a high energy form of DNA that is formed by negative supercoiling generated by RNA polymerases [31-33].
Recently another cytosolic Z-DNA binding sensor termed DAI (DNA-dependent activator of IFN regulatory factors) was reported [34]. DAI detects cytosolic DNA and induces IFN transcription through the transcription factor IRF-3 [34-37].

1.3.2 Toll-like receptors

The TLRs are a class of pattern recognition receptors that detect a broad range of pathogens, including bacteria, fungi, protozoa and viruses [38-41]. The TLRs 1, 2, 4, 5 and 6 seem specialised in detecting mainly bacterial products such as di- and tri-lipopeptides, lipoproteins, peptidoglycan, LPS and flagellin (reviewed in [40]). In contrast, the TLRs 3, 7, 8 and 9 specialize in viral detection and recognize single and double stranded nucleic acids [40]. Another unique feature of these receptors is that only TLR 3, 4, 7 ,8 and 9 mediate expression of IFN α/β genes [42]. The ability of the nucleic acid sensing TLRs to discriminate between self and non-self is not mediated by the nature of the receptors or the ligands, but is due to the cellular localization of the receptors in the cell. These TLRs are located to and detects nucleic acids in endosomal compartments where they normally have no accessibility to the cells own DNA or RNA [43-48].

1.3.2.1 TLR3

Many viruses possess a genome that consist of dsRNA, or dsRNA is produced as an intermediate by many viruses during their replication cycle [8]. TLR3 is localized to endosomes in the cell and is a receptor for synthetic dsRNA such as poly I:C and its mismatched analogue poly I:C_{12}U [49,50]. TLR3 is expressed by DCs, NK, macrophages and epithelial cells [49,51-55]. NK cells possess the strongest expression of TLR3, and stimulation with poly I:C augments NK cell-mediated cytotoxicity and results in production of proinflammatory cytokines like IL-6 and IL-8 [55,56].

Binding of synthetic dsRNA to TLR3 results in expression of IFNs and subsequent resistance against various viral pathogens [6], but at the same time it seems that TLR3 is less important for the initial, cell-autonomous recognition of viral infection that results in the first wave of IFN α/β production [57]. In accordance to these findings TLR3- deficient mice were

no more susceptible to infections of dsRNA, ssRNA and DNA virus infections than normal mice [58]. In the case of West Nile virus (WNV) it appears that this virus in fact benefits from the interactions with TLR3 [59]. The biological significance of TLR3 in fighting virus infection appears rather obscure and some authors have raised the question if it has a role at all [58]. Other studies have in addition reported that TLR3 preferentially recognizes synthetic dsRNA rather than virus-derived dsRNA [60] and that TLR3 trigger transcription of IFN after delivery of extracellularly dsRNA [61,62]. TLR3 is located to endosomal compartments in immune-cells, and this might explain the preference for extracellularly delivered ligands [48,63].

The function of TLR3 in immunity is now identified as a linkage between innate immunity and adaptive, cell-mediated immunity. Dendritic cells (DC) is a specialized type of antigen presenting cells (APC) that acquire novel antigens, presents these in MHC-II complexes and then direct the activation and proliferation of antigen-specific T-cells [64]. In addition, DCs are able to present peptides in MHC-I complexes derived from exogenous antigens in a process known as cross-presentation [65]. This ability resides almost exclusively with the $CD8\alpha^+$ DCs [66], a specific subset of DCs in which TLR3 is predominantly expressed [67].

As $CD8\alpha^+$ DCs phagocytose foreign antigen, the antigen is transferred to endosomes for processing. The presence of TLR3 in endosomes enables the receptor to recognize antigen derived dsRNA, which in turn activate maturation of the cell. The maturation process activates and enhances the $CD8\alpha^+$ DCs ability to display antigen in MHC I, which in turn are recognized by specific CD8 T-cells. The onset of $CD8\alpha^+$ DCs maturation and presentation of exogenous antigen on MHC I are shown to be dependent of TLR3 [68], and it has been shown that stimulation of $CD8\alpha^+$ DCs by IFN is important to boost this process [69].

1.3.2.2 TLR7, 8 and 9

The TLRs 7, 8 and 9 are receptors that detect viral and bacterial RNA and DNA, and similar to TLR3 they are localized to endosomes in the cells [63,70-72]. In both human and mice TLR7 is activated by viral ssRNA from influenza, vesicular stomatitis virus and Sendai virus [43,46,73-75]. In addition synthetic ssRNA nucleotides and several synthetic nucleotide analogues called imidazoquinolines represented by Imiquimod, S-27609 and R-848 are also reported to function as ligands [43,76,77]. Apparently TLR7 is not able to discriminate between foreign and self RNA, but studies have shown that this receptor has affinity for uracil repeats normally found in the 3´UTR of the genomes of RNA viruses [43,75,78]. TLR7 is found in many tissues, but expression is predominantly in B-cells, monocytes and pDCs [55,79].

TLR8 appears to recognize the same ligands as TLR7, but there are some peculiarities regarding the affinity for synthetic agonists and the natural ligand ssRNA between TLR7 and TLR8 in human and mice. Human TLR7 and TLR8 and murine TLR7 all detect viral and synthetic ssRNA, in contrast to murine TLR8 that need additional stimulation by CpG rich DNA [75,80,81]. TLR8 appear to have a broader cellular and tissue expression than TLR7 and TLR9. In humans, the strongest TLR8 mRNA expression was observed in monocytes and it was also readily detected in myeloid DCs (mDC), macrophages and Langerhans cells [55,82,83].

TLR9 detects unmethylated CpG-rich DNA from bacteria and viruses [84,85]. Unmethylated CpG-rich DNA is mainly present in genomes of prokaryotes and viruses. Generally, vertebrate DNA is methylated wherever a cytosine is followed by a guanosine thus providing the DNA with a unique signature compared to prokaryote organisms. The ability to distinguish between pro- and eukaryote DNA renders TLR9 the only TLR with the ability to

recognize specific nucleotide motifs. The distribution of TLR9 is limited to pDC and B-cells and is therefore localized to the same cells as TLR7 [54,55,86].

1.4 Signalling pathways

1.4.1 The RIG-I/MDA5 signalling pathway

Upon recognition of viral RNA by RIG-I and MDA5, both receptors initiate signalling pathways that regulate the transcription of IFN genes [17] (Fig. 1). Both receptors have two tandem N-terminal caspase activation and recruitment domains (CARD) which is important for the transduction of signals through the signalling pathways resulting in activation of specific transcription factors [87]. After ligand recognition, the CARD-domains are ubiquitinated by the tripartite motif protein 25α (TRIM25α) [88]. This modification is critical for the ability of RIG-I to induce signal transduction and for recruitment of the downstream adaptor protein MAVS [87,89-91]. MAVS (mitochondrial antiviral signalling adaptor, also called IPS-1, VISA or CARDIF) is associated to the mitochondrial outer membrane, and possess a CARD-domain important for its recruitment by RIG-I and MDA5. The interactions between RIG-I and MAVS triggers recruitment of a regulatory component known as NF-κB essential modifier (NEMO, also known as IKKγ), an adaptor that bridges the activation of IRF-3/IRF-7 and NF-κB [92]. Another important factor recruited by MAVS is tumor necrosis factor receptor-associated factor 3 (TRAF3) which activates the two kinases IKKε and TBK1 [93].

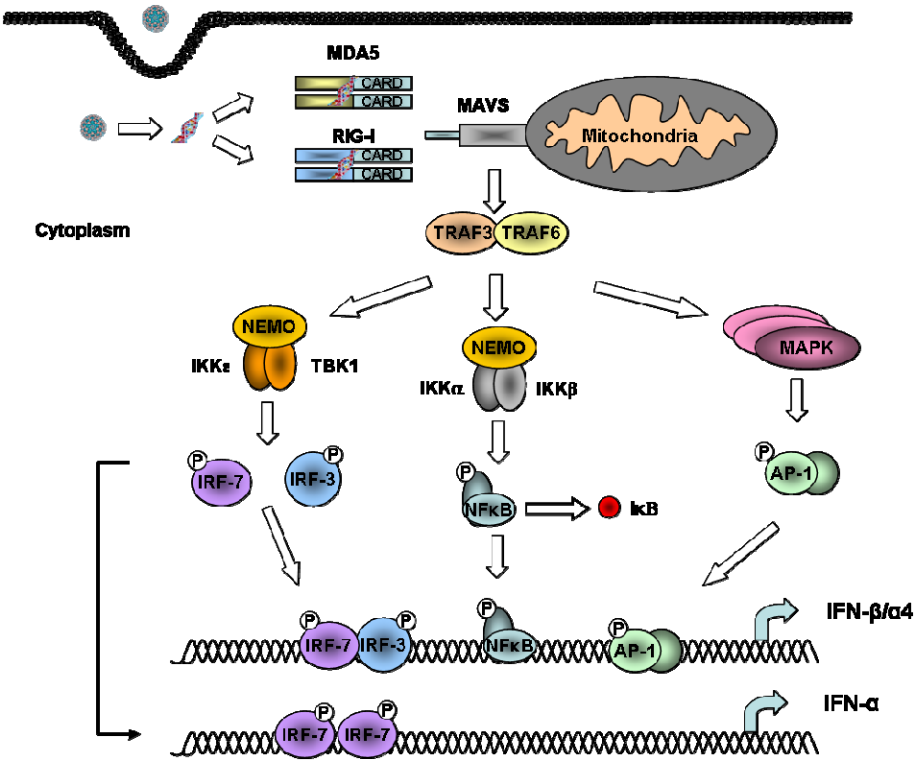

Figure 1. The RIG-I/MDA5 signalling pathway. RIG-I and MDA5 recognize viral RNA and interact through their CARD-domains with the mitochondrial-associated adaptor MAVS. MAVS activates several signalling pathways leading to phosphorylation of the transcription factors IRF-3, IRF-7, NF-κB and AP-1 via IKKε/TBK1, IKKα/IKKβ and MAP kinases. Modified from [92,94].

