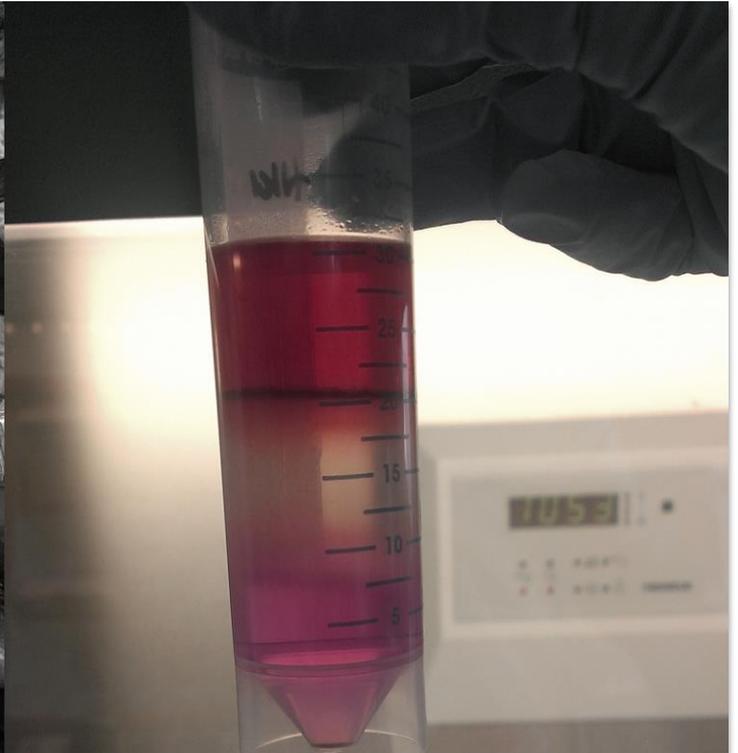


Adjuvant combinations with the Toll-like receptor ligand CpG

Protective immune responses in Atlantic salmon

Hanna Leena Thim

A dissertation for the degree of Philosophiae Doctor – Fall/Winter 2016



*EITHER WRITE SOMETHING WORTH READING,
OR DO SOMETHING WORTH WRITING*
BENJAMIN FRANKLIN

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List of papers

Paper I: Immuprotective activity of a salmonid alphavirus vaccine: Comparison of the immune responses induced by inactivated whole virus antigen formulations based on CpG class B oligonucleotides and poly I:C alone or combined with an oil adjuvant.

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Vaccine (2012) ¹authors contributed equally

Paper II: Vaccine adjuvants in fish make a difference: Comparing three adjuvants (Montanide ISA763A Oil, CpG/poly I:C combo and VHSV glycoprotein) alone or in combination formulated with an inactivated whole salmonid alphavirus antigen

Hanna L. Thim, Stéphane Villoing, Marian F. McLoughlin, Karen E. Christie, Søren Grove, Petter Frost, Jorunn B. Jørgensen

Vaccines (2014)

Paper III: Homing of antigen-presenting cells in head kidney and spleen – salmon head kidney hosts diverse APC types

Dimitar B. Iliev, Hanna L. Thim, Leidy Lagos, Randi Olsen, Jorunn B. Jørgensen

Frontiers in Immunology (2013)

Paper IV: Profiling B cell populations in Atlantic salmon – Toll-like Receptor expression and responsiveness to CpG stimulation

Shiferaw Jenberie, Hanna L. Thim, Ingvill Jensen, Jorunn B. Jørgensen

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Formulations combining CpG containing oligonucleotides and polyI:C enhance the magnitude of immuneresponses and protection against pancreas disease in Atlantic salmon

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Developmental and comparative immunology (2010)

Immune parameters correlating with reduced susceptibility to pancreas disease in experimentally challenged Atlantic salmon (*Salmo salar*).

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Fish and shellfish Immunology (2013)

Comparison of transcriptomic responses to pancreas disease (PD) and heart and skeletal muscle inflammation (HSMI) in heart of Atlantic salmon (*Salmo salar* L).

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Fish and shellfish Immunology (2015)

Summary of thesis

Aquaculture is in need of improved viral vaccines to meet the demands of a growing sustainable industry. Fish viral vaccines are often based on less immunogenic antigen (Ag) formulations that require adjuvants to elicit sufficient protection. Toll-like receptor (TLR) agonists holds high immunostimulatory properties and are in mammals well explored vaccine adjuvants. The potential of synthetic TLR-agonists as adjuvants lie in their ability to provide signals engaging both innate and adaptive immunity that in turn may prime an array of immune responses.

In this thesis, the main aim has been to increase our knowledge regarding the immunostimulatory properties of the nucleic acid-sensing TLR-agonists CpG and poly I:C in Atlantic salmon. First, the TLR-agonists were included as an adjuvant combo to inactivated whole salmonid pancreas disease virus (SPDV/SAV) Ag formulations where effects on protection against SAV was investigated both by injection and co-habitant challenge. The work show that the combo greatly potentiated neutralizing antibody responses. In line with the challenge studies, it was further investigated if cells in the periphery could take up the soluble antigens CpG and ovalbumin (OVA) and home to the immunological tissues head kidney (HK) and spleen. Indeed, leukocytes homed to the HK and displayed traits common for maturing Ag-presenting cells (APCs). In that study, a B-lymphocyte like IgM positive cell population was described in HK that downregulated early B cell marker transcript, while secreted IgM transcripts were upregulated upon *in vitro* CpG stimulation. This suggests that the HK harbor antibody secreting cells (ASCs). Based on that, method optimization to characterize Atlantic salmon B cells functionally were initiated. Flow cytometry analysis of naïve salmon using trout monoclonal antibodies show that IgM⁺ B cells are the dominating Ig class in salmon systemic lymphoid tissues. Moreover, mRNA transcript analyzes of sorted IgM⁺ B cells from HK, peripheral blood and spleen leukocytes show that they express a range of nucleic acid-sensing TLRs. In addition, CpG stimulation alone was enough to induce IgM secretion in sorted HK and spleen IgM⁺ B cell cultures. Our findings suggests that these TLRs are promising 'model-agonists'.

To further support the use of these TLR-agonists as adjuvants in finfish viral vaccines, research regarding their direct and indirect effects, particularly on salmon B cells, should be emphasized. This requires markers as well as *in vitro* and *in vivo* assays that are yet not in place.

Abbreviations

AP1: activator protein 1	IPN: infectious pancreatic necrosis
APC: antigen-presenting cell	IPNV: infectious pancreatic necrosis virus
ASC: antibody-secreting cell	IRAK: IL-1R associated kinase
CD: cluster of differentiation	IRF: IFN regulatory factor
CFA: complete freund's adjuvant	ISCOM: immunostimulating complex
CMC: cell mediated cytotoxicity	ISG: IFN stimulating gene
CMS: cardiomyopathy syndrome	ISA: infectious salmon anemia
CREB: cyclic AMP-responsive element binding protein	ISAV: infectious salmon anemia virus
CTL: cytotoxic T lymphocyte	ITAM: immunoreceptor tyrosine-based activation motif
DC: dendritic cell	JAK/STAT: janus activated kinase/signal transducers and activators of transcription
dpi: days post infection	LPS: lipopolysaccharide
dpv: days post vaccination	LRR: leucine rich repeat
DTH: delayed type hypersensitivity	mAb: monoclonal antibody
GALT: gut-associated lymphoid tissue	MAC: membrane attack complex
GIALT: gill- associated lymphoid tissue	MAL: MyDD88-adaptor like
HLB: hydrophilic:lipophilic balance	MALT: mucosa-associated lymphoid tissue
hvp: hours post vaccination	MDA5: melanoma differentiation-associated protein-5
HSMI: heart and skeletal muscle inflammation	MHC: major histocompatibility complex
ICOS: inducible costimulatory	MPL: monophosphoryl lipid A
ICR: immune cell recruitment	MyD88: myeloid differentiation primary response protein 88
IFA: incomplete freund's adjuvant	nAb: neutralizing antibody
IFN: interferon	NALT: nasopharynx-associated lymphoid tissue
Ig: immunoglobulin	NCC: non-specific cytotoxic cell
IHN: infectious haematopoietic necrosis	NF- κ B: nuclear transcription factor kappa B
IHNV: infectious haematopoietic necrosis virus	NK: natural-killer cell
IL: interleukin	NLR: nod-like receptor
ILT: interbranchial lymphoid tissue	nsP: non-structural protein
i.m: intramuscular	
i.p: intraperitoneal	

NVI: Norwegian veterinary institute

ODN: oligodeoxynucleotide

OIE: office international des epizooties

ORF: open reading frame

OVA: ovalbumin

o/w: oil-in-water

PAMP: pathogen associated molecular pattern

PAX5: paired box protein 5

PD: pancreas disease

pDC: plasmacytoid DC

PLA: poly(lactic acid)

PLGA: poly(lactic-co-glycolic acid)

PM: plasma membrane

PMCV: piscine myocarditis virus

PO: phosphodiester

poly I:C: polyinosinic: polycytidylic acid

PRR: pattern-recognizing receptor

PRV: piscine reovirus

PTO: phosphothiorate

QTL: quantitative trait locus

RAG: recombination-activating gene

RC: replicase complex

RIG-I: retinoic acid-inducible gene-1

RLR: RIG-I like receptors

RT-qPCR: real-time quantitative polymerase chain reaction

SALT: skin associated lymphoid tissue

SARM: sterile α - and armadillo-motif-containing protein

SAV: salmonid alphavirus

SD: sleeping disease

SDV: sleeping disease virus

SPDV: salmon pancreas disease virus

ssRNA: single stranded RNA

SVCV: spring viraemia of carp virus

TcR: T cell receptor

TDB: trehalose dibehenate

T_h: T helper cell

TIR: toll/IL-1 receptor

TLR: toll-like receptor

TNF: tumor necrosis factor

TRAF: TNF-receptor associated factor

TRAM: TRIF-related adaptor protein

T_{reg}: induced regulatory T cell

TRIF: TIR-domain containing adaptor protein inducing IFN β

VHS: viral haemorrhagic septicaemia

VHSV: viral haemorrhagic septicaemia virus

VN: virus neutralization

VPL: virus-like particle

WGD: whole genome duplication

w/o: water-in-oil

w/o/w: water-in-oil-in-water

Introduction

1 A summarized introduction to present day aquaculture

Present day, aquaculture is booming, growing more rapidly than the other food animal-producing sectors combined. With a decline in the capture fishing industry together with diminishing wild stock, aquaculture has risen as an important seafood source. In 2012, an all-time production high was set, when nearly fifty percent of all fish for human consumption came from aquaculture (FAO 2014). That same year Norway produced more than 1.2 million tonnes of Atlantic salmon to the domestic and global market; contributing to more than fifty percent of the world total production of that specific fish (Guttormsen 2015). Parallel to an increased production demand, is the continuous struggle to handle disease outbreaks. For the aquaculture industry, development and implementation of efficient viral vaccines, alongside controlled and predictable disease handling is of crucial importance to meet future needs (Brudeseth et al. 2013, Gudding 2014, Gudding & Van Muiswinkel 2013).