1.4.2 TLR3 signalling pathway

TLR3 is sole member of the TLR receptors that utilize the TIR domain containing adapter inducing IFN-β (Trif, also known as TICAM1) as an adaptor to activate transcription [95,96] (Fig. 2). While all the other TLRs can utilize MyD88 as adaptor for downstream signalling, the latter is not involved in TLR3 activation, and this pathway is thus referred to as the MyD88-independent pathway. Trif relay signals from TLR3 to Rip1 and TRAF6 for the activation of NF-κB and AP-1 [97,98] and Trif is also able to interact with TBK1 and IKKε in order to phosphorylate IRF-3 and IRF-7 [96]. Critical in the activation IRF-3 and IRF-7 through Trif is the recruitment of TRAF3 as a regulator of TBK1 [93]. Trif is also utilized by

TLR4, but TLR4 apply an additional adapter called Trif-related adapter molecule (TRAM) that connects TLR4 to Trif. [95,99].

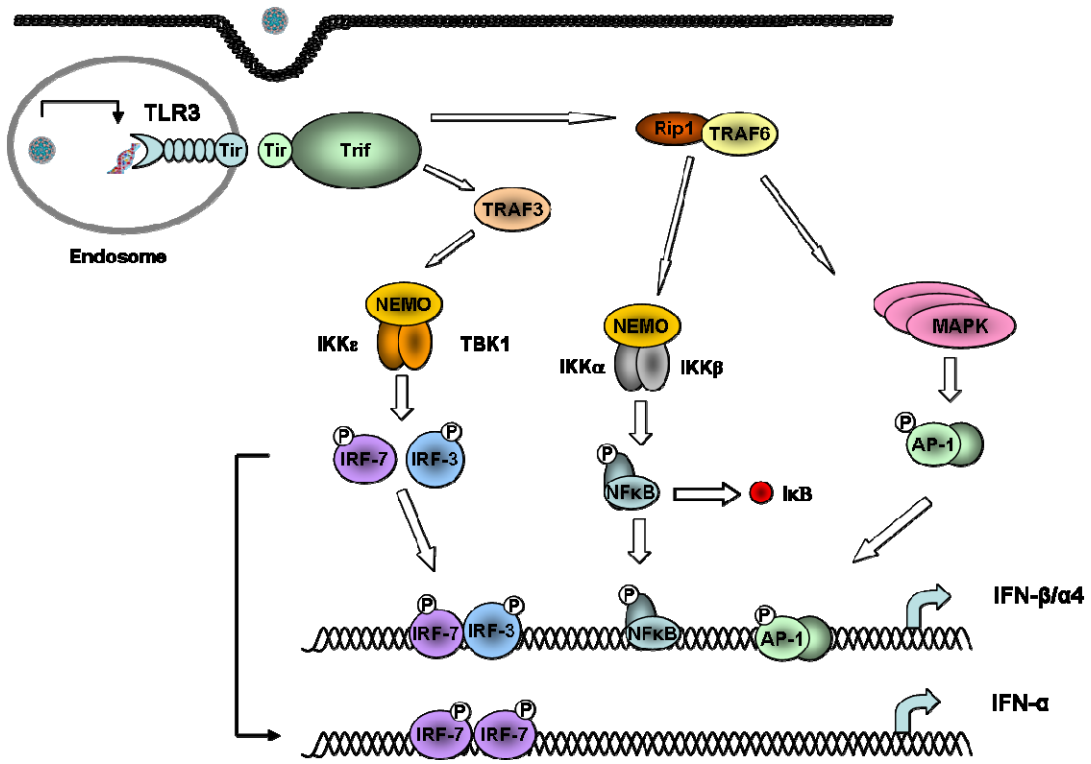

Figure 2. The TLR3 signalling pathway. TLR3 is located to endosomal vesicles and recruits the adapter Trif upon detection of dsRNA. Trif recruits the adaptor TRAF3 which interact with IKKε/TBK1, which activate IRF-3 and IRF-7 through phosphorylation. Trif also relay signals to Rip1 and TRAF6. This results in phosphorylation of NF-κB via IKKα/IKKβ and AP-1 via the MAP kinase pathway. Modified from [94].

1.4.3 TLR7, 8 and 9 signalling pathway

The signalling pathway activated by ligand binding to TLR7, 8 and 9 is specific for immune cells and is part of what is referred to as the MyD88-dependent pathway. As the name implies MyD88 is the central adaptor that is associated to the TLRs, and it is used to a

different extent by all TLRs except TLR3 [94]. Whereas TLR5, 7, 8, 9 and 11 is associated directly to MyD88, TLR1, 2, 4, and 6 use additional adaptors as a linker to MyD88 [94].

In pDCs, MyD88 is associated directly to the TLRs, and upon ligand binding MyD88 recruits members of the IL-1 receptor-associated kinase (IRAK) family and TRAF6 [100-103]. MyD88, IRAK1, IRAK4 and TRAF6 form a signalling complex that activates IRF-7 by phosphorylation and ubiquitination (Fig.3).

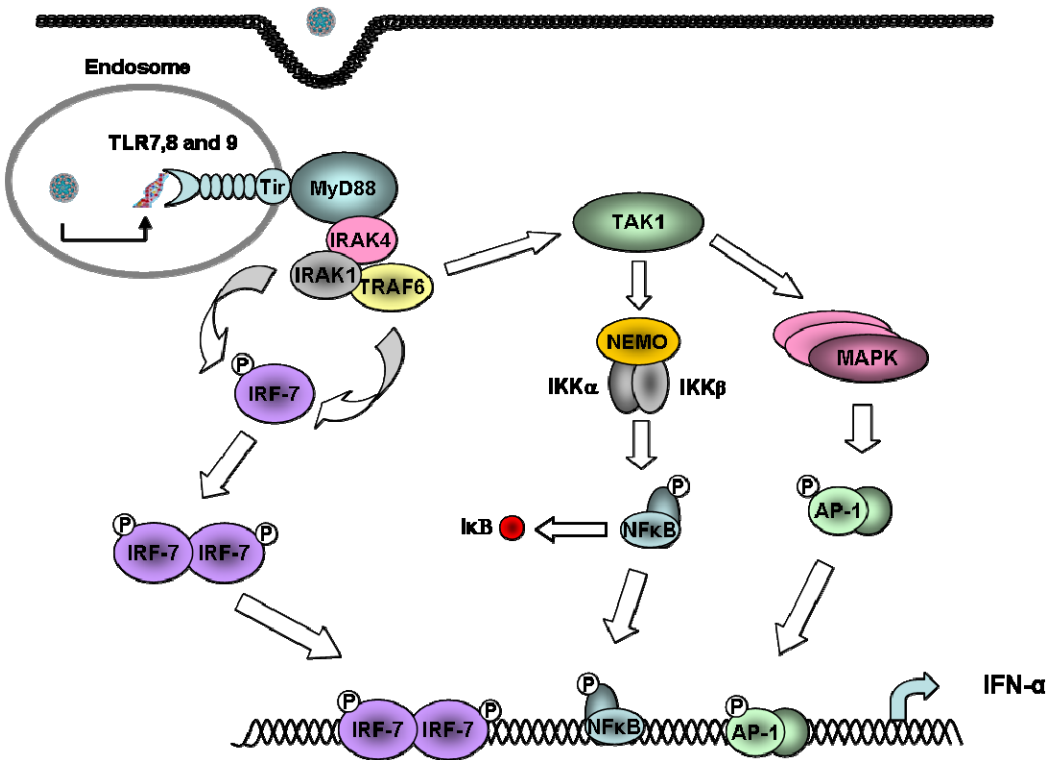

Figure 3. The TLR7, 8 and 9 signalling pathway. TLR7, 8 and 9 reside in endosomal vesicles in pDCs. Upon detection of ssRNA (TLR7 and 8) or CpG-rich DNA (TLR9) the respective TLRs form a complex with Myd88, IRAK1, IRAK4 and TRAF6. This complex phosphorylates IRF-7, which dimerize and translocates to the nucleus. In addition, the MyD88 complex activates the pathways leading to activation of AP-1 and NF-κB. Modified from [94].

1.5 Induction of IFN transcription

Expression of IFN is induced rapidly in cells as a response to invading pathogens through the signalling pathways described above. Transcription of IFN-β is regulated by three different transcription factors known as IFN regulatory factors (IRFs), nuclear factor-κB (NF-κB) and c-Jun/ activated transcription factor-2 (ATF-2) [8]. C-Jun/ATF2 is generally referred to as AP-1. These transcription factors interact and form a multiprotein transcription-promoting complex called the enhanceosome that bind to regulatory domains of the IFN promoter [104]. The promoter of IFN-β contains specific sites recognized by the different transcription factors. Two IFN-stimulated response elements (ISRE) are recognized by IRF-3 and IRF-7, one site by AP-1 and a κB site is recognized by NF-κB [104,105].

Activation of AP-1 is activated by multi-kinase complexes through the Jun kinase pathway and will not be addressed further [106].

1.5.1 Interferon regulatory factors

The IRFs constitute a family of transcription factors in which nine members are found in mammals. Similar for all these proteins is the N-terminal DNA binding domain that contains five tryptophan residues. Through this domain they are able to recognize ISRE sites found in the promoters of IFN-β and the IFN stimulated genes (ISG) [107-109]. In general, all IRFs are constitutively expressed in most cells, but some of these are inducible by IFN, IFN-γ and TLR ligation. Gene disruption studies on most of the IRFs have been performed, and these studies show that they have distinct roles in modulating immune responses and in the development and function of immune cells [108,110,111]. Of the IRFs that participate in transcription regulation of IFN genes, IRF-3 and IRF-7 are the most important.

1.5.1.2 IRF-3 and IRF-7

IRF-3 and IRF-7 are highly homologous genes and they are considered to be the key regulators of IFN gene expression in response to virus infection [112]. In non-immune cells IRF-3 is constitutively expressed and is localized to the cytosol of unstimulated cells. IRF-7 has a weak constitutive expression sufficient to participate in activation of the IFN-α/β promoter, but can also be induced by IFN [113-115] . Activation of the IRF-3 and IRF-7 in non-immune cells are initiated by ligand binding to cytosolic receptors (RIG-I and MDA5), TLR3 or TLR4. This results in the activation of TBK1 and IKKε which phosphorylate specific serine residues in the C-terminal region of IRF-3 and IRF-7 [116,117]. This phosphorylation elicits a conformational change that allows the formation of IRF dimers and translocation to the nucleus where they interact with other transcription factors to initiate the transcription of IFNs. The heterodimer of IRF-3 and IRF-7 appears to be important for the initial transcription of the IFN genes upon virus infection of the cell.

In mammals, IRF-3 is a potent activator of the IFN-β and the IFN-α4 gene, but not on the remaining IFN-α genes [112,115,118]. In contrast, IRF-7 regulates transcription of both IFN-β and IFN-α genes, and is required for both the initial IFN-β induction and the late induction of IFN-α subtypes after virus infection [118,119]. These properties have resulted in a model where IRF-7 is the key regulator of the IFN transcription in non-immune cells [120,121].

IRF-7 also has an important function in pDCs, known for their ability to produce high amounts of IFN in response to ligand binding of TLR7 and TLR9 [7,43,100]. In the pDC, IRF-7 is constitutively expressed and is activated through the MyD88-dependent pathway. The ability of pDCs to rapidly produce IFN is not only due to the ready state of IRF-7, but also to their possession of TLR7 and TLR9 [122]. In pDCs it is observed that RNA and DNA localizes to endosomes for longer time than what is observed for other DCs. The retention of

ligands thus enables the TLRs to detect them more efficiently than in the other DCs and macrophages, in which RNA and DNA are translocated to lysosomes and degraded [122].

1.5.1.3 Other IRFs

IRF-1 was the first IRF to be discovered and was characterised as regulator of IFN responses. IRF-1 has several important functions including regulation expression of important ISGs such as 2', 5' oligoadenylate synthetase (OAS) and PKR [123-125]. In addition it is involved in development of NK-cells [126], in promotion of T_1H-cell responses[127,128] and apoptosis induced by DNA damage [129]. IRF-2 is a negative regulator of the transcription of ISGs. It binds the same promoter regions as IRF-1, and these two transcription factors compete for the same DNA-binding sites [130,131]. The most important role for IRF-2 may thus be to attenuate the IFN response in most cells.

IRF-4 is involved in the development of DCs, B- and T-cells, and it controls the transcription of IL4 [132-134]. Many of its function is in cooperation with IRF-8 [135-137]

IRF-5 is inducible by IFN and TLR ligation, and it was suggested that it was involved in regulation of IFN expression. However, a recent study has shown that IRF-5 is required for the induction of proinflammatory cytokines [138].

IRF-6 is the only gene where function is not determined, but recent studies suggest that it is involved in proliferation and differentiation of keratinocytes [139].

IRF-8/IFN consensus sequence-binding protein (ICSBP) is an immune system-specific member of IRF-family [113,137,140] and the protein is expressed at high amounts in the different subtypes of DCs in response to TLR ligation and virus infection [136]. Recent studies have also shown that IRF-8 is implicated in the direct regulation of IFN expression in DCs, but does this in a post-induction phase and as a response to the initial synthesis of IFNs

[141]. In addition, IRF-8 is essential for the development of the pDCs and the CD8α^+ DCs [136,142-145] and it is involved in the development of T_H1-cell responses [146-148].

IRF-9 is part of the transcription complex ISGF-3, which is responsible for the induction of the majority of ISGs [149].

1.5.2 NF-κB

NF-κB consists of a family of proteins that comprises several dimeric transcription factors that bind to discrete DNA sequences known as κB sites in promoter and enhancer regions of various genes [150]. In mammalian cells the NF-κB family is composed of five members, but the most frequently activated form of NF-κB involved in transcription of IFN is a heterodimer composed of RelA and p50 [151]. The RelA-p50 hetero-dimer is kept in an inactive state in the cytoplasm of unstimulated cells by the inhibitory actions of a family of proteins known as IκB-proteins (reviewed in [152]). Upon virus infection, the IκB proteins are phosphorylated by a protein complex composed of IκB kinase α (IKK α), IKKβ and NEMO. The phosphorylation of IκB-proteins by the IKK complex results in degradation by the 26S proteasome, releasing the RelA-p50 from the inhibition and thus allowing it to move to the nucleus.

1.6 IFN signalling through the Jak/STAT pathway

Binding of the secreted IFN to the IFN-α/β receptor (IFNAR) activates a signalling pathway that results in the expression of a specific set of genes called IFN stimulated genes (ISGs) [6] (Fig. 4). The IFNAR is composed of the two subunits IFNAR1 and IFGNAR2 and they are associated to two kinases, Jak1 and Tyk2, of the Janus tyrosine kinase (JAK) family [5]. IFN binds to IFNAR2 and this leads to recruitment of IFNAR1. The activation of the

receptor facilitate a cross-reaction between Jak1 and Tyk2, and these factors will phosphorylate the signal transducer and activator of transcription 1 (STAT1) and STAT2 at specific tyrosine residues. Upon phosphorylation STAT1 and STAT2 form a heterodimer that dissociates from the receptor and is translocated to the nucleus where they bind to IRF-9 to form a heterotrimeric transcription factor known as IFN-stimulated gene factor 3 (ISGF3) (reviewed in [153]). In the nucleus ISGF3 will bind to ISRE sites found in the promoters of ISGs. Among the ISGs are many proteins with antiviral properties such as Mx, ISG15 and 2′-5′ oligoadenylate synthetase (2′-5′ OAS) [6]. In addition transcription of other factors that enhance the IFN transcription are induced, the most important being IRF-7. In non immune cells IRF-7 will participate and enhance the late transcription of the IFN-α genes [118].

The importance of IFN signalling in the innate antiviral immune response is emphasized by studies in mice with deficient IFNAR genes [154]. This study showed that the IFNAR-deficient mice were extremely sensitive to viral infections and unable to control virus infections.

The pathways described above involve pathogen recognition by several receptors that result in the subsequent induction of an antiviral state in the cells by IFN signalling. However, synthesis of ISGs can be induced independent of IFN signalling and the key regulator of this alternative pathway is IRF-3 [155]. The first reported mediator of IFN-independent activation of ISGs was dsRNA, which activates IRF-3 and other factors to form a transcription complex called dsRNA activated factor 1 (DRAF1) that induces transcription of ISGs [156-158].

More recent studies have identified a novel pathway that is independent of IFN and induces an antiviral response upon entry of enveloped viral particles from both RNA and DNA viruses [159,160]. The receptor(s) that initiates this signalling pathway remains unknown, but the outcome is the activation of TBK1 which participates in hyper-phosphorylation of IRF-3 [160,161] (Fig. 4). Importantly, the TLR and RIG-I pathways are

apparently not involved because Trif, MyD88 and NF-κB are dispensable for the induction of ISGs in response to virus particles in the cell [161]. The ability to directly induce an antiviral state in the cell may serve as the actual first line of defence against invading pathogens, thus providing the infected cell with enough time to implement an effective immune response.