1.1 Disease prevention in aquaculture

For wild fish, disease outbreaks are considered a part of a normal biological process. In aquaculture, the disease situation for farmed fish gradually changed for the worse in large-scaled farming. With higher densities, combined with the effectiveness of pathogen transportation through water, diseases in fish farming became problematic. Worldwide, infectious bacterial diseases are the most abundant causative agents (54.9%), followed by viral diseases (22.6%), parasites (19.4%) and fungi (3.1%) in today's finfish aquaculture (McLoughlin & Graham 2007). Historically, until the seventies immunoprophylaxis mainly consisted of antimicrobial compounds, when vaccines were introduced to commercial aquaculture (Gudding & Van Muiswinkel 2013). With the introduction of bacterial vaccines in Northern Europe and North America, bacterial diseases in these areas are, per today, well controlled, and the use of antibiotics limited (Somerset et al. 2005). Since the 1990s in Norway, the yearly antibiotic use for fish has typically been kept below one metric ton (Gudding 2014, NORM/NORM-VET 2014). The introduction of water-in-oil (w/o) emulsion vaccines for bacterial diseases (1980-90s) are seen as the key reason for the positive development of Norwegian aquaculture (Somerset et al. 2005). Antigen-components available in the industry today are based on classical fermentation, cultivation and/or recombinant

technologies. Worldwide, there are vaccines for more than 17 fish species, protecting against more than 20 bacterial diseases and 6 viral diseases (Brudeseth et al. 2013). Most of these vaccines provide an acceptable protection against disease, yet they do not provide sterile immunity, meaning that they fail to protect against infection (Gudding 2014). Due to a scarcity of efficient anti-viral therapeutics and a difficulty developing efficient viral vaccines, viral outbreaks render catastrophic economical losses and a reduced animal welfare status across the world (Dhar et al. 2014). Section 3.0 provide an overview of viral vaccines in aquaculture.

1.2 Viral diseases in aquaculture – present day status

Kibenge et al. (2012) argue that the fish strains used in aquaculture that often recently have been derived from wild species, may account for the high infectious pressure present; by insufficient adaptation to the high-density confinements, chronic stress increases. Hence, viruses that are harmless under natural conditions are given the opportunity to propagate to variants that are more virulent. This occurrence, named “local effect”, is of crucial economic importance (Kibenge et al. 2012). No clear evidence of transmission from wild to farmed fish can be stated, still, studies show that it may vary depending on the viral disease (Johansen et al. 2011). Worldwide, aquaculture has had a growth rate of about 6% over the past decade. The industry is increasing in types of farmed species and as an unfortunate result, an increased number of diseases needs to be controlled (Dhar et al. 2014, Kibenge et al. 2012).

Some of the best-known infectious finfish viruses in aquaculture are viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV), spring viraemia of carp virus (SVCV), all belonging to the Rhabdoviridae family, and the aquatic orthomyxovirus, infectious salmon anemia virus (ISAV). VHSV and IHNV are both known for outbreaks originating from wild fish reservoirs (Johansen et al. 2011), while the spread of SVCV and ISAV have been linked to imports of carp in the US and to imported virus infected salmon eggs in Chile, respectively (Crane & Hyatt 2011, Kibenge et al. 2012). OIE (Office International des Epizooties), the world organization for animal health, has in 2016 listed ten notifiable infectious fish diseases (OIE 2016). Among them, along with the viruses listed above, is salmon pancreas disease virus (SPDV) commonly referred to as Salmonid alphavirus (SAV), causing pancreas disease (PD). The Norwegian veterinary institute (NVI), state PD as the most critical infectious disease in the Norwegian aquaculture industry in their 2015 annual fish health report (Hjeltnes 2016). Two other major viral diseases in Norwegian aquaculture are heart and

skeletal muscle inflammation (HSMI; where the causative agent is believed to be *Piscine reovirus*, PRV) and cardiomyopathy syndrome (CMS; where *Piscine myocarditis virus*; PMCV, has been described as the likely causative agent). Both are treated with great concern due to constant high outbreak reports. Infectious pancreatic necrosis virus (IPNV), which was a major concern in Atlantic salmon farming a decade ago, with more than 200 yearly outbreaks, has been declining and is now more scarcely reported in 15 out of about 600 locations (Hjeltnes 2016). The use of QTL (quantitative trait loci)-resistant fish, i.e. fish with a gene-marker for IPNV-resistance, combined with a greater effort to eliminate “house strains” of IPNV are stated as the major incentives for the significant decrease in IPNV outbreaks in the report.

1.3 Pancreas disease is a major concern in Norwegian aquaculture

That PD causes both a detrimental welfare and economical loss is clear, albeit to state a precise cost for an outbreak is difficult. Estimates demonstrate an average direct cost of 55.4 million NOK for a SAV3 (SAV has six known subtypes presented in section 1.3.5) outbreak nine months after sea transfer at a facility with 1E06 smolt (Hjeltnes 2016). PD has been documented in Scotland and Ireland since the late seventies and early eighties. In Norway, the first registration of PD came in 1989 in Hordaland. Until 2003, NVI received scarce PD reports from the southwest area of Norway. Ever so gradually, both the geographical spread and disease reports increased. PD was, in 2007, stated as endemic in a large region of Western Norway and became a notifiable disease. Actions were implemented to prevent further spread north, and successfully, only sporadic outbreaks have been reported in northern Norway since, see Fig.1 (Jansen et al. 2016). In 2011, a marine SAV2 was identified, causing outbreaks in mid Norway (Hjortaas et al. 2013). Due to a rapid spread of SAV2 further north, a second endemic zone was legislated in 2012 north of the first, SAV3, endemic zone (Jansen et al. 2016).

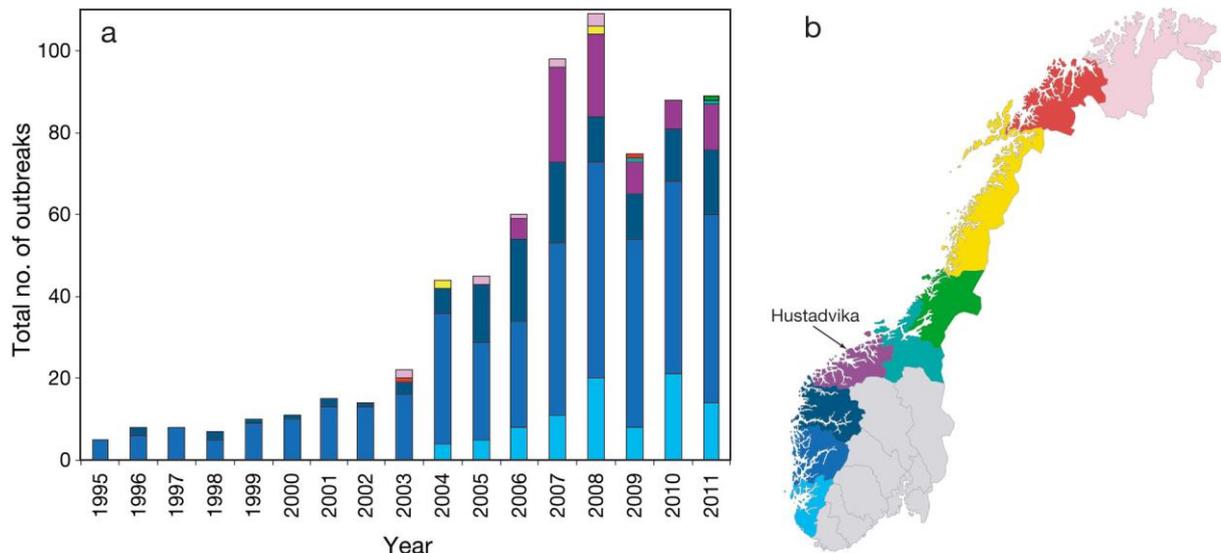


Figure 1. PD outbreaks in Norway from 1995 to 2011, before characterization of SAV2. a: annual number of outbreaks for each county, color coded as in b. Grey: no reported outbreaks. Figure from (Bang Jensen et al. 2012).

Diagnostic recordings of PD follow the seawater phase and most PD disease outbreaks occur between May and October. A disease outbreak can last over a long period and the economic loss after an outbreak manifests in unsatisfying growth and reduced slaughter quality. There is one commercial PD vaccine on the market, although with a debated effect. PD vaccination have reduced the amount of outbreaks and likely reduced mortality and, in addition, vaccination against PD may also have contributed to less virus shedding from infected fish (Bang Jensen et al. 2012).

The first sign of an imminent PD outbreak is a sudden drop in appetite a week or two in advance. Clinically diseased fish are often lethargic, observed close to the surface, swimming against the current, crowding close to the corners of the cage or may be resting at the bottom of the cage. Mortality vary greatly, and can range from 5 to 60% (McLoughlin & Graham 2007). Months or more after onset of mortality, many survivors fail to grow and become runts. Yellow mucoid gut contents is a common macroscopic sign and typical for a lack of appetite. SAV mainly infect the exocrine pancreas, heart and skeletal muscle, and are the tissues where PD induced microscopic lesions and abnormalities are observed. The most prominent changes occur as the disease develops, with a severe or total loss of exocrine pancreas, myocarditis, skeletal muscle degeneration and myositis as main findings (McLoughlin & Graham 2007, OIE 2013). Still, a SAV infection does not have to induce clinical signs (Graham et al. 2006). PD survivors develop a strong and long lasting immunity for the disease (Houghton & Ellis 1996)

with detectable neutralizing antibodies (Graham et al. 2006) that provide protection over time (Paper II). In addition, a relevant immune gene expression is upregulated after experimental infection (Johansen et al. 2015, Grove et al. 2013).

1.3.1 SAV classification and alphavirus structure

SAV belongs to the genus *Alphavirus* in the *Togaviridae* family and are enveloped, positive-sense single-stranded (+ss)RNA viruses. The SAV genome is roughly 12kb with a 5'-terminal cap and a 3'- polyadenylated tail divided into two open reading frames (ORFs). The nonstructural proteins (nsPs; nsP1-4) are encoded on the larger ORF and the structural proteins (E3, E2, 6K, TF and E1) on the smaller ORF with a subgenomic mRNA promoter that divides the two ORFs (Ryman & Klimstra 2008).

A fully transcribed and functional SAV virus particle has a diameter of 55 to 65nm and is spherical in shape (OIE 2013) (Fig. 2). An alphavirus nucleocapsid consists of one +ssRNA genome copy complexed with 240 capsid protein copies, where each individual capsid protein is arranged as pentamers and hexamers to form a T=4 icosahedral symmetry (Cheng et al. 1995, Paredes et al. 1993). The lipid bilayer of the virion resemble the phospholipid composition to that of the host plasma membrane. Eighty copies of the viral glycoprotein spikes (trimers of E1/E2 or E1/E2/E3 depending of the specific alphavirus) are anchored in the virion bilayer (Gaedigk-Nitschko & Schlesinger 1990).

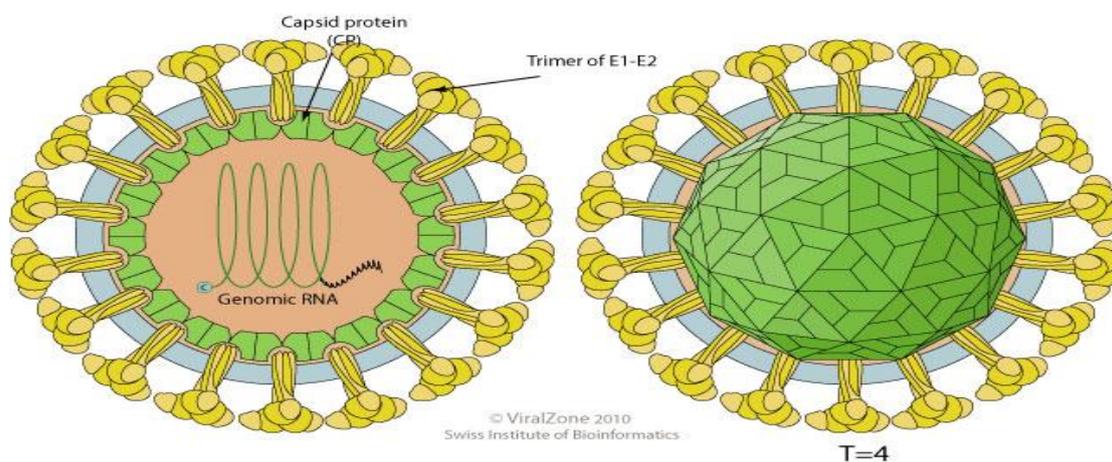


Figure 2. Alphavirus. Alphaviruses are enveloped, spherical and icosahedral viruses. The capsid has a T=4 icosahedral symmetry consisting of 240 monomers. The envelope has 80 spikes, where each spikes is a trimer of E1/E2 or E1/E2/E3 proteins. Figure from (Hulo et al. 2011).