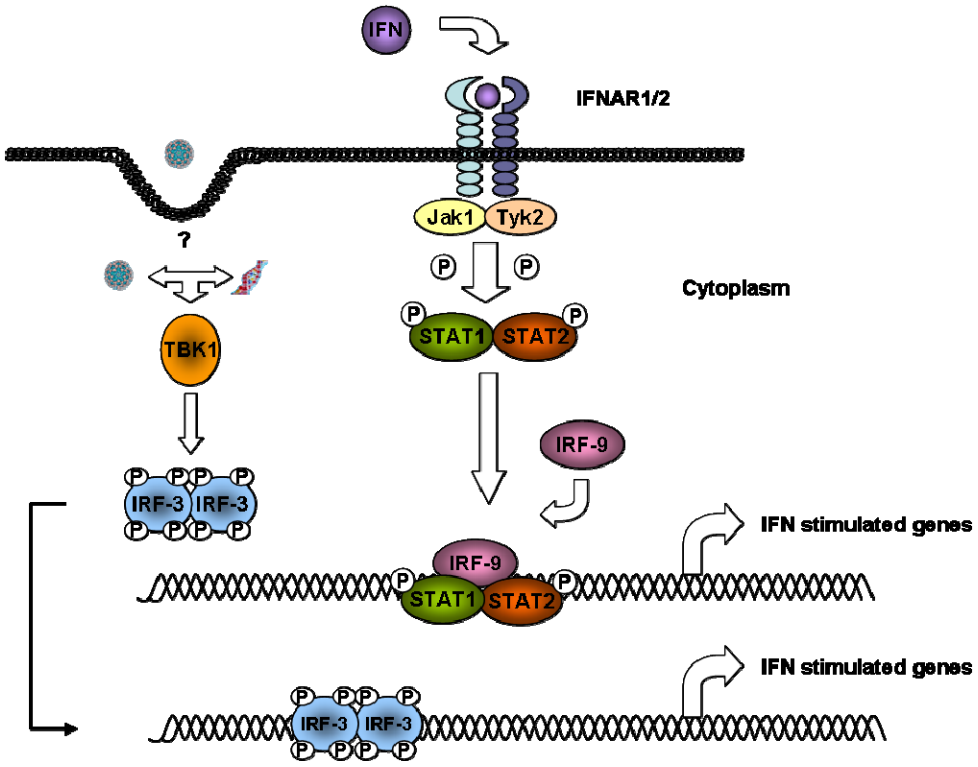

Figure 4. The Jak/STAT signalling pathway. The binding of IFN to the IFN-α/β receptor (IFNAR) results in cross-reaction between the two receptor-associated kinases Tyk1 and Jak2, which in turn phosphorylate STAT1 and STAT2. STAT1 and STAT2 form a hetero-dimer which in the nucleus binds IRF-9 to form the transcription factor complex ISGF3. ISGF3 binds to promoters with ISRE elements and induce transcription of ISGs such as Mx, ISG15 and OAS. Viral entry also induce expression of ISGs independent of IFN stimulation through novel receptors and/or pathways that results in hyper-phosphorylation of IRF-3. Modified from [6,161].

1.7 Antiviral proteins induced by the IFN system

The human genome has been estimated to contain somewhere between 600 and 2000 ISGs, but only a fraction of them encodes proteins with antiviral properties [162]. Of the known antiviral ISGs, some of the best characterized are Mx, ISG15, PKR and OAS.

1.7.1 Mx

Mx proteins belong to the dynamin superfamily of high molecular weight GTPases, and the name was given because of their ability to provide resistance against myxo- and orthomyxoviruses such as influenza A [163-167]. The human genome contains two related Mx genes (MxA and MxB), the protein size range from 70 to 80 kDa and both are induced by IFN [164,168,169]. MxA accumulates in the cytoplasm upon IFN stimulation or virus infection, and the antiviral activity has been extensively studied [164,168-170]. On the other hand, MxB appears to have important functions in transport between the nucleus and the cytoplasm, which is not involved in defence against virus infections [169,171-173]. Mouse also have two Mx genes encoding the Mx1 and Mx2 proteins, but different from humans both proteins have antiviral activity [169,174]. Mouse Mx1 is located to the nucleus and selectively inhibits the replication of orthomyxoviruses, while Mx2 accumulates in the cytoplasm and confer resistance against rhabdoviruses (vesicular stomatitis virus (VSV)) and bunyaviruses (LaCrosse virus (LACV)) [169,174-176]. While mouse Mx1 and Mx2 inhibit viral replication in the respective cellular compartments they accumulate, human MxA have a broad antiviral spectrum and inhibits viruses irrespective of where they replicate in the cell. So far human MxA is reported to confer resistance against members of the bunyaviruses, orthomyxoviruses, paramyxoviruses, rhabdoviruses, togaviruses, picornaviruses and reoviruses [177].

The antiviral function of Mx proteins depends on several factors. Mx proteins are known to aggregate and form homo-oligomers, and they are also known to make complexes with viral proteins [164,169,174]. The ability to make aggregates and to recognize viral components are dependent of the intrinsic GTPase activity of the Mx proteins [178]. Studies on mutant MxA lacking the GTPase domain failed to form oligomers, and although the antiviral activity was retained the overall stability of the protein was severely diminished [179].

The interaction between human MxA and viruses has been thoroughly studied, and in the case of thogoto virus (THOV) the mechanism has been described. MxA interacts directly with THOV nucleocapsids and retain them in the cytoplasm, preventing viral transport and replication in the nucleus [177,180,181]. Similar mechanisms have been reported for other viruses as well, such as LaCrosse virus [182].

1.7.2 ISG15

ISG15 was one of the first ISGs to be characterised based upon the inducibility by IFN stimulation and virus infection [183-186]. It was also identified as the first member of the family of protein-modifiers today known as the ubiquitin-like proteins [187].

Human ISG15 is expressed as an inactive precursor (17 kDa) that is cleaved by specific proteases in order to expose a C-terminal motif that is central for the active protein (15 kDa) [188,189]. Through the active C-terminal domain ISG15 is able to bind covalently to proteins in a process called conjugation or ISGylation. Over 200 ISGylated proteins of different functions have been identified, but how this conjugation of ISG15 to its target proteins affect their function is not yet understood [190-192].

ISG15 is one of the earliest and strongest induced ISGs, and is expressed in most cells by dsRNA and upon infection of virus and bacteria [185,193-195]. Several observations have

led to the notion that ISG15 has a function in the cell upon IFN stimulation and infections by pathogens. Many important factors involved in the IFN signalling pathway are targets for ISG15, and one of these studies concluded that ISGylation extended the lifespan of IRF-3 during virus infections [191,196]. The ISGylation of IRF-3 appears to have an indirect effect on its function, and this might be similar for the other cellular proteins that are targeted by ISG15. Among the proteins involved in the innate antiviral response includes PKR, MxA, STAT1, Jak1 and RIG-I, but there is no evidence of a defect in the IFN signalling in ISG15 deficient mouse when it comes to the Jak/STAT pathway [191,192,197].

Several *in vitro* studies have shown that the IFN system and ISG15 is involved in inhibition of retrovirus replication by targeting of virus assembly [198-200]. The studies showed that human immunodeficiency virus – 1 (HIV-1) release from the cell was impaired upon IFN stimulation, and that this effect was mimicked by transient expression of ISG15 [199,201]. Similar to the cellular targets for ISGylation, the mechanism utilized by ISG15 to inhibit HIV-1 remains elusive.

ISG15 also appear to play a role in influenza virus infections. All influenza viruses produce a protein that interferes with the IFN system. This protein is known as the non-structural protein 1 (NS1), and the NS1 of influenza B virus specifically inhibits ISG15 conjugation during infection [202]. Even though no direct effect of this interference by NS1 has been observed, it is a strong indication that ISG15 has a possible antiviral role during infection of influenza B.

1.7.3 PKR

PKR is a serine/threonine protein kinase that is activated by dsRNA, IFN stimulation and viral infections (reviewed in [124]). Binding of dsRNA results in auto-phosphorylation of PKR, and this enable the protein to phosphorylate other proteins where the most prominent is the protein synthesis initiation factor eIF2α [203-205]. eIF2α is protein essential for

translation of mRNA, and PKR-dependent phosphorylation prevents recycling of this protein and thus results in translational inhibition of cellular and viral mRNA.

PKR appears to have important antiviral effects because several viruses have evolved mechanisms to block activation of the protein during infection (extensively reviewed in [8,206]). For instance, influenza viruses are shown to activate a cellular inhibitor of PKR, poliovirus induce degradation of PKR and HIV-1 inhibits the catalytic activity of PKR [207-210]

1.7.4 2´-5´ Oligoadenylate synthetase (OAS)

2´-5´- Oligoadenylate synthetase (OAS) is a dsRNA dependent enzyme that uses ATP as a substrate to synthesize 2´-5´-oligoadenylates. Three different forms of OAS are found in humans that are inducible by IFN and these oligoadenylates activate a latent endoribonuclease known as RNase L (reviewed in [211]). The primary function of RNase L is to cleave ssRNA and the enzyme has specific affinity for ssRNA with regions of secondary structures [212]. However, the precise mechanism behind the antiviral activity of RNase L still remains elusive, but several studies shown that RNase L deficient mouse display enhanced susceptibility against infection of picornaviruses, Herpes Simplex 1 and WNV [213-216].

1.8 Viral antagonism of the IFN system

Most viruses have adopted mechanisms that interfere with the hosts IFN system response. Generally viruses use non-structural (NS) proteins for this purpose, and these are referred to as IFN-antagonists. The NS-proteins are often multifunctional proteins, and they interact with both cellular and viral proteins. As the functions of these viral proteins are important for the virus to successfully replicate in the host, an increasing number of studies

have concluded that viral IFN antagonists target virtually all components of the IFN- system [217].

One of the best described IFN antagonistic mechanisms in viruses is exerted by the influenza A NS1 (NS1A) protein, which is a multifunctional protein capable of inhibiting the host IFN response at many levels. The influenza virus has an obvious requirement for proteins with these qualities, because the virus is highly sensitive to IFN stimulation and particularly the actions of MxA [218-220].

One property of NS1A is the ability to bind and sequester dsRNA and thus avoid detection by the many intracellular receptors that induce IFN upon virus infection of the cell [221]. Masking of dsRNA has been linked to inhibition of several factors important for induction of IFN including NF-κB, IRF-3 and PKR [222-226]. More recently it has been suggested that the primary function of dsRNA binding by NS1A was to inhibit the 2`-5´ OAS pathway and thus avoid degradation of the viral genome by RNaseL [227]. Masking of the viral genome is an indirect method to avoid detection by the host, and several studies have shown that influenza A through NS1A in addition apply more direct strategies to inhibit the IFN system. One of these strategies is to inhibit the machinery that perform 3` end processing of cellular pre-mRNA [228,229]. NS1A targets the cleavage and polyadenylation specificity factor (CPSF) and thus efficiently block transport of cellular mRNA from the nucleus. As a consequence the host cell synthesis of IFN and antiviral proteins is severely impaired during the initial stages of the infection [230].

Direct interaction between NS1A and RIG-I leading to disruption of RIG-I mediated induction of IFN has recently been reported [231,232]. RIG-I is the major receptor detecting influenza virus infection in non-immune cells, and inhibition of this pathway results in equivalent inhibition of IRF-3 activation [232]. NS1A appears to bind both RIG-I and MAVS because all three proteins are found in the same complexes after virus induced cell lysis [231].

The ability to at least reduce the initial IFN response is a great advantage for all replicating viruses. First of all the virus may avoid the direct actions by antiviral proteins such as Mx, which could inhibit viral replication at an early stage. Secondly it may delay the effects exerted by cytokines such as IFN which not only activates neighbouring cells but also act in an autocrine fashion to induce an antiviral state in the cell.

2.1 The IFN system of fish

2.1.1 IFN genes in fish

IFN genes have been cloned from several fish species such as Atlantic salmon, rainbow trout, pufferfish and zebrafish [233-236]. So far two IFN genes known as IFN-α1 and IFN-α2 have been found in Atlantic salmon. In addition three IFN genes have been found in rainbow trout and channel catfish, but the presence of more genes has been suggested by genetic studies in channel catfish and pufferfish [235-238]. Interestingly, fish IFN genes possess a similar five exon/four intron structure as the mammalian IFN-λ genes, in contrast to the mammalian IFN-α/β genes that lacks introns [239]. The inducible nature of fish IFNs is also well established. Several fish viruses have been shown to induce IFN and IFN like activity [236,240-242] and poly I:C is widely used to induce an antiviral state in fish [233,235,237,243-246]. Furthermore, CpG-rich oligonucleotides is established as an inducer of IFN and antiviral activity in salmonids [247-250].

Cloning of fish IFN genes have made it possible to produce recombinant IFN from Atlantic salmon, spotted green pufferfish and channel catfish by transfection of eukaryotic cell lines, and recently salmon and rainbow trout IFN was produced in E. coli [234-237,251]. Recombinant IFN has proved to be a valuable tool to investigate antiviral activity in fish,

which has been demonstrated in Atlantic salmon, catfish and rainbow trout against IPNV, channel catfish herpesvirus (CCV) and viral hemorrhagic septicemia virus (VHSV) respectively [235-237]. In addition, recombinant IFN has been shown to induce expression of ISGs such as Mx and ISG15 in Atlantic salmon, rainbow trout and pufferfish [234-236,251,252].

2.1.2 The salmon IFN promoter

The promoter sequences of salmon and zebrafish IFN genes have previously been reported, and they share some features with the mammalian IFN promoters [253,254]. The salmon IFN promoter has a rather unique organization and appears to have two major regulatory regions called promoter region (PR) - I and PR-II [253]. PR-I possesses two IRF-binding motifs (marked as IRF-E in fig. 4) and a putative NF-κB – binding motif (Fig. 5). PR-II contains three to four IRF-binding motifs and an AP-1 element. This promoter sequence is present in both of the two salmon IFN-α1 and IFN-α2 genes, and the different promoter motifs suggest that salmon IFNs are regulated by the same transcription factors as mammalian IFNs. What is unique about the organization and regulation of the salmon IFN genes is that both encode two transcripts of different length. The short transcript is controlled by PR-I, while the long transcript is controlled by PR-II and in fact also contains PR-I.

To validate the potential to induce transcription of the IFN genes, the different promoter regions were fused in front of a promoterless luciferase reporter gene in order to make an IFN-promoter luciferase construct. The constructs were transfected into two different salmonid cell-lines, and their ability to transcribe the luciferase gene upon poly I:C stimulation was measured. These studies showed that the PR-I promoter gave high luciferase activity upon poly I:C stimulation whereas the PR-II promoter gave a weak induction. Transcript analysis also showed that the short transcripts by far was strongest induced by poly

I:C. These results indicate that the short promoter is the main control region of IFN transcription and also that cells produce the short IFN-transcripts upon poly I:C stimulation and virus infection. The exact function of the long IFN transcripts remain elusive, whereas the proteins encoded by the short transcripts of IFN-α1 and IFN-α2 were shown to have antiviral activity [235,251]. The short IFN promoter is most similar to promoter regions of mammalian IFN-β, because it contains binding motifs for NF-κB and different IRFs.

The IFN-promoter luciferase construct containing the PR-I region has proved to be a valuable tool to investigate how cellular and viral proteins activate the IFN promoter *in vitro*, and we have applied this methodology in paper III and IV.

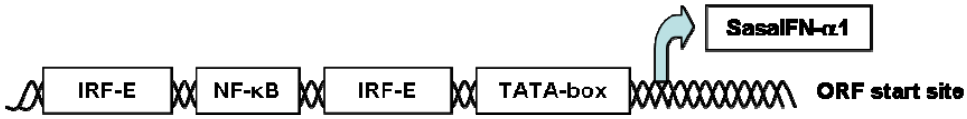

Figure 5. The promoter region (PR)-1 of the SasaIFN-α1 gene. The potential binding sites for transcription factors are boxed and the transcription start site is marked with a bent arrow. The PR-I was fused to a promoterless luciferase gene in the IFN-promoter luciferase construct. Modified from [253].

2.1.3 IFN signalling pathways in fish

Many of the kinases, adaptors and transcription factors that are involved in regulation of IFN transcription has been found in fish. A recent genomic survey identified most of the important kinases (e.g; TBK1, IKKs and IRAKs), adaptors (MyD88, NEMO, Trif) and IRFs in zebrafish, fugu and pufferfish [255]. Similar surveys have discovered TLR9 from several fish species, and TLR8, TLR9 and MyD88 have been studied in Atlantic salmon ([256-258] and personal communication with Skjæveland, I). The IRFs have received special attention and several members of this family have been studied in fish. IRF-1 has been cloned from

several species [259-263], IRF-2 from rainbow trout and mandarin fish [259,264] and IRF-7 like genes from mandarin fish and crucian carp [262,265]. The latter studies revealed that transcription of fish IRF-7 was inducible by poly I:C, virus infection and stimulation by supernatants with IFN-like activity.

There are limited data on the presence of IFN receptor genes from fish, but two separate surveys have demonstrated several possible receptor genes in the genomes of pufferfish and zebrafish [234,255]. One knock-down study using zebrafish identified an IFN-λ like receptor as a possible candidate [266]. However, this study failed to use IFN to confirm these observations and it is possible that the different IFNs may use additional receptors as well.

Most of the members in the Jak-STAT pathway have been identified in fish, including Tyk2, Jak1, STAT1, STAT2 and IRF-9 [255,267-270]. Functional studies have only been performed with crucian carp and zebrafish STAT1, and the latter was shown to rescue IFN signalling in a STAT1-deficient human cell line [270]. Similar as observed with fish IRF-7, goldfish STAT1 was inducible by poly I:C, virus infection and stimulation by supernatants with IFN-like activity [269].

2.1.4 ISGs in fish

Several fish ISGs have been identified and Mx and ISG15 are the most thoroughly studied. Mx genes have been cloned from numbers of different species, and these studies have shown that both Mx transcription and protein synthesis is inducible by poly I:C, IFN and virus infection [271-278]. As previously described, mammalian Mx proteins display potent antiviral activity against many different viruses, and this seems also to be the case with fish Mx proteins. Antiviral activity of Mx against IPNV is reported in Atlantic salmon, against

rhabdoviruses in Japanese flounder, against IPNV and infectious haematopoietic necrosis virus (IHNV) in brown trout and against nodaviruses in grouper [279-282].

The first ISG15 gene in fish was identified as a response to cell injury induced by nephrotoxin in gold fish, and similar to the mammalian homologue this protein had the ability to conjugate to cellular proteins [283]. ISG15 was also found in fugu, zebrafish and catfish, and the presence of conserved ISRE elements and NF-κB responsive sites in the promoter of pufferfish suggest that it is an ISG [283]. The same study also showed that goldfish ISG15 was induced by infection with mycobacteria. Recently ISG15 genes have been found in Atlantic salmon, Atlantic cod and crucian carp, and the expression of these genes are strongly induced by virus infection [252,284,285]. In Atlantic salmon ISG15 was induced by both IPNV and ISAV, poly I:C and recombinant IFN, and it was also able to make conjugates with an unidentified protein from ISAV [252].

2.2 Infectious salmon anemia virus (ISAV)

Orthomyxoviruses are enveloped RNA viruses with a genome consisting of six to eight single stranded segments of negative polarity. Among the viruses that belong to this family of viruses are influenza viruses and THOV. THOV viruses are arboviruses that infect both mammals and ticks, the Influenza B and C infect primarily humans, while influenza A can infect humans, birds and several other species.