1.3.2 Alphavirus replication

Alphavirus replication starts when a virus enters the cell through receptor-mediated endocytosis (RME), facilitated by E2, and the intact virus is pH-dependently delivered to an endosome, facilitated by E1 (Metz et al. 2011, Strauss & Strauss 1994, Wahlberg et al. 1989, White & Helenius 1980). Alphaviruses have many hosts and target a diversity of the host's cell types, hence it is reasonable that the viruses use an entry receptor present on a large selection of cells or possess the capacity to use different cell surface receptors (Lanzrein et al. 1994, Griffin 2007). A hypothesized acidic pH-dependent envelope-endosomal fusion releases the nucleocapsid to the cytoplasm. A process seemingly dependent on that the target membrane has a presence of cholesterol, provisional of the specific alphavirus (Leung et al. 2011). In the cytoplasm, the alphavirus binds to ribosomes and directly serves as the mRNA template for protein synthesis of the complementary 42S minus strand containing the polyproteins (Strauss & Strauss 1994). See Fig. 3 for a schematic organization of the alphavirus genome.

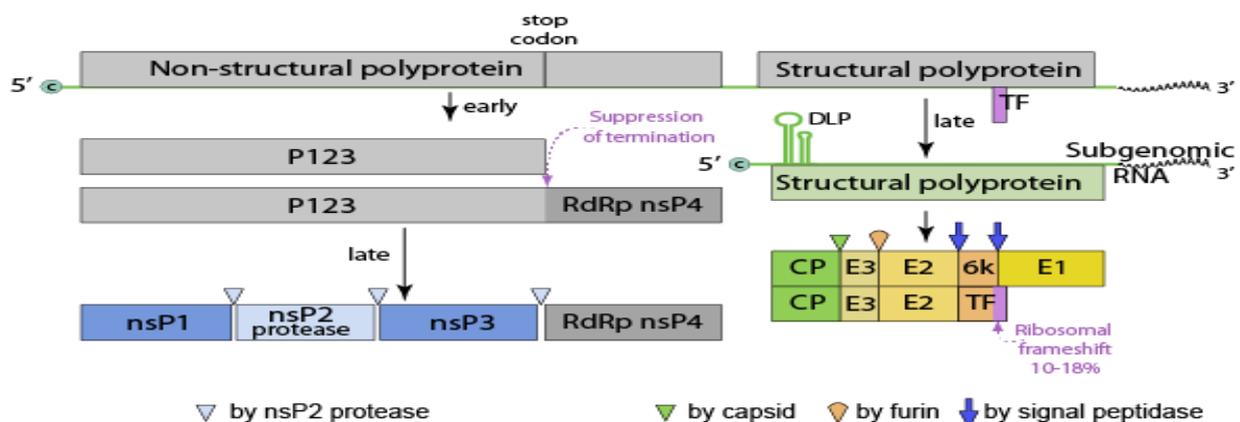


Figure 3. Diagrammatic presentation of an alphavirus genome. The replication cycle is depicted as a series of temporally regulated steps further described in the text. 1: translation and processing of the non-structural polyprotein (nsP). 2: transcription of complementary, negative-sense RNA. 3: synthesis of progeny genomes. 4: synthesis of subgenomic RNAs. 5: translation and processing of structural polyprotein CP/E2/6KE1. 6: packaging of progeny genomes into nucleocapsids. 7: budding at the plasma membrane to release progeny virions. Figures from (Ryman & Klimstra 2008, Hulo et al. 2011).

Genome replication is instigated by translation of a polyprotein that contains the four nsPs from the mRNA-like genome. Proteolytic cleavage of the non-structural polyprotein (P1234), by a papain-like serine protease of the nsP2 component, produces the mature individual viral proteins; nsP1-4 (Karlsen et al. 2009, Ryman & Klimstra 2008). The nsPs constitute the replicase complex (RC) that, together with the host proteins, facilitates replication (Karlsen et al. 2009, Mérour et al. 2016), see Fig. 3. In addition to possessing the necessary functions to replicate the viral genome, the nsPs can antagonize the host's innate immune system and

modulate cellular transcription and translation (Snyder et al. 2013), although less about these processes are known in regards of piscine alphaviruses. The second ORF is also translated as a polyprotein, where the capsid protein (C) cleaves itself in the cytoplasm where it associates with viral genomic RNA and forms the nucleocapsid core that later is enveloped by the viral protein-enriched plasma membrane (PM) envelope. The remaining polyprotein bulk consisting of the envelope proteins pE2, 6K and E1 is cleaved to individual proteins by host signal peptidases in the secretory pathway before packaging and egress to the PM. At the PM; the site for virus budding, pE2/E1 heterodimers are assembled to trimers (spikes) and pE2 is further cleaved to E2 and E3 (Hikke et al. 2014, Karlsen et al. 2009, Snyder et al. 2013). For terrestrial alphaviruses, it is implied that both the 6K and TF proteins play an important role in the release of the virus (Snyder et al. 2013).

1.3.3 Six known SAV subtypes

SAV display a genetic heterogeneity in Europe (Fig 4). Partial sequencing of nsP3 and E2 from SAV isolates across Europe in 2008 revealed three additional subtypes (Fringuelli et al. 2008) to the previously three known (Hodneland et al. 2005, Weston et al. 2005), completing the list as known today, with six SAV subtypes (SAV1-6). The coding sequence of nsP3 retains a greater divergence compared to E2 across the subtypes. However, true effects of the insertions or deletions is unknown (Fringuelli et al. 2008, Karlsen et al. 2009). SAV1 induces PD in Scotland and Ireland. SAV2 has been known as a freshwater rainbow trout pathogen causing sleeping disease (SD) in continental Europe, until recently, when a marine SAV2 was detected in the seawater phase,

infecting Atlantic salmon both in Scotland and Norway (Fringuelli et al. 2008, Hjortaas et al. 2013). SAV3 is endemic along the Norwegian southwest coast and causes outbreaks in both Atlantic salmon and rainbow trout. SAV4-6

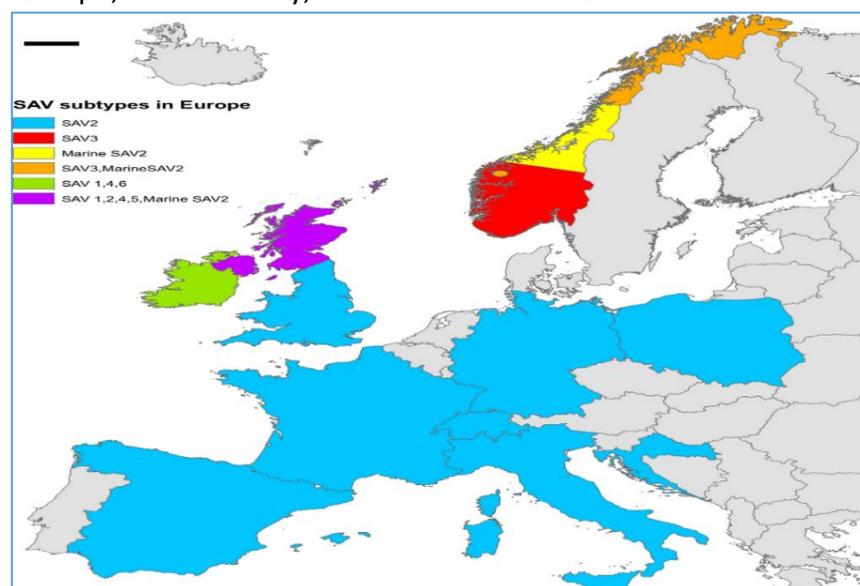


Figure 4. European SAV subtype distribution map. From Jansen et al (2016).

have been detected in Atlantic salmon in overlapping regions along the Irish, Northern Irish and Scottish coast (Graham et al. 2012, Hjeltnes 2016, Karlsen et al. 2009).

1.3.4 SAV diagnostics

To confirm a SAV diagnosis, cell-culture assays, transcript analyses and immunostaining assays with monoclonal antibodies (mAbs) are necessary complements to traditional diagnostic criteria (clinical signs and histopathology). However, to obtain legible results of SAV replication in cell lines can be challenging. Detectable cytopathic effect (CPE) can appear after approximately one week in culture, at when virus titers most often peak (Gahlawat et al. 2009, Graham et al. 2008). CPE may not always be present or can be indistinct, which can lead to false-negative reads (Desvignes et al. 2002, Karlsen et al. 2006, Nelson et al. 1995) and several passages may be needed before CPE is detectable (Graham et al. 2003, Jewhurst et al. 2004). Passaging may result in decreased virus titer (Graham et al. 2008) and induction of mutations in regions important for host cell entry and viral packaging (Karlsen et al. 2006, Moriette et al. 2006). RT-qPCR of nsP1 or E1 allow for earlier viral detection at two to four days post infection (dpi) with a peak after three to seven dpi (Christie et al. 2007, Gahlawat et al. 2009). Present day, cultivation of SAV as a reliable diagnostic tool has receded and RT-qPCR (Hodneland & Endresen 2006) has taken over. Further, detection of SAV neutralizing antibodies (nAbs) is a well-implemented method (Jewhurst et al. 2004, OIE 2013, Todd et al. 2001, Christie et al. 1998). However, a virus neutralization (VN) test does not necessarily confirm the presence of virus and it must be noted that a VN assay require sero-conversion for the test to be positive (McLoughlin et al. 1996).

1.3.5 SAV neutralizing Ab and serological cross-reactivity between subtypes

Atlantic salmon surviving a SAV infection, both natural and experimental infection, was early shown to be protected against subsequent infections (Houghton 1994, McVicar 1987). This indicated an ability to mount a protective immunological response against the disease. A successful passive immunization study with kidney homogenate raised from naturally infected Atlantic salmon gave no PD pathology in experimentally challenged fish, showing up to 100% neutralizing effect (Houghton & Ellis 1996) and it was concluded that the fish were able to produce protective nAbs against SAV. In Norway, nAbs have also been detected from SAV3 field outbreaks and these nAbs were shown to cross-react with the Irish SAV1 reference isolate F93-125 (Christie et al. 1998, Nelson et al. 1995). Confirmation of serological cross-reactions

between subtypes from Norwegian, Scottish and Irish SAV isolates are evident through both field and experimental studies (Graham et al. 2003, McLoughlin et al. 1998, Taksdal et al. 2007, Weston et al. 2002). However, most of these studies have been one directional, i.e. using a single subtype, often SAV1, as the virus in the VN assay. In 2013, a comprehensive neutralization study by Graham et al (2014) included homologous virus/serum pairs from all six subtypes, both experimentally obtained and from field outbreaks. The study documented broad cross-reactivity between all subtypes, with SAV6 showing the lowest heterologous cross-neutralization.

1.3.6 How to successfully infect Atlantic salmon with SAV

A crucial aspect of challenge experiments is how to expose the fish to the causative agent. Intraperitoneal (i.p.) and intramuscular (i.m.) injections hold an advantage that they, to a certain extent, can guarantee a successful infection where viral dose and time of infection are precisely controlled. Albeit, if the desire is a more natural route of exposure, the option is a cohabitant challenge model by i.p. injecting so-called shedder fish with the causative agent and rear them together with the experimental fish. SAV i.p. injected fish have been shown to shed virus into their environment 4-10 dpi (Andersen et al. 2010, McLoughlin et al. 2006) at when the experimental fish will be exposed to the causative agent and can be 'naturally' infected. The downside is that time and infectious dose may vary for each individual. Both methods are commonly applied for experimental SAV studies (Christie et al. 2007, Xu et al. 2012, Andersen et al. 2010) and recently bath immersion has been presented as a valid option (Jarungsriapisit et al. 2016). SAV nAbs have been detected as early as 10 dpi after experimental challenge (McLoughlin et al. 1996). Common acceptance is that the fish seroconvert three to six weeks post-injection challenge at 12-15°C (Christie et al. 2007, Desvignes et al. 2002, McLoughlin & Graham 2007) and that a lag effect is to expect for a co-habitant challenge using shedders (Houghton & Ellis 1996, Graham et al. 2011). Note that most SAV infection models rarely induce mortality (Karlsen et al. 2006).

2 Organization and execution of teleost immunity

There is a never-ending battle in multicellular organisms between intruding pathogens and the host's defense mechanisms. Throughout evolution, these defense mechanisms have been

refined and the intruders have found new ways to evade them. Here follows a brief introduction to teleost immunity, mainly against viral pathogens.

2.1 Teleost morphology and immune system organization

Teleost (bony fish) represent a transition point on the phylogenetic spectrum in regards of how their immune system is organized. Teleost resemble higher vertebrates more regarding key innate and adaptive immunological mechanisms compared to invertebrates (lacking adaptive immunity). Immune cells equivalent to mammalian macrophages/monocytes, neutrophils, eosinophils, dendritic-like cells, B cells, plasma cells, T cells and NK-like cells (or non-specific cytotoxic cells) are present in teleost (Rauta et al. 2012, Whyte 2007). However, a knowledge gap exists regarding functionality. Noteworthy, several rounds of whole genome duplications (WGDs) in the largest animal phyla (>20.000 different fish species) that also is known for its high species gene variation, impede the mapping of individual genes and their practical function (Volf 2004). Salmonidae proposedly have gone through four WGD events, which importantly can have led to both sub- and neo-functionalization of ancestral genes (Pietretti & Wiegertjes 2014, Lien et al. 2016).

Despite morphological similarities to mammals, the immune tissue organization in fish differ. The most distinct difference being the lack of bone marrow and lymph nodes. In fish, the anterior kidney (head kidney or HK), the thymus and the spleen are the major lymphoid organs (Rombout et al. 2005). As the primary lymphoid organs; the HK execute functions comparable to those of the mammalian bone marrow regarding hematopoiesis (Rombout et al. 2005) and the thymus stand as the main site for T cell lymphogenesis (Bowden et al. 2005, Koppang et al. 2010), respectively. The HK also holds the highest numbers of developing B cells (Zwollo et al. 2005, Zwollo et al. 2010). In addition, both HK and spleen serve as secondary lymphoid organs (Whyte 2007). In teleost four mucosa-associated lymphoid tissues (MALTs) have been characterized (Gomez et al. 2013, Sunyer 2013). The gut-associated lymphoid tissue (GALT; Salinas et al. 2007, Zhang et al. 2010), the skin-associated lymphoid tissue (SALT; Leal et al. 2016, Xu et al. 2013), the gill-associated lymphoid tissue (GIALT) that harbor the interbranchial lymphoid tissue (ILT) (Koppang et al. 2010, Dalum et al. 2015) and the most recent, nasopharynx-associated lymphoid tissue (NALT; Tacchi et al. 2014).

2.2 Innate immunity – first but not least

As free-living organisms, fish are already early during their embryonic development, depending on non-specific, innate, immunity for survival (Rombout et al. 2005). Through a distinct evolutionary ‘immunological big-bang’, approximately 450 million years ago, several rounds of gene duplication gave rise to recombination-activating genes (RAGs) and adaptive immunity emerged with lymphocytes as we define them today. Hence, jawed fish (cartilaginous and bony fish) are the earliest vertebrates known to possess an innate and a ‘modern’ adaptive defense (Agrawal et al. 1998, Schluter et al. 1998, Watts et al. 2001). In fish, innate immunity stands as a fundamental defense system that through a large set of receptor proteins contribute to homeostasis and to shape adaptive immune responses (Uribe et al. 2011). There is a whole arsenal of innate factors directing the innate immune response, both by sensing intruders and executing measures to limit the harm against the host. Those factors can be divided into physical, cellular (i.e. specific surface receptors) and humoral (i.e. complement factors, cytokines) (Uribe et al. 2011).

2.2.1 Innate immunity – first waves of defense

If a virus manage to breach the very first wave of defense; the innate constitutive factors such as epithelial surfaces and mucus layers, the second wave of innate immunity, with so called inducible factors, stand alert (Watts et al. 2001). As the ‘primitive’ arm, innate immunity respond to conserved common pathogen structures called pathogen-associated molecular patterns (PAMPs). PAMP-recognition is enabled through soluble and cell-associated germline encoded pattern recognition receptors (PRRs). PRRs can functionally be classified as i: soluble bridging ii: endocytic or iii: signaling PRRs (Aoki et al. 2013). Of the signaling PRRs, the Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-I like receptors (RLRs), all are present in fish (Aoki et al. 2013, Purcell et al. 2006, Rebl et al. 2010). TLRs are the most studied PRRs in teleost and highlighted in section 2.2.4. Common for all PRRs sensing viral RNA is the activation of antiviral responses mediated by interferon (IFN) production and inflammatory cytokines (Hansen et al. 2011). These are effector functions initiated through different signaling pathways regulating activation of specific transcription factors, such as interferon regulatory factors (IRFs) and nuclear transcription factor kappa B (NF- κ B). IRFs contribute to an antiviral effect by induction of IFNs and shape the full immune response in a wider sense. NF- κ B activation promotes an inflammatory response and can switch an immature DC (dendritic cell)

phenotype to an inflammatory phenotype that is capable of inducing adaptive immunity (Li & Verma 2002).

2.2.2 A pro-inflammatory response to kick-off innate immunity

Cytokines are a family of low molecular weight proteins, secreted from activated immune cells that aid in inflammatory modulation, development and maintenance of adaptive responses (Chabalgoity et al. 2007). The cytokine family is divided into IFNs, interleukins (ILs), tumor-necrosis factors (TNFs), colony stimulating factors and chemokines (Savan & Sakai 2006). Chemokines are a superfamily within the cytokines, essential for mediation of immune effector cell movement to sites of inflammation/infection (Plouffe et al. 2005, Whyte 2007).

For any type of infection, the inflammatory response is crucial in higher vertebrates for an efficient innate and subsequent adaptive response (Hussell & Goulding 2010). Studies support that also in fish, an inflammatory response is critical to mount an efficient antiviral reaction. However, it is not known if the inflammatory response can mediate the subsequent adaptive response or have a direct antiviral effect (Collet 2014). In fish, macrophages (resident in the peritoneal cavity) and granulocytes (attracted by inflammatory response) are the most important phagocytic inflammatory cells, mobilized by infection or tissue injury (Dijkstra et al. 2001, Dixon & Stet 2001, Jørgensen 2014). Macrophages are potent producers of the pro-inflammatory cytokines tumor-necrosis factor alpha (TNF- α) and interleukin-1 beta (IL-1 β) (Collet 2014). TNF- α regulate leukocyte trafficking as well as inflammation (Shimizu et al. 1992, Zou et al. 2003) and gene homologs have been identified in a diversity of fish species, with a variable number of genes depending on species (Haugland et al. 2007, Zou et al. 2002). In trout, IL-1 β transcripts are upregulated early after viral infection (Collet 2014, Secombes et al. 2011) and recombinant IL-1 β affect leukocyte proliferation, peritoneal leukocyte phagocytosis, chemotaxis and induce relevant immune gene transcript levels (Secombes et al. 2011, Hong et al. 2001, Peddie et al. 2001, Plouffe et al. 2005). As for TNF- α , IL-1 β has been cloned from many fish species (Secombes et al. 2011) and in some species several isoforms exist (Engelsma M Y. 2003, Husain et al. 2012).

2.2.3 Mediators of teleost innate immunity

The complement system plays a central part in innate immunity and, as it is known from mammals, it can be activated in three ways: through the classical, the alternative or the lectin pathway. Activities and homologs of mammalian complement components from all three

routes are documented in teleost (Nakao et al. 2011). The alternative pathway induces a five to ten times more pronounced response in teleost compared to mammals, arguably due to the high isotype variation of factor C3 (Nakao et al. 2006, Watts et al. 2001). Functionally and structurally diverse teleost complement components, compared to other vertebrate species, suggests an importance as a rapid response system against intruders (Plouffe et al. 2005, Zhu et al. 2013). Lorenzen and LaPatra (1999) showed that in presence of complement, salmonid antibodies can neutralize IHNV and VHSV. Complement-mediated phagocytosis (Schraml et al. 2006), lysis of microbes through MAC (Boshra et al. 2006) and chemotactic functions (Watts et al. 2001) are also documented in teleost.

Upon binding of some PAMPs to its PRR, specific transcription factors (the IRFs) induce the so-called first line of antiviral defense, the interferons (IFNs). IFNs got their name for their ability to interfere with viral infections, this although IFNs *per se* possess no antiviral activity. Secreted IFNs communicate with neighboring cells through the JAK/STAT pathway that in turn can activate a grand repertoire of IFN-stimulated genes (ISGs). IFNs are generally divided in three chief groups; type I, II and III. In fish, type I (a, b, c, d, e and f) IFNs induces specific antiviral immune defenses, while type II (γ) IFN promotes cell-mediated immunity as well as antiviral and -bactericidal responses (Zou & Secombes 2011, Zou et al. 2014). The teleost IFN arsenal vary greatly between species and salmonids possess several virally inducible IFN genes (Zou et al. 2014, Sun et al. 2009). Atlantic salmon type I IFN genes resides in a multiple gene cluster with two IFNa (IFNa1 and a3), four IFNb (IFNb1-b4) and five IFNc (IFNc1-c5) genes (Sun et al. 2009). IFNa2 and IFNd are found outside the cluster (Svingerud et al. 2012). In trout, seven IFNe (IFNe1-e7) genes and two IFNf (IFNf1 and f2) genes have been described. The initial six known salmonid type I IFN groups are further divided to group I (IFNa, d and e) and II (IFN b, c and f) based on the cysteine pattern of the mature peptide (Zou et al. 2014). All components of the JAK/STAT signaling pathway have been identified in fish (Zhang & Gui 2012, Zou & Secombes 2011), as well as a large number of ISGs providing antiviral activities (Langevin et al. 2013). Reports show that Atlantic salmon and trout type I IFNs have been upregulated after IFN stimulation (Chang et al. 2009, Sun et al. 2009), displaying an ability to act as ISGs themselves. Further, ISGs such as Mx also possess an antiviral effect in Atlantic salmon against IPNV (Larsen et al. 2004) as well as in grouper (Chen et al. 2008) and barramundi (Wu et al. 2010) against nodavirus.