Infectious salmon anemia virus (ISAV) is the first aquatic virus designated to the *orthomyxoviridae* and is so far the only member of the new virus genus *Isavirus* [286-288]. The virus is a major causative agent of mortal disease in farmed Atlantic salmon globally. Outbreak of ISAV infection is reported in other salmonids, but the disease generally causes lower mortalities compared to Atlantic salmon [289-292].

The number of fish farms with outbreaks in Norway is relative low, but a recent study found avirulent strains of the virus in smolts from 22 of 24 investigated production sites throughout the country [293]. This implies that most Norwegian salmon farms could be ISAV positive and that a change from avirulent to virulent ISAV strains is the mechanism behind the sporadic outbreaks. Recent publications have identified several factors that may have influence on the virulence of the different strains of the virus, some of them similar to that described for the highly pathogenic Influenza A H5 strains [294,295].

Similar to the other influenza viruses, ISAV has a genome consisting of eight segments encoding at least ten proteins [288,296,297]. Two of these proteins may be involved in interference of the innate immune response in Atlantic salmon. The first proteins (s7ORF1) is encoded from a reading frame on segment 7, which is shown to produce one collinear and one spliced transcript [288,298-300]. The collinear transcript appears to encode either a non-structural minor structural or a structural protein involved in antagonism of the IFN system [298,299,301]. One study has suggested that the s7ORF1 protein interferes with the Jak/STAT pathway because of its inhibitory activity of the Mx promoter [301].

The gene encoding the second IFN antagonist candidate is located to segment 8 of the ISAV genome. Different from segment 7, segment 8 contains two overlapping reading frames in which the smaller ORF1 encodes the matrix protein of the virus capsid [297,298]. The structure and function of the larger ORF2 protein (s8ORF2) has not been characterized yet.

Previous studies have shown that ISAV induces expression of Mx and ISG15 proteins in cultivated salmon cells [245,252]. These studies concluded that Mx and ISG15 displayed no ability to protect cells from ISAV infection, in contrast to IPNV which is severely inhibited by Mx [280]. In addition, stimulation with poly I:C and recombinant salmon IFN-α1 failed to block ISAV replication and this suggest that ISAV have evolved strategies to avoid interference bye the IFN system during replication. However, poly I:C stimulation of salmon

prior to ISAV infection resulted in reduced cumulative mortality compared to mock-treated salmon, and this suggests that poly I:C can induce some protection *in vivo* against ISAV infection [243] .

The present status on the interactions between salmon and ISAV is that salmon is able to mount an IFN system response against ISAV, but at the same time ISAV has evolved strategies to avoid interference by the immune system. However, what mechanisms salmon use to suppress ISAV replication and how ISAV antagonize this immune response still remains elusive.

Aims of study

The general purpose of this project was to study the regulation of the Atlantic salmon IFN system in response to recombinant salmon IFN-α1, poly I:C, the imidazoquinoline S-27609 and ISAV infection. In addition we have studied functions of two candidate IFN antagonist proteins from ISAV, and we have performed structural and functional studies of a salmon IRF-7 like protein.

The major aims were:

- Study transcription of IFN, Mx and ISG15 genes in response to ISAV infection both *in vivo* and *in vitro*, and to elucidate the protective potential of recombinant IFN-α1 against ISAV infection.
- Study the induction of IFN, Mx and ISG15 genes in response to the imidazoquinoline S-27609, a specific ligand for the mammalian Toll-like receptor 7.
- Functional study of candidate IFN-antagonist proteins of ISAV, and how transient expression of these genes inhibits activation of IFN-α1/ α2 promoter.
- Cloning, sequencing and functional studies of an Atlantic salmon IRF-7 like gene.

Summary of papers

I. Infectious salmon anemia virus is a powerful inducer of key genes of the type I interferon system of Atlantic salmon, but is not inhibited by interferon

In paper I we have investigated the role of the IFN system of Atlantic salmon in response to ISAV infection. Generally ISAV was shown to be a strong inducer of IFN, Mx and ISG15 transcripts both *in vivo* and *in vitro*. The *in vitro* studies was performed in salmon TO cells, and the induction patterns of the selected IFN system genes in ISAV infected cells was compared to that of poly I:C stimulated cells. Poly I:C stimulation resulted in a early peak of IFN transcription followed by a later peak of Mx transcription. In contrast, ISAV infection resulted in early expression of Mx and ISG15 while IFN transcription was considerably delayed and did not increase until late in the infection. A cohabitant infection trial with ISAV in Atlantic salmon resulted in 100% mortality, and transcript levels and protein expression where monitored in the organs. ISAV induced transcription of IFN, Mx and ISG15 genes in liver and head kidney, and immunoblotting confirmed the presence of Mx and ISG15 proteins in liver. In order to elucidate the protective potential of the IFN system against virus infections, TO cells were stimulated with recombinant salmon IFN–α1 (rSasa IFN–α1) and subsequently infected with either ISAV or IPNV. Whereas rSasa IFN–α1 provided full protection against IPNV, no protection of the TO cells were observed against ISAV. This study clearly describes that ISAV possess mechanisms that enable the virus to evade the IFN system response in Atlantic salmon.

II. Induction of interferon system genes in Atlantic salmon by the imidazoquinoline S-27609, a ligand for Toll-like receptor 7

Imidazoquinolines are synthetic low molecular compounds with antiviral and antitumor activity, and they are demonstrated to be ligands for TLR7 and TLR8. The antiviral properties of imidazoquinolines have much been ascribed to induction of IFN–α from innate immune cells in the blood, especially the plasmacytoid dendritic cells (pDC). In this study we show that imidazoquinolines, represented by S-27609, has potent stimulatory effect on the Atlantic salmon IFN system. Injection of S-27609 induced transcription of IFN–α1/ α2, Mx and ISG15 in several organs of salmon, which suggest that salmon respond to this treatment through TLR7-like receptors. The stimulatory effects of S-27609 were compared to poly I:C, which is a ligand for MDA5 in most cells and for TLR3 in some of the immune cells. Transcript levels were measured in head kidney and liver, and the treatments induced expression of IFN-α1/ α2, Mx and ISG15 in both organs. In liver poly I:C proved to induce a stronger and more prolonged expression of IFN-α1/ α2 than S-27609. Surprisingly this was not reflected in the kinetics of Mx and ISG15 transcription, which were comparable after both treatments. In head kidney poly I:C induced IFN-α1/ α2 transcription early with a peak after 24 hours, and Mx and ISG15 showed a prolonged induction after 14 hours. S-27609 induced a biphasic expression of IFN-α1/ α2, with a low peak after 14 hours and a continuous strong increase after 48 hours. Mx levels were observed to increase steadily from 14 hours until the end of the study. ISG15 increased steadily until peak levels after 48 hours, after which transcript levels started to decline. A possible explanation for the differences displayed by the two treatments could be that S-27609 induce IFN through immune cells such as pDCs, while poly I:C induce a general response in most nucleated cells.

III. Molecular and functional characterization of two infectious salmon anaemia virus (ISAV) proteins with type I interferon antagonizing activity

In this study we characterize two proteins encoded by the two smallest genomic segments of the piscine orthomyxovirus infectious salmon anaemia virus (ISAV). Both proteins, encoded by the un-spliced ORF from genomic segment 7 (s7ORF1) and the larger ORF from segment 8 (s8ORF2), are involved in modulation of the type I interferon response. The data suggests that the s7ORF1 protein is collinearly encoded, non-structural, contains no nuclear localisation signals, localises mainly to the cytoplasmic perinuclear area and does not bind single- or double-stranded RNA. On the other hand, genomic segment 8 uses a bicistronic coding strategy and the encoded s8ORF2 protein is a structural component of the viral particle. This protein contains two nuclear localisation signals, has a predominantly nuclear localisation, binds both double-stranded RNA and poly-A tailed single-stranded RNA, but not double-stranded DNA. In poly I:C stimulated salmon cells both ISAV proteins independently down-regulate the type I IFN promoter activity. Thus, ISAV counteracts the type I IFN response by the action of at least two of its gene products, rather than just one, as appears to be the case for other known members of the *Orthomyxoviridae*.

IV. An Atlantic salmon IRF-7 gene identified by analysis of sequence and function and its expression compared with a STAT1 gene

Interferon regulatory factor 7 (IRF-7) plays a crucial role in virus-induced activation of interferon-α/β transcription in mammals. This work describes a structural and functional homologue of mammalian IRF-7 from Atlantic salmon. The cloned gene encodes a putative protein of 415 amino acids, which in phylogenetic analysis groups with other fish IRF-7 like

proteins in the vertebrate IRF-3/IRF-7 family. Using an IFN promoter-luciferase assay we showed that salmon IRF-7 gave increased promoter activity after poly I:C stimulation. Transcript levels of IRF-7 were measured by real-time RT-PCR and compared to those of signal transducer and activator of transcription 1 (STAT1), which is important for transcriptional activation of IFN stimulated genes. Recombinant salmon IFN-α1 and poly I:C proved to be potent inducers of IRF-7 and STAT1 in Atlantic salmon TO cells, and poly I:C also induced both genes in head kidney and liver of Atlantic salmon. However, differences in transcription kinetics between IRF-7 and STAT1 indicate that the genes are regulated through different pathways. Finally, infectious salmon anemia virus (ISAV) infection of TO cells induced early synthesis of STAT1, whereas IRF-7 transcription was up-regulated much later. This indicates that IRF-7 synthesis is a possible target for antagonism of the IFN system by ISAV in Atlantic salmon.