2.2.4 TLRs – the most studied PRRs in fish

TLRs are expressed in a variety of immune cells such as phagocytes, DCs and B cells. Since TLRs recognize a large variety of PAMPs, they can trigger a rapid inflammatory response and prime adaptive immunity (Iwasaki & Medzhitov 2010, Takeuchi & Akira 2010). Toll-like homologs of the 'original' Toll receptor identified in fruit fly (*Drosophila melanogaster*) have since been identified in more or less all animal kingdom classes, including several orders of fish (Pietretti & Wiegertjes 2014). TLRs can be classified based on their location (cell surface/type I: the human TLR1, 2, 4, 5, 6 and 10 or endosomal/type II: the human TLR3, 7, 8, 9) (Palti 2011) or which general class of PAMPs they recognize (6 major families; the mouse TLR1, 3, 4, 5, 7 and 11) (Roach et al. 2005). Homologous fish TLR genes, based on both classification systems has been discovered (Palti 2011, Pietretti & Wiegertjes 2014, Rebl et al. 2010). The TLR arsenal is much greater in fish, with nearly 20 types identified in about a dozen teleost species; TLR 1, 2, 3, 4, 5M, 5S, 7, 8, 9, 13, 14, 18, 19, 20, 21, 22, 23, 24, 25 and 26 (Rauta et al. 2014) including isoforms such as TLR8a1-2 and TLR8b1-2 in Atlantic salmon (Palti 2011).

The immunological importance of TLRs are reflected by their presence in early stages of evolution and their conservation through both invertebrate and vertebrate lineages (Medzhitov & Janeway 2000). Structurally they comprise an extracellular N-terminus with leucine-rich repeat regions (LRRs), a transmembrane domain and an intracellular C-terminus with a Toll/IL-1 receptor (TIR) domain (Akira et al. 2006). The TIR domain is central for attracting adaptor molecules and is highly conserved in TLRs within a species as well as across animal species (Werling et al. 2009). The LRRs are less well conserved and variations in repeat number, sequences and length of the LRR regions determine the individual TLR's classification (Aoki et al. 2013). Although orthologues in the TLR superfamily can be established based on structure and synteny, the function of TLRs may not be conserved across different vertebrate classes (Pietretti & Wiegertjes 2014). Based on the documented, conserved positions of LRR insertions between human and fish (Aoki et al. 2013) and phylogenetic studies of both TIR domains and LRRs, fish TLRs may still be functional orthologues' of TLRs from other species (Pietretti & Wiegertjes 2014).

TLR ligand recognition in the LRR region, initiates intracellular conformational changes by homo- or heterodimerization of two proximate TIR domains and a successive activation of TIR domain-containing adaptor proteins (Akira et al. 2006). Depending on the pathogen and which

TLR it activates, the specific adaptor proteins, signaling pathway and cytokine profile induced vary. Known mammalian adaptor proteins are myeloid differentiation primary response protein 88 (MyD88), MyD88-adaptor-like (MAL or TIRAP), TIR-domain-containing adaptor protein inducing IFN β (TRIF or TICAM1), TRIF-related adaptor protein (TRAM or TICAM2) and sterile α -and armadillo-motif-containing protein (SARM) (O'Neill & Bowie 2007). Downstream signaling, triggered by the adaptor proteins, involves interactions between IRAKs (IL-1R-associated kinases) and TRAFs (TNF-receptor associated factors) that eventually lead to activation of transcription factors such as IRFs, NF- κ B, CREB (cyclic AMP-responsive element binding protein) or AP1 (activator protein 1) (Akira et al. 2006, O'Neill & Bowie 2007). There are two predominant intracellular TLR pathways known from mammals; MyD88-dependent and MyD88-independent, where all known TLRs signal through the dependent pathway except TLR 3 (independent pathway) and TLR4 that can trigger both pathways (Bagchi et al. 2007). MyD88 has been identified in several fish species (Poynter et al. 2015), including Atlantic salmon (Skjavelevand et al. 2009). Genes of other key mediators (TRIF, IRAK-4, TRAF-6, NF- κ B, IRF3 and IRF7) have been identified in various fish species (Poynter et al. 2015). Even though the pathways seem conserved, further functional characterization is needed (Rebl et al. 2010).

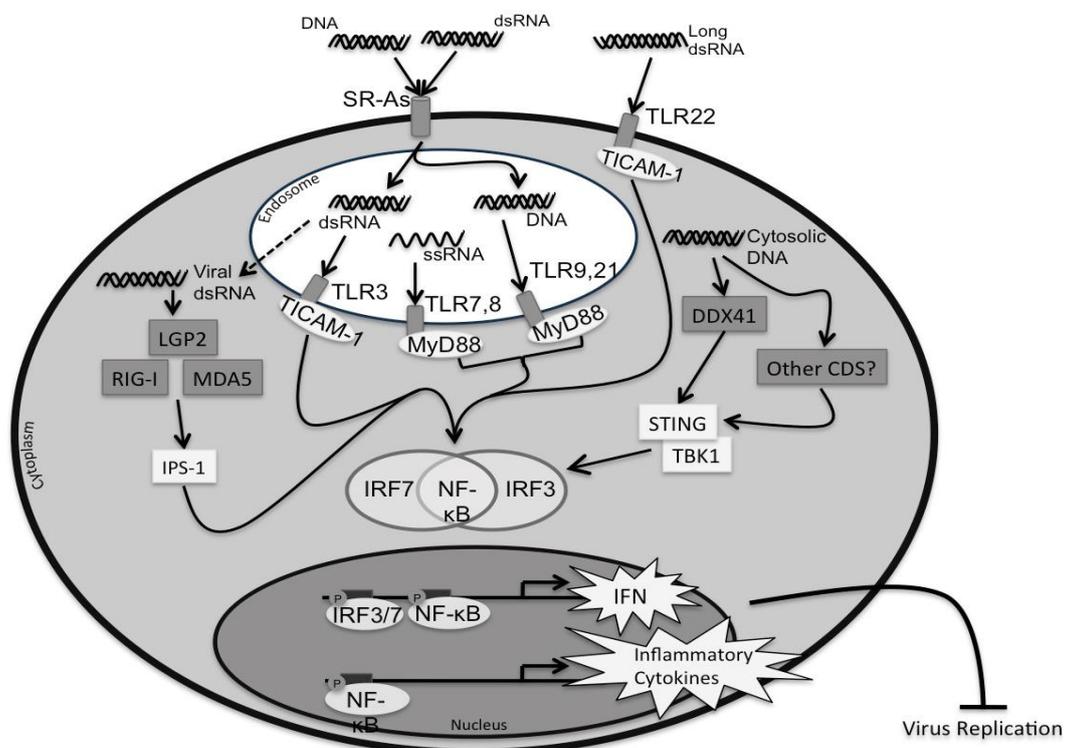


Figure 5. Suggested pathways of fish nucleic acid PRR signaling. (Poynter et al. 2015)

Description of TLR functionality in fish is mainly deduced from studying immune responses triggered by known ligands from mammalian literature and ligand specificity remain unclear for many fish TLRs (Poynter et al. 2015). Nucleic acid-sensing fish TLRs that have been determined experimentally, are TLR3 and TLR22 (dsRNA), TLR7 and TLR8 (ssRNA) and TLR9 and TLR21 (bacterial/viral DNA) (Poynter et al 2015). See Fig. 5 for suggested signaling pathways of nucleic acid PRRs in teleosts, which are loosely described in the text. TLR3 seem to mimic what is observed in mammals, with increased TLR3 expression after both viral infection or stimulation with synthetic dsRNA (polyinosinic:polycytidylic acid or poly I:C) (Purcell et al. 2006, Svingerud et al. 2012). Although, transcripts of both TLR7 and TLR8 are detected in various fish tissues and species (Chen et al. 2013, Arnemo et al. 2014, Skjæveland et al. 2009), the role of TLR7 and TLR8 in fish is less clear. Upregulation of both TLR7 and TLR8 have been described after *in vitro* stimulation with type I IFNs in Atlantic salmon (Lee et al. 2014, Svingerud et al. 2012), while no significant effect of TLR8 expression were present *in vivo* after SAV3 injection in Atlantic salmon (Skjæveland et al. 2009). Note that in human and mice both the ligand specificity and induced pathways for TLR 7 and TLR8 vary even though they belong to the same TLR-family (TLR7) (Gorden et al. 2005, Poynter et al. 2015). Mammalian TLR9 ligands; unmethylated CpG dinucleotides (further described in section 3.2.3) have been applied as immune stimulants in various fish species (Meng et al. 2003, Tassakka & Sakai 2004, Jørgensen, Zou, et al. 2001). In Japanese flounder, TLR9 activated a TNF promoter upon CpG stimulation, suggesting TLR9 to hold a similar function to its mammalian orthologues (Takano et al. 2007). Further, TLR9 transcripts have been both upregulated or insignificantly affected upon CpG treatment (Ortega-Villaizan et al. 2009, Skjæveland et al. 2008, Strandskog et al. 2008). Important innate and adaptive cytokines (pro-inflammatory, type I IFNs and IFN γ) and ISGs (i.e. Mx, VIG1) have been upregulated after CpG stimulation of Atlantic salmon both *in vitro* and *in vivo* (Jørgensen et al. 2003, Strandskog et al. 2008, Strandskog et al. 2011). The fish-specific TLR21 and TLR22 are suggested to have the same ligands as their mammalian counterparts; TLR9 and TLR3, respectively (Yeh et al. 2013, Matsuo et al. 2008). TLR22 is, interestingly, a surface expressed PRR that also signal through TICAM1, similar to TLR3 (Matsuo et al. 2008). Of the cellular TLRs, some exist both as membrane and soluble versions (TLR5) and two putative soluble isoforms of TLR20 have been found in Atlantic salmon (Lee et al. 2014). TLR4 is a well-studied human TLR, extremely sensitive to LPS (Beutler et al. 2001, Płóciennikowska et al. 2015). Fish are tolerant to relatively high LPS doses and resistant to

endotoxic shock (Iliev et al. 2005). Fish TLR4 is, so far, chiefly described in cyprinids (Jault et al. 2004, Meijer et al. 2004, Kongchum et al. 2010, Su et al. 2009). Counterparts of three mammalian TLR4 co-proteins have not been found in fish (Pietretti & Wiegertjes 2014, Iliev et al. 2005) and it can thus be implied that this absence may contribute to the endotoxic resistance in fish.

2.3 Adaptive immunity in fish – a slightly temperature sensitive story

Higher vertebrates vitally depend on adaptive immunity to rid viral infections. The lymphocytes are the main adaptive mediators, separating the adaptive immunity into humoral (B cells) and cellular (T cells and NK cells) responses. Through cross-communication with innate markers, cells and cytokines, antigen-specific B and T cells expand and produce high-affinity antibodies and generate cytotoxic T lymphocyte (CTLs), providing sterile immunity and ensuring a memory response upon re-infection. Many of the key factors from mammalian adaptive immunity are described in fish (CD4, CD8, MHC I and II, T cell receptor and immunoglobulin genes) (Laing & Hansen 2011, Sunyer 2013), albeit functionally less is known. See Table 1 for a comparison between fish and mammalian adaptive factors and functions.

Table 1. Key features of adaptive immunity in teleosts and mammals

Factor/Function	Teleosts	Mammals
Immunoglobulin	M, D, T/Z	M, G, A, E, D
AID	Y	Y
Class-switch	N	Y
Somatic hypermutation	+++	+++
Affinity maturation	+	+++
Memory responses	+	+++
TcR, CD3, CD4, CD8	Y	Y
MHC I, MHC II	Y	Y
CD28, CD40, CD80, CD86, ICOS	Y	Y
Th1, Th2 and Th17 cytokines	Y	Y
Spleen, thymus, bone marrow (BM)	Y (no true BM)	Y
MALT	Y	Y
Germinal centers and lymph nodes	N	Y

Comparison of key lymphocyte factors and functions in teleosts and mammals. MHC; major histocompatibility complex. ICOS; inducible costimulatory. Th; T helper subset. Adapted from (Laing & Hansen 2011, Sunyer 2013).

2.3.1 Fish immunoglobulins and B cell subsets

Hitherto, three fish immunoglobulin (Ig) isotypes are described. IgM is the major systemic teleost Ig, mediating primary and secondary Ab responses, since teleost lack a class switch mechanism and an IgG homolog (Kamil et al. 2013). IgMs heavy chain is similar to that of mammals (Hordvik 2015, Solem & Stenvik 2006), which holds a tetrameric structure compared to the mammalian pentameric structure (Fig. 6) (Solem & Stenvik 2006). Due to WGD events,

two functional IgM heavy chain isoforms (IgH-A and -B) exist in Atlantic salmon (Yasuike et al. 2010). The A/B ratio is often near 40/60 and upon prolonged immune triggering, skewed ratios may be observed (Kamil et al. 2013, Hordvik 2015), although the functional consequences of the two isotypes remain unknown. Rainbow trout IgT (Hansen et al. 2005), named IgZ in zebrafish (Danilova et al. 2005), has been suggested a mucosal important Ig and a functional equivalent to mammalian IgA (Zhang et al. 2010, Xu et al. 2013, Xu et al. 2016). The concentration of IgT is 100 to a 1000 times lower in sera compared to IgM (Castro et al. 2013, Zhang et al. 2010); while in mucosa IgT is more abundant (54%) than IgM (Zhang et al. 2010). IgD is mainly described at the genetic level for most teleost, however two IgD⁺ populations (IgM⁺/IgD⁺ and IgM⁻/IgD⁺) have been described in catfish, a species where IgT seem to be lacking (Edholm et al. 2010). In zebrafish and rainbow trout, two B cell populations have been described; IgM⁺/IgD⁺/IgT⁻ and IgM⁻/IgD⁻/IgT⁺ (Zhang et al. 2010) and recently also an IgD⁺/IgM⁻ subset in trout (Castro et al. 2014).

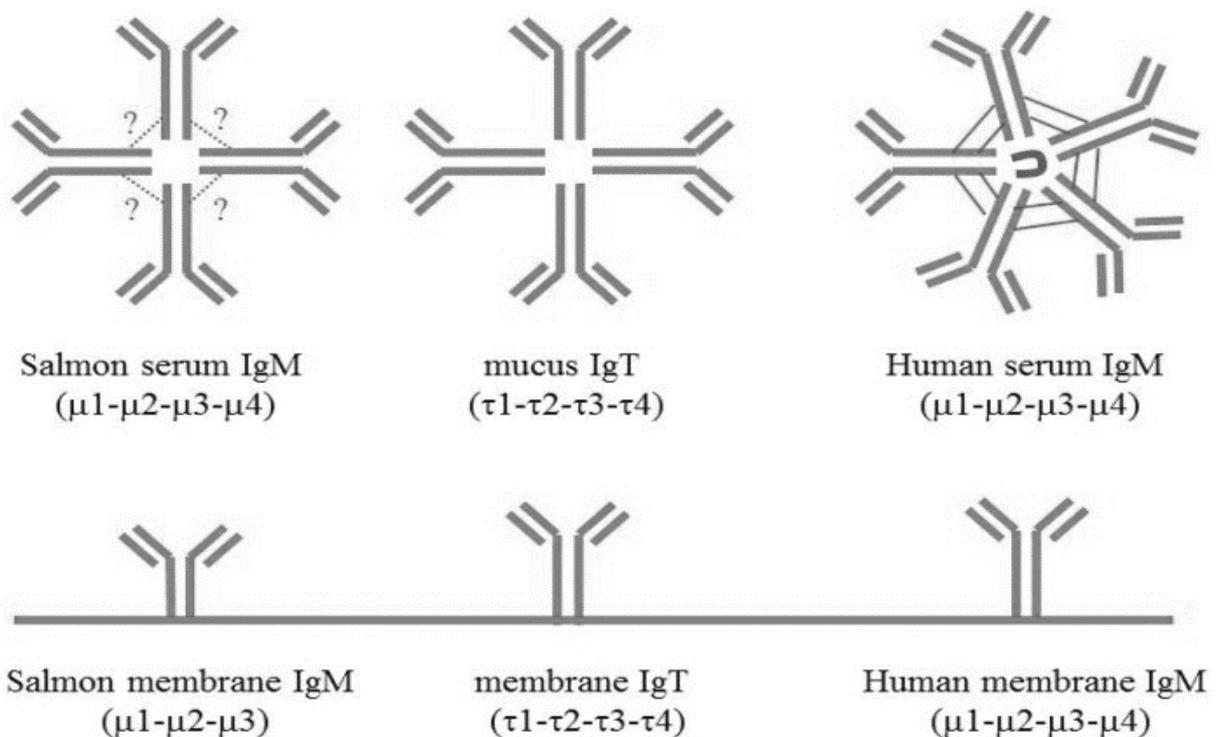


Figure 6. Schematic overview of membrane bound and secreted IgM and IgT in salmonid fish, compared to human. Serum IgM is a tetramer in teleosts. In contrast to the IgM pentamer in mammals, the monomer units in teleost IgM are loosely linked by disulfide bonding. Membrane anchored IgM in teleosts is one domain shorter than in mammals because of a special splicing pattern which excludes the entire fourth constant domain. Salmonid IgT appears to be non-covalently bonded tetramers in mucus. The similarity between the first constant domains of IgM and IgT is relatively high (52%) in Atlantic salmon, whereas the remaining domains of the heavy chains are only 13%–24% similar. Figure from (Hordvik 2015).

Studies in rainbow trout indicate a B cell subtype arrangement similar to that known in mammals (Bromage et al. 2004, Ye et al. 2011, Zwollo et al. 2008) and B cells with bactericidal and phagocytic activity have been described (Sunyer 2012). In trout B cells, a correlation between a greater Ab affinity and increased disulfide polymerization is shown and for example suggested to compensate for a lack of IgG during secondary Ab responses (Ye et al. 2011, Ye et al. 2010, Ye et al. 2013). Further, the loss of membrane bound IgM during B cell development seems conserved compared to mammals (Zwollo et al. 2008, Barr et al. 2011, Zwollo 2011) and a shift towards Ab-secreting cells (ASCs) is also described by Zwollo et al (2010). In their comparative study between mouse and rainbow trout, the authors revealed a B cell maturation profile in trout kidney by the use of differentially expressed B cell markers (Fig. 7) after dividing the kidney into five segments (K1 to K5). In K1 (HK) early maturing B cells were abundant, while K5 (posterior kidney) had a high frequency of late developing B cells and APCs. Several vaccination and challenge experiments show that fish are capable of inducing strong antibody responses, with antibody titers seemingly correlated to protection (Munang'andu et al. 2013, Solem & Stenvik 2006, Steine et al. 2001, Thim et al. 2012).

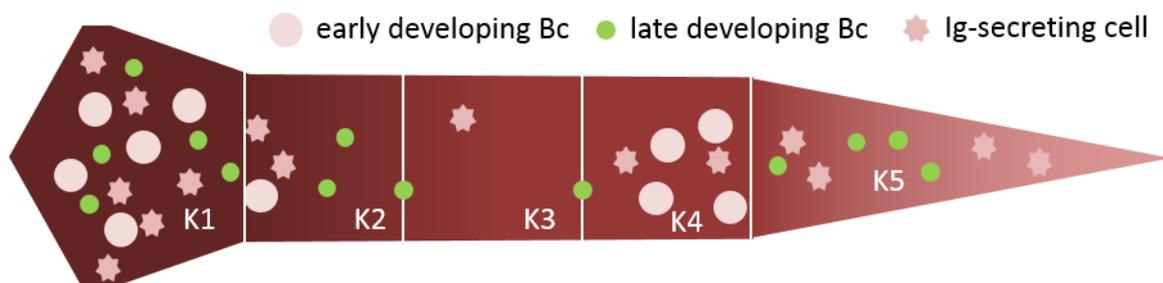


Figure 7. Illustrative model of trout kidney B cell maturation gradient. By dividing the kidney in five segments (K1 to K5) three main B cell populations have been described by FACS using antibodies against differentially expressed B cell markers. A maturation gradient was suggested, indicating an abundance of early maturing B cells in head kidney (K1) and unexpectedly in K4. The posterior kidney (K5) harbored a high frequency of late developing B cells and Ab-secreting cells. Figure adapted from (Zwollo et al. 2010).

2.3.2 Fish T lymphocytes

Sterile immunity against a viral infection manifests through eradication of virus and to achieve this, intracellular immune effector mechanisms are fundamental. These mechanisms are dependent on membrane-to-membrane binding and subsequent pore-formation leading to destruction of virus-infected cells. This destruction can be antigen-specific (T cells) or unspecific (NK cells). Antigen-specific T cell activation requires antigen-presentation through MHC I or II on APCs that bind to CD8 or CD4 on T cells, respectively. CD4 and CD8 receptors are

co-expressed with the T cell receptor (TcR), which recognize the specific antigens presented by MHC I or II. Specific cell mediated cytotoxicity (CMC), mediated by CTLs (CD8⁺ cells) has been described in various fish species as reviewed by Nakanishi et al (2015). Studies in gibel carp show that CD8α⁺ cells contribute to protection and kill virus infected targets by utilizing perforin and granzyme displaying an important role of fish CTLs as antiviral immune mediators (Somamoto et al. 2013, Somamoto et al. 2002, Toda et al. 2011). Recent publications, demonstrate functional CD4-1⁺ T cells in salmonids (Maisey et al. 2016, Takizawa et al. 2016), where antigen-specific stimulation of isolated CD4-1⁺ T cells, induces proliferation and upregulation of key CD4⁺ cytokine (IL-4, IL-15, IL-17D and IFN-γ1) transcripts (Maisey et al. 2016). Takizawa et al (2016), explores the function and tissue distribution of three distinct CD4⁺ populations (CD4-1⁺/CD4-2⁺, CD4-1⁻/CD4-2⁺, CD4-1⁺/CD4-2⁻) based on that salmonids harbor two CD4 genes (CD4-1 and -2) (Laing & Hansen 2011, Moore et al. 2009). They identify two CD4⁺ populations with anticipated lymphocyte traits, as well as a single positive CD4-1⁺ population displaying myeloid traits and high phagocytic capacity and similar myeloid CD4-1⁺ populations have been described in humans.