3. Discussion

The type I IFN system is evolutionary conserved among the vertebrates, and despite certain differences it appears to have similar functions in fish and mammals. At the functional level fish appear to utilize similar receptors as mammals to recognize conserved pathogen structures such as RNA and DNA in order to induce the IFN system. We have shown in paper II that salmon responds to the imidazoquinoline S-27609, a specific ligand for TLR7 in mammals, by inducing expression of IFN and several ISGs with known antiviral activities. Even though stimulation with poly I:C also induced the same set of genes in salmon, there where certain discrepancies between the two treatments that suggest that these stimulants are recognized by different receptors or cell types. Plasmacytoid DCs use TLR7 to detect ssRNA, and in this cell type the transcription factor IRF-7 plays a vital role. In paper IV we have characterised an IRF-7 like protein from salmon. IRF-7 is the major regulator of IFN transcription in all mammalian cells, and structural and functional studies suggest that the salmon protein has similar tasks. As one of the important functions of the IFN system is to inhibit viral infections, we have investigated the interaction between salmon and ISAV (paper I, III and IV). These studies show that the virus is a strong inducer of the IFN system if salmon, but at the same time ISAV has developed efficient mechanisms to avoid the countermeasures from the host.

The results are discussed in detail in paper I-IV, but some selected and updated topics will be discussed further in the following sections.

3.1 Poly I:C and S-27609 as modulators of the IFN response

The innate receptors involved in activation of the IFN system have been extensively studied in mammals and a general picture of their distribution and function is now emerging. RIG-I and MDA5 are regarded as the most important receptors for viral RNA in non-immunological cells [21]. Immune cells detect viral RNA and DNA through TLR3, 7, 8 and 9 [39].

In mammals, poly I:C is detected by MDA5 and TLR3 [22,49]. Both receptors signal through pathways that regulate the transcription factors IRF-3, IRF-7 and NF-κB, which in turn activate transcription of the IFN-β gene [112,120,151]. Rainbow trout possesses TLR3 and many of the downstream signalling partners for TLR3 is found in zebrafish [255,302,303]. In addition, sequence data in Genbank suggest that zebrafish also possess RIG-I (Accession number cd594255) and MDA5 (accession number xm_689032) [303]. It is thus very likely that fish detect poly I:C through similar mechanisms as mammals.

As described earlier and in paper I, II, III and IV, poly I:C is the most frequently used stimulator of the IFN system in fish and it is equally suited to induce IFN system genes both *in vivo* and *in vitro* [239]. The expression of IFN-α1/α2, Mx and ISG15 is comparable in liver and head kidney of salmon after poly I:C stimulation, and this suggest that most cells respond to this treatment by activation of the IFN system. In paper I we have performed a time-scale study to observe the kinetics of IFN-α1/α2 and Mx transcription upon poly I:C stimulation in TO cells. Expression of IFN-α1/α2 was induced after 6 hours, displayed a biphasic kinetic with the strongest peak after 18 hours and a second and weaker peak after 48 hours. In comparison Mx transcripts increased after 30 hours and reached peak levels after 48 hours, indicating that TO cells responds to poly I:C by synthesis of IFN which in turn induce expression of Mx. A similar picture is observed (paper IV) concerning IRF-7 and STAT1 kinetics after poly I:C and rSasaIFN-α1 stimulation. IRF-7 and STAT1 transcript levels did

not increase until 16 hours after poly I:C stimulation, in comparison to rSasaIFN-α1 which induced both genes as early as three hours after stimulation. In mammals both genes are induced by IFN, and this seems to be the case in salmon and other fishes.

The ability to induce IFN and ISGs both in cultivated cells and salmon indicate that poly I:C is detected by receptors with an ubiquitous distribution. The most obvious receptor for poly I:C in fish is MDA5, but also immune cells with TLR3 is likely to participate in activation of the IFN system *in vivo*.

S-27609 is a ligand for TLR7 in mammals [76,77]. TLR7 is found in specific immune cells such as pDCs, and ligand recognition activates a signalling pathway through MyD88 and IRF-7 which enable these cells to produce high levels of IFN-αs [84]. Although pDCs constitute a small fraction of the total number of cells in the blood, these cell types are responsible for a major part of the IFNs produced in response to virus infection [304].

Fish appear to possess both TLR7, MyD88 and DCs, it is thus reasonable to assume that salmon respond to the TLR-7 ligand S-27609 by similar mechanisms as mammals does [255,305-311]. In paper II we show that S-27609 induces high levels of IFN, Mx and ISG15 in head kidney and liver in salmon, but in contrast to poly I:C stimulation the expression was most pronounced in head kidney. Head kidney is one of the primary immunological organs in fish and the presence of activated immune cells at this site can explain the differences between the two treatments. One point that should be mentioned is that most immunological cells (e.g DCs) upon encounter of antigens mature and migrate to immunological organs. When we observe high levels of IFN and ISGs after S-27609 stimulation, this could also reflect increased migration of effector cells to the head kidney. It is also possible that additional IFN-genes not detected by our assays affect ISG expression by S-27609. These assays are designed to recognize the only two salmon IFN genes characterized so far, thus

other IFN-genes are not likely to be detected. Salmon possess two other classes of IFN genes that may be involved in S-27609 signalling (Robertsen et al, unpublished). The kinetics in head kidney of S-27609 stimulated salmon indicate that additional IFNs are induced, because Mx and ISG transcripts are increasing early without any IFN transcription. This is also evident in liver, where S-27609 induces a weak transcription of IFN compared to poly I:C, but at the same time induce an equivalent expression of Mx and ISG15 mRNA as poly I:C.

Our studies show that both poly I:C and S-27609 induces expression of key genes of the IFN system in salmon. One important difference between these stimulants is that TO cells are insensitive to S-27609, whereas poly I:C is a strong inducer of the IFN system in these cells. This observation support the hypothesis that S-27609 is detected by immune cells such as pDCs while poly I:C is recognized by receptors present in all nucleated cells.

3.2 The role of IRF-7 in IFN-dependent immune responses in salmon

In mammals, IRF-7 is essential for the induction of IFN-α/β genes in response to virus infection [120]. In most non-immunological cells IRF-7 display a weak constitutive expression sufficient to participate in the initial induction of IFN-β during virus infection [112,312]. However, IRF-7 transcription is also induced by IFN stimulation and is thus involved in enhancement of the IFN induction [115,118]. IRF-7 is constitutively expressed in pDCs, and the robust synthesis of IFNs in response to virus infections by these cells is totally dependent of IRF-7 [120].

We have investigated the properties of a salmon IRF-7-like gene in paper IV. Our studies show that salmon IRF-7 is induced by poly I:C, rSasaIFN-α1 and ISAV infection, and transient transfection of IRF-7 *in vitro* shows that the protein up-regulate IFN promoter activity. Even though IRF-7 was induced by ISAV infection, we observed that IRF-7 transcripts appeared at a late stage (48 hours) after infection. In contrast, STAT1 was induced

four hours after infection, and poly I:C and rSasaIFN-α1 treatment induced IRF-7 and STAT1 at similar time points. Thus, ISAV functions as a negative regulator of IRF-7 during infection, whereas STAT1 transcription is unaffected by the virus.

These observations points out that the salmon IRF-7 is involved in regulation of IFN expression and possesses similar properties as described in mammals. As ISAV have evolved a strategy to inhibit induction of IRF-7, this strongly suggests that the gene has an important role in the cell during viral infections. ISAV also inhibit transcription of other genes during infection, thus this subject will be discussed further in section 3.3. However, it still remains elusive whether fish IRF-7 is the main regulator of IFN transcription as observed with mammalian IRF-7 [120].

3.3 *In vitro* interactions between salmon and ISAV

As described in paper I, infection of ISAV induces high levels of IFN, Mx and ISG15 transcripts both *in vivo* and *in vitro*. The insensitivity of TO cells to S-27609 suggests that ISAV is recognized by other receptors than TLR7 *in vitro* and cytoplasmic receptors such as RIG-I and MDA5 are obvious candidates because these are the main receptors that detect pathogens in non-immune cells. In TO cells, Mx and ISG15 transcripts levels were increasing as early as four hours after ISAV infection. This contradicts the hypothesis put forward in a previous study that suggests that the ISAV S7ORF1 protein inhibits signalling through the Jak/STAT pathway [301]. On the other hand, this is consistent with earlier work showing that TO cells respond to ISAV infection by producing high levels of Mx and ISG15 proteins as early as 24 hours after infection [245,252]. TO cells are thus able to induce transcription of ISGs and synthesize these proteins in response to infection by the virus. We also observed elevated levels of ISAV transcripts immediately after infection and it is likely that the cell induce expression of ISGs in response to viral replication. Similar mechanisms is described in

human cells upon entry of enveloped viruses and result in transcription of ISGs independent of IFN stimulation [159,160]. This IFN-independent mechanism is dependent of ongoing viral replication and results in hyper-phosphorylation of IRF-3.

Whereas Mx and ISG15 were induced immediately after infection and transcript levels continued to increase throughout the study, IFN displayed different kinetics by being induced at considerably later time points. The first 36 hours after infection no transcription of IFN was observed, while the period from 48 to 96 hours was marked by a pronounced increase of IFN transcripts. These observations show that ISAV is a strong inducer of IFN in TO cells, but compared to Mx and ISG15 it is also obvious that the virus inhibit or delays IFN transcription. This is in agreement with a previous study, where we show that the IFN promoter is first induced 48 hours after infection and increases to peak levels after 96 hours [253].