2.3.3 Mediators of teleost adaptive immunity

Cytokines are key regulators within the entire immune system and contribute greatly in shaping the adaptive responses. B and T cells are particularly sensitive to cytokines, which affect effector functions and homing properties of B and T cells, as well as their differentiation into memory cells. Mammalian CD4⁺ T cells massively proliferate and differentiate into at least four specific effector T helper cell (T_h) subsets upon TcR/CD3 activation; T_h1, T_h2, T_h17 and T_{reg}, (induced regulatory T cells) (Sallusto & Lanzavecchia 2009). Each specific subset of T_h cells in higher vertebrates is linked to a unique cytokine profile as well as specific transcription factors (Fig. 8; Chabalgoity et al. 2007). A skewing towards a T_h1 response is important for elimination of intracellular pathogens and IFNγ and IL-12 are its key cytokines; and T-bet, STAT1 and STAT4 the major transcription factors necessary for full T_h1 differentiation. In turn, for a T_h2 profile, IL-4/13 and GATA3 are the key cytokines and transcription factor, respectively. Cytokines from mammalian T_h subsets have been identified also in teleost (Laing & Hansen 2011, Wang & Secombes 2013, Wang et al. 2016). Whether T_h-specific subsets function in fish in a similar manner as in mammals is still an open question. In fish, a higher constitutive T_h2 environment have been described at mucosal surfaces, gills, heart, muscle and brain. This suggests a

protective environment against parasites that may limit overwhelming inflammatory reactions from constant contact with water-borne antigens (Wang et al. 2016, Fischer et al. 2013). Through functional studies of three active IL-4/13 proteins (IL-4/13A, IL-4/13B1 and B2), Wang et al. (2016) show that trout IL-4/13B1 and B2 isoforms are highly induced by viral and parasitic infections. *In vivo*, IL-4/13B1 and B2 seemingly contributed to cell-mediated immunity, while IL-4/13A held a high constitutive expression in most tissues, suggested to provide a basal Th2-immunity.

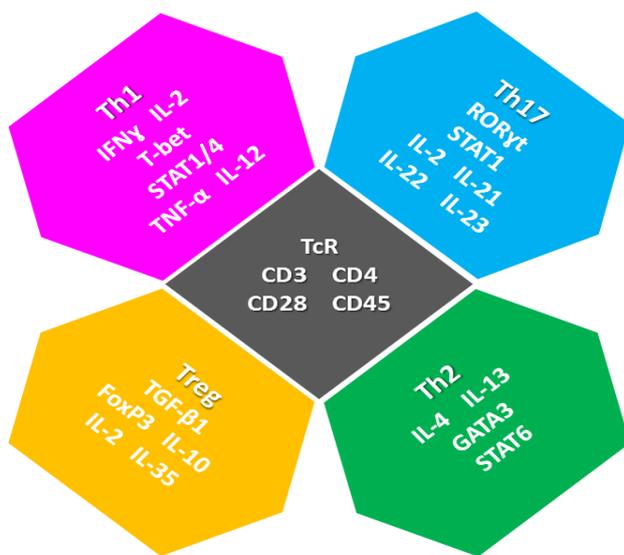


Figure 8. Shared components of T cells (grey) and specific components of T helper subsets (color) Th₁, 2, 17 and regulatory T cells (Treg) in mammals. T helper cells are characterized based on common surface markers (TcR, CD3, CD4, CD28 and CD45), while each Th subset is distinguished by specific cytokines and transcription factors. Putative forms of all mentioned components have been characterized in various teleost species (Laing & Hansen 2011, Wang & Secombes 2013).

Further, non-specific cytotoxic cells (NCCs) and NK-like cells proposedly mediate non-specific CMC (Fischer et al. 2006, Nakanishi et al. 2015) and are characterized by a spontaneous ability to recognize and kill altered target cells (Frøystad et al. 1998, Praveen et al. 2004). In channel catfish, NCCs were cloned from alloantigen-stimulated blood leukocytes and killed both the alloantigenic stimulated cells and unrelated allogenic targets by a perforine/granzyme-mediated apoptosis pathway (Shen et al. 2004).

2.3.4 Sudden temperature changes can affect immune responses

The body temperature of most bony fish are constantly equilibrated with the surrounding water through the large surface of the gills (Fry 1967). While an optimal immune response for a given fish species is obtained at its normal summer temperature, a sudden drop in temperature seems more detrimental for the immune system than the temperature itself (Roberts & Rodger 2001). Importantly, the adaptive immunity seems more temperature dependent than the innate factors (Ellis 2001, Magnadóttir 2006). Especially T cells are assumed to have a less efficient 'homeoviscous' adaptation to reduced temperatures than B

cells (Bly & Clem 1992, Vallejo et al. 1991), related to the fatty acid composition of the plasma membrane. Several studies have shown that during this adaptation period, innate factors, such as neutrophils, macrophages, alternative complement factors and NK-like cells, are upregulated (Alcorn et al. 2002, Bly & Clem 1992, Le Morvan et al. 1998, Lorenzen et al. 2009, Raida & Buchmann 2007). Due to the general sensitivity in fish to temperature (poikilothermic nature) and the high degree of pathogen exposure imposed by the aquatic environment it is suggested that fish rely more on innate than adaptive responses.

3 Viral vaccines in aquaculture - present day status

3.1 Teleost viral vaccine design – fishy business

A vaccine, human or piscine, exploits the recipient's capacity to develop immunological memory against a given pathogen. This with the intent to prevent an infection to take hold or substantially shorten the course of an infection by the authentic pathogen. There are known, established means to high vaccine efficacy, such as live vaccines that engage the recipient's full immunological memory. Although, because of risks related to reversion to virulent strains and ecosafety, strict legislation reside regarding live virus vaccines for farmed fish. Based on the unsatisfying protection elicited by present vaccines against fish viral diseases and known side effects from licensed aquaculture adjuvants - finfish viral vaccine development are, undoubtedly, in need to lay new routes to higher vaccine efficacies.

3.1.1 Vaccine administration and formulations

Oral or immersion vaccine routes are the most feasible ways to vaccinate fish in bulk (Gudding & Van Muiswinkel 2013). Currently, salmonid vaccine administration by injection is the main method used, as it provides the highest and most durable efficacy. However, the injection route is indeed more labor intensive and more stressful for the fish. Commercial immersion vaccines are predominantly diluted formalin inactivated bacterial suspensions or live bacterial vaccines, in which the fish are dipped (often at low dilutions) for a given period (often 30 to 60 seconds). With the advantage being that the fish can be immersion vaccinated at developmental stages when they are still too small for injection administration (Brudeseth et al. 2013). For oral vaccines, antigens are supplied either as suspensions for coating the feed, or mixed into the feed during production. For oral administrations, the challenges are to prevent gastric degradation and/or uptake in the gut to maintain a high efficacy. Injection

vaccination has two main routes, i.p. or i.m., at where emulsion vaccines are i.p. injected and DNA vaccines i.m. injected (Brudeseth et al. 2013).

Immersion vaccines are water based formulations containing antigen, while an emulsion vaccine is a two-component vaccine with a dispersed phase and a continuous phase, where the former phase is not miscible in the latter. For vaccine formulations, these phases are water and oil, respectively, and the water phase (mainly) contain the antigen (Tafalla et al. 2013, Guy 2007). To facilitate stabilization, a surfactant needs to be included, which is a compound exhibiting a polar hydrophilic group and a non-polar hydrophobic group that often possess a fatty chain. The surfactant's affinity for either phase is determined by its hydrophilic: lipophilic balance (HLB). The HLB define the emulsions as w/o (water-in-oil; low HLB), o/w (oil-in-water; high HLB) or w/o/w (water-in-oil-in-water; intermediate HLB) (Ascarateil & Dupuis 2006). The w/o is a common vaccine emulsion believed to exert a depot effect, with slower antigen release, providing longer-term immune responses (Sommerset et al. 2005). Oil emulsions are further defined as a classical adjuvant, introduced in section 3.2.

3.1.2 Approved finfish viral vaccines and vaccine regimen

During the 1980s-90s Norwegian aquaculture vaccine development was allowed to boost. The industry was quite freely regulated, with the attempt to control a growing problem with bacterial diseases (Gudding & Van Muiswinkel 2013). In the 1980s, introduction of immersion vaccines gave satisfactory protection against diseases such as cold-water vibriosis (*Vibrio salmonicida*) and vibriosis (*Vibrio anguillarum*). Although, when furunculosis (*Aeromonas salmonicida*) appeared, the developed immersion vaccine was inefficient in providing the wanted efficacy. Hence, an emulsion vaccine against furunculosis was implemented and proved fruitful (Brudeseth et al. 2013, Sommerset et al. 2005). Unarguable, due to the development of multivalent bacterial emulsion vaccines that also contained antigens against *Vibrios*, aquaculture successfully increased sustainability through reduced use of antibiotics. In addition, this resulted in increased fish welfare and lengthening of the production cycle ultimately leading to increased economic growth (Fig. 9). Yet, achieving satisfactory protection by vaccination against viral diseases have proven difficult. Since the introduction in 1995 of a recombinant viral vaccine that targets IPNV (Frost & Ness 1997), vaccination strategies remain fairly unchanged and still rely on emulsion technology. Emulsion vaccines can only be administrated through injection and they are known to cause serious side effects, for example

in salmon, affecting both welfare and slaughter quality (Gudding & Van Muiswinkel 2013, Kibenge et al. 2012).

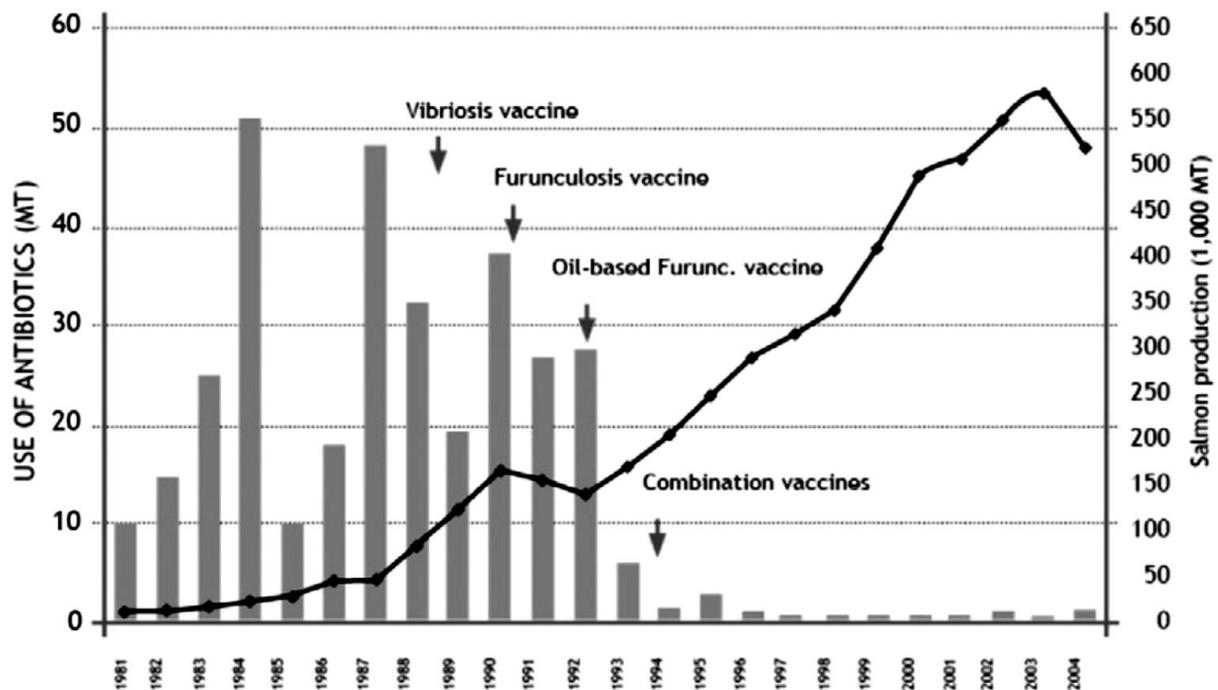


Figure 9. Norwegian Atlantic salmon production (black dotted line) in relation to antibiotic use in fish (grey bars), from 1981-2004. Arrows indicate introduction of bacterial vaccines, shortly after a dramatic drop in antibiotic use is visible (1993). (Håstein et al 2005).