In paper IV we investigated the expression patterns of IRF-7 and STAT1 after stimulation of rSasaIFN-α1 and poly I:C in addition to ISAV infection. We observed that poly I:C and IFN stimulation of TO cells induced transcription of both genes at an early stage and with similar kinetics. ISAV infection had a different influence on the transcription of these genes. STAT1 was induced after three hours infection and had similar kinetic as Mx and ISG15. IRF-7 transcription was absent until 36 hours after infection and appeared to be inhibited in a similar fashion as IFN.

In paper I, we have also investigated the ability of rSasaIFN-α1 to protect TO cells against ISAV and IPNV infection. As ISAV appears to have evolved a strategy to inhibit transcription of IFN and IRF-7, it is reasonable to assume that the IFN system of salmon possess some mechanisms to inhibit replication of the virus. In this study TO cells were stimulated with rSasaIFN-α1 prior to infection with ISAV and IPNV. rSasaIFN-α1 induces an antiviral state in the cells that provide strong protection against IPNV and previous studies

have shown that Mx is one of the proteins that inhibit replication of the virus [280]. In contrast, rSasaIFN-α1 was unable to provide any protection against ISAV in TO cells.

The observation that ISAV is not inhibited by the antiviral state induced by rSasaIFN-α1 stimulation in TO cells suggests that the virus have evolved mechanisms to evade or antagonize the IFN system responses in salmon. Because TO cells synthesize Mx and ISG15 in response to ISAV infection, it is evident that the virus is not affected by the presence of these proteins in the cells [245,252]. Nonetheless, a multitude of ISGs are synthesized by the cell and there is still limited knowledge about how they participate in the response against virus infections. Thus, it is possible that ISAV targets other ISGs that may interfere with virus replication, but these are at the time not known.

What we do know from our studies is that IFN and IRF-7 transcription is affected by ISAV infection. One possible explanation is that IFN and IRF-7 have similar promoter regions and that ISAV targets transcription factors that are common for these genes and not for Mx, ISG15 and STAT1. It is also possible that ISAV inhibits transcription of other important antiviral ISGs with similar promoter regions as IFN and IRF-7, because rSasaIFN-α1 stimulation does not inhibit ISAV replication.

Orthomyxoviruses such as influenza and THOV have different strategies to inhibit IFN transcription, but a common trait for these viruses is that they block transcription of IFN-β through inhibition of IRF-3 during infection [313-315]. In human, transcription of IRF-7 is regulated through different pathways [316,317]. One of these pathways is independent of IFN signalling, is activated directly by virus infection and involves formation of the virus-activated factor (VAF) which is composed of IRF-3, IRF-7 and additional co-activators [104,316]. VAF bind efficiently to ISRE sites in the promoters of both IFN-β and IRF-7 during virus infection, and not only initiate IFN-β transcription but also enhance this step by

increased synthesis of IRF-7. If similar mechanisms are present in salmon, this could explain why both IFN and IRF-7 transcription is inhibited during ISAV infection.

3.4 *In vivo* interactions between salmon and ISAV

We have shown in paper I that salmon responds to ISAV infection by activating the IFN system. Even though transcription of IFN system genes is induced and the antiviral proteins Mx and ISG15 are synthesized in organs of infected salmon, these countermeasures are insufficient to limit ISAV replication. The ISAV isolate used in this study is highly virulent and originate from an outbreak in Norway [318,319]. This was confirmed by the cohabitee infection trial, where all the salmon injected intraperitoneally with 100 ISAV particles were dead after 22 days and all the cohabitees after 38 days. Even though salmon responds to ISAV infection by inducing a powerful IFN system response, these countermeasures are insufficient to inhibit viral replication. However, a previous study has shown that poly I:C stimulation provides some protection against ISAV infection *in vivo* [243]. This indicates that the IFN system of salmon interferes with ISAV replication, even though the virus has evolved efficient mechanisms to circumvent the hosts' immune response. The immune response *in vivo* involves different effector cells such as macrophages, NK-cells and DCs and also results in activation of adaptive immune responses. The immune response *in vivo* is thus considerably more complex than *in vitro*. Nonetheless, our studies of ISAV infection in TO cells could provide some clues about virus-host interactions in general. ISAV inhibits transcription of IFN and IRF-7, and these genes are involved in bridging innate and adaptive immune responses [320,321]. The ability to inhibit transcription of IFN and IRF-7 could have more far-reaching implications than just inhibit IFN responses in the infected cell. Antagonism of the IFN system may therefore serve to delay or inhibit activation of adaptive immune responses by the host.

3.5 ISAV S7ORF1 and S8ORF2- proteins with IFN antagonistic properties

Using the IFN-promoter luciferase assay we have previously shown that ISAV activates the IFN promoter in TO cells [253]. Similar to the transcription kinetics of IFN described above, ISAV induces the IFN promoter at a late stage of infection. We have used the IFN-promoter luciferase assay to elucidate the properties of two ISAV proteins encoded by S7ORF1 and S8ORF2. Our results show that both S7ORF1 and S8ORF2 had a negative effect on the IFN promoter after poly I:C stimulation. The strongest impact was observed with S7ORF1 which reduced IFN-promoter activity with a 7.6 fold decrease, while S8ORF2 resulted in a 3.1 fold decrease. One interesting observation is that co-transfection of the ISAV genes does not have a cumulative effect on the IFN-promoter compared to effect of the genes alone. This suggests that the two proteins inhibit different steps of the signalling pathways that induce the expression of IFN-genes. In addition to our work, a previous study has showed that the ISAV S7ORF1 protein is a negative regulator of the Mx promoter [301]. We did not study the effect on the Mx promoter, but this contradicts our results because we show that ISAV is a potent inducer of Mx and ISG15 in TO cells.

The apparent existence of two proteins with IFN-antagonistic properties makes ISAV a unique member of the orthomyxovirus family, because the other influenza-viruses characterized so far possess only one IFN-antagonistic gene in their genome. As an example, the NS1 of influenza A (NS1A) viruses is a multipurpose protein capable of both sequestering dsRNA to avoid detection by the PKR and/or OAS and also capable of blocking the signalling pathway that activate IFN transcription [313]. These properties are also found in the two ISAV proteins. S8ORF2 has the ability to bind dsRNA and is localized to the nucleus, and similar to NS1A it possesses two nuclear localization signals (NLS) that are important for translocation [322]. Because ISAV and orthomyxoviruses generally replicate in the nucleus,

the presence of these proteins in this compartment is likely to have an important function for the virus [286].

The exact function of S7ORF1 remains elusive, but we and others have shown that the protein has cytoplasmic localization, does not bind RNA and have a strong inhibitory effect on the IFN-promoter [301]. These properties could indicate that S7ORF1 interacts with proteins located to the cytoplasm that are involved in signalling pathways that regulate IFN and IRF-7 transcription. However, what cellular factors ISAV targets to antagonize induction of IFN is at present still unknown.

3.6 Future perspectives

Our studies show that the IFN system of salmon is unable to limit ISAV infections *in vitro* and *in vivo*. ISAV inhibit induction of IFN and IRF-7 and replicates efficient in IFN treated TO cells. The ability to evade the IFN response indicates that ISAV targets other antiviral ISGs than Mx and ISG15, because our and previous studies show that transcription and protein synthesis of the latter is not affected by the virus [245,252]. S8ORF2 binds and possibly sequester dsRNA, making PKR a probable target as seen with influenza A which inhibit activation of both PKR and OAS [222,227]. Thus, interactions between ISAV proteins and salmon ISGs such as PKR need to be studied in more details. S7ORF1 on the other hand may interact with proteins involved in the signalling pathways of IFN and IRF-7 as it is localized in the cytoplasm and as it lacks RNA-binding properties. Candidates for this interaction could be the RNA-binding receptors and associated adaptors, since influenza A appears to bind both RIG-I and MAVS [231,232]. Other options are the transcription factors IRF-3, IRF-7 and NF-κB, as several orthomyxoviruses are shown to regulate the activity of IRF-3 [313-315]. However, this calls for more functional studies of the pathways that regulate IFN transcription and how ISAV possibly interact with these. Our studies show that IRF-7 is involved in the regulation of IFN in salmon and it is inhibited by ISAV. What remains to be elucidated is the role IRF-7 has in salmon compared to the other IRFs.

Fish IFN genes appear to have a different organisation from that of higher vertebrates, as salmon seems to possess three classes of IFN (personal communication with Robertsen, R). These could have a specific tissue distribution, as S-27609 stimulation induces expression of ISGs without any prominent induction of IFN-α1/ α2 in organs of salmon. Thus, there are still gaps that need to be filled in order to fully understand the IFN system in fish and how this interacts with virus infections.

Main conclusions

- The synthetic ssRNA analogue S-27609 activates the IFN system of salmon, possibly through specialised immune cells similar to the mammalian pDCs.
- Salmon possess an IRF-7 like protein involved in regulation of the IFN system.
- ISAV is a powerful inducer of some key genes of the salmon IFN system, but inhibits induction of others.
- ISAV have evolved mechanisms to antagonize the IFN system, because ISAV replication is not inhibited in TO cells treated with IFN.
- The ISAV genome encodes two proteins with IFN antagonistic properties. Our studies show that both of these proteins are negative regulators of the IFN promoter.

5. References

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Paper I

Paper II

Paper III

Paper IV