An overview of commercial salmonid viral vaccines available on the world market is given in Table 2. ISA vaccination (w/o; inactivated whole virus, subunit) has been deployed since 1999 in North America and since 2004 in the Faroe Islands (Kibenge et al. 2012). In 2010 it was introduced in Norway, certified for most parts of the country (Johansen et al. 2011). In Chile, ISA vaccination was first introduced in 2007, when their market was massively hit with ISA outbreaks. This led to development of improved vaccine products in Chile (Brudeseth et al. 2013, Kibenge et al. 2012). Both recombinant and inactivated whole virus IPN antigens, are part of several mono- and multivalent vaccines (w/o) worldwide (Gomez-Casado et al. 2011, Brudeseth et al. 2013). For IHN, a DNA vaccine has proved highly efficacious and is licensed for use in British Columbia, Canada (Kurath 2008). An inactivated whole virus (SAV1) vaccine against PD (w/o) is commercially available and in use in Norway, Scotland and Ireland and confer cross-protection between subtypes (Metz et al. 2011). Another inactivated SAV (SAV3) vaccine has been developed (not commercial available) and it has shown a promising efficacy tested by i.p. challenge, co-habitant challenge and in the field (Karlsen et al. 2012). Further,

the immunogenicity of subunit and DNA vaccines against the E1 and E2 envelope proteins have been evaluated, where an E2 subunit design seems most promising (Xu et al. 2012).

Table 2.
Viral antigens in licensed commercially available vaccines for anadromous and freshwater salmonids.

Species	Region	IPNV	ISAV	SAV	IHNV
Atlantic salmon	Norway and Faroe islands	Inj	Inj	Inj ^a	
	Chile	Inj, Ora	Inj, Ora		
	UK/Ireland	Inj		Inj	
	N. America		Inj		Inj ^b
Rainbow trout	Norway				
	Europe (excl. Norway)	Inj			
	Chile	Inj, Ora			
	N. America				
	Japan				
Coho salmon	Chile	Inj, Ora	Inj, Ora		
	Japan				
Ayu	Japan				

Inj; injectable vaccine. Ora; oral vaccine. a; No injectable PD vaccine in Faroe islands. b; DNA vaccine.

Adapted from (Brudeseth et al 2013).

Norway is regarded a 'high cost' aquaculture nation, where development and introduction of vaccines has been regarded as a justified cost to keep the industry profitable (Tafalla et al. 2013). China covered more than 60% of the world total aquaculture production in 2011 (Brudeseth et al. 2013). Even so, historically in China, vaccines have been scarcely implemented, where one factor has been cost effectiveness (i.e. cost of vaccine production vs. fish value) (Sommerset et al. 2005). Over the past few years, China has experienced an intensified vaccine development. With this, large-scale vaccination has spread to other species than salmonids (Brudeseth et al. 2013). It is crucial to keep the fish healthy through the entire production cycle and specific vaccination regimen may need several administration rounds to boost sufficient protection, expediting various administration routes. However, this increases fish handling and production costs and are thus often not preferred. Present day, multivalent emulsion vaccines are the main vaccination regimen of salmonids across Europe (Brudeseth et al. 2013, Kibenge et al. 2012, Tafalla et al. 2013), since emulsion formulations enables multiple antigens (commonly six to seven) in the same formulation. The fish are vaccinated in the fingerling state (30-40g), while still in freshwater and before transfer to the much more pathogen exposed seawater stage (Sommerset et al. 2005). In Norway during 2012, 95%, or approximately 300 million Atlantic salmon smolt were vaccinated with a six-component emulsion vaccine containing antigens protecting against furunculosis, vibriosis, cold-water

vibrosis, winter-ulcer (*Moritella viscosa*; all bacterial diseases) and IPN (viral disease) (Brudeseth et al. 2013). The first commercially available PD vaccine, a monovalent w/o vaccine should preferably be injected 230 degree days (approximately 2-3 weeks) before the multivalent vaccine, which increased fish handling (Brudeseth et al. 2013). However, a seven-component vaccine with antigens against the above mentioned diseases, including PD, is now commercially available (Hjul 2015, Felleskatalogen 2015).

3.1.3 Viral vaccination trends and technologies – a fish-eye perspective

There are five key vaccine categories; attenuated/modified live, inactivated, subunit, recombinant and nucleic acid/naked DNA vaccines, where inactivated virus and subunit vaccines are the two main strategies for finfish vaccines today (Kibenge et al. 2012). For any host, live attenuated vaccines holds a great advantage by being cost effective and generally provide high efficacy and induces long-lived immunity after a single dose. Poliomyelitis, mumps, measles, rubella and yellow fever (Minor 2015) represent human attenuated vaccines. Serial passages contribute to attenuation, such as for the yellow fever virus. The virus was first propagated on mouse embryo tissue and further propagated on chicken embryo tissue that, after 176 passages, lead to the safe Yellow Fever-17D (YF-17D) vaccine (Roukens & Visser 2008). YF-17D mimics a natural infection, able to induce an immune response that provides subsequent protection upon encounter with the pathogen. YF-17D is the only vaccine commercially available against the disease (Roukens & Visser 2008). Safety concerns for this type of vaccines; such as the occasional false-positive detection of disease, the concern against reversion to a virulent strain, the ability to cause disease in immunocompromised individuals or spread of the attenuated strain into the environment, impede further development of attenuated veterinarian vaccines (Chambers et al. 2014, Tafalla et al. 2013). However, reverse genetics, a recombinant technique, is promising for creating live attenuated viral vaccines. Intriguingly, a first, genetically modified live bacterial vaccine against an avian pathogenic *E. coli* has recently been deployed (La Ragione et al. 2013). A recombinant, fully attenuated live SDV vaccine has shown promising protection in trout infection studies (Moriëtte et al. 2006). Romero and co-authors (2011, 2008) have designed an avirulent recombinant rIHN-V-Gvhsv GFP strain by reverse genetics that replicates as efficiently as wild type IHN and induce protective responses against IHN and VHSV in trout. This kind of recombinant techniques,

holds an advantage by abolishing a prerequisite for additional oil or other adjuvants to confer satisfying efficacy (Mérour & Brémont 2014).

Viral subunit or inactivated vaccines, does not confer the same immunogenicity due to faster clearance from the injection site and lack of replication, which introduce the need for a booster or adjuvant (see section 3.2). Nonetheless, they are more storage stable and provide an improved safety aspect. Concerns against viral subunit vaccines regards correct folding and lack of glycosylation, since the viral proteins often are produced in bacteria (Wright et al. 1989, Vidal et al. 1989). For inactivated virus vaccines, up-concentration of large quantities of the given virus is required for antigen production. This can prove challenging in aquaculture, since some viruses are difficult to culture. Subsequent inactivation of the pathogen is done by for example physical or chemical agents (Mahdy et al. 2015). Subunit vaccines are distinguished by containing only one or a set of pathogen components, such as a protein, glycoprotein or outer membrane vesicle and the specific components are chosen based on their antigenic capacity. Recombinant DNA technologies aid in designing subunit vaccines against pathogens that for example are difficult to culture, although requiring antigenic proteins to be known (Karch & Burkhard 2016).

DNA vaccines are very promising future alternatives to traditional viral finfish vaccines, where selected antigens are inserted into a non-pathogenic delivery vector. The advantage being that the host itself produce the antigens delivered by the vaccine to muscle or skin and subsequently present them through MHC class I and II to B and T cells, triggering the much sought for, antiviral T_H1 response, abolishing a need for adjuvants. However, reasons such as low immunogenicity or expression of the transgene or a high degradation rate of the plasmid DNA may lead to failed induction of protection. Hence, to combine adjuvants with the plasmid DNA may provide sufficient protection (Hølvold et al. 2014). Further, a study by Chang and colleagues (2015) show that to include a plasmid encoding type I IFNs (IFN α , β or γ) to a hemagglutinin-esterase DNA vaccine model potently enhance protection against ISAV in salmon. No mammalian DNA vaccines are licensed, however DNA vaccines against West Nile virus for use in horses and the IHNV for salmon are commercially available (Chambers et al. 2014). Clynav, against PD in salmon, is the first DNA vaccine recommended for marketing authorization in the European Union (Bensetter 2016).

3.2 Adjuvants

3.2.1 Introduction to adjuvants – function and categories

Since a majority of vaccines developed today, such as subunit vaccines, display a weaker immunogenicity, substances that aid by increasing the immunogenicity are required. Such helpers are called adjuvants, from the Latin word *adjuvare* – to aid/help. Table 3

Table 3. Adjuvanted advantages

1	Enhance speed, magnitude and length of response
2	Dose sparing effect
3	Protective effect on antigen
4	Increased cross protection and functional antibody titer
5	Decrease non-responsiveness in specific target groups
6	Reduce number of immunizations
7	Facilitate mucosal immunity
8	Induce robust cell-mediated immunity
9	Overcome antigenic competition

Table adapted from (Lee and Nguyen 2015, Levast et al. 2014, Reed, Orr, and Fox 2013)

presents a number of adjuvant advantages. Adjuvants have been used as vaccine components empirically for many decades, thus, only recently, knowledge regarding their modes of action have unraveled and still much is unknown (Gerdtz 2015). Although many known adjuvants potently increase immunogenicity, some of them inflict adverse effects to the host, such as immunopathogenicity at the injection site. In aquaculture, this is highly unwanted due to reduced fish welfare, muscle or slaughter quality and hence, lower production value. Major efforts within both human and animal vaccinology have focused on safe adjuvant regimens and with increased knowledge on how to initiate a desired immune response, such technologies have gradually reached the commercial market or are in the last phases of clinical trials.

Adjuvants can be characterized based on their carrier status (administration system) and/or their immunostimulatory effect. It is important to distinguish between an immunostimulant and an adjuvant. An immunostimulant is generally a compound that independently possess immunostimulant and/or immunomodulatory properties, while an adjuvant may consist of different elements that exert specific immune-functions to increase immunogenicity (Guy 2007, Salgado-Miranda et al. 2013). Fig. 10 provide an overview of adjuvant categories, along with classification, mechanisms, receptors and the type of immune response they induce. A limited number of adjuvant formulations are licensed for human and animal husbandry. Oil emulsions and aluminum salts are most common within the veterinary sector, where mineral oils are well-represented within aquaculture (Salgado-Miranda et al. 2013). The use of mineral oils as adjuvants facilitate the inclusion of PAMPs against several pathogens, which facilitates

the creation of multivalent vaccines that reduces fish handling. Human licensed adjuvants are; aluminum salts, o/w formulations (MF59, squalene, ISA51 AS03 and AF03), virosomes and AS04, which is a monophosphoryl lipid A preparation (MPL) (Reed et al. 2013, Di Pasquale et al. 2015). AS04 (Adjuvant Systems, a trademark by GlaxcoSmithKline) belong to the most advanced commercial adjuvant compounds due its combination of alum and MPL, which is a TLR4 agonist shown to generate a T_H1 response (Lee & Nguyen 2015). The benefit of TLRs as adjuvants are introduced below (3.2.2). First, a general introduction to adjuvants, demonstrating their versatility, which is important to consider when choosing adjuvant to shape a targeted response against any given pathogen.

Aluminum salts are extensively used as an adjuvant in human vaccines and are TLR-independent, acting through NLRs (Apostólico et al. 2016). Aluminum salts does not seem to function by depot effect, induces antibody responses and a T_H2 skewed environment, thus limiting their use towards pathogens that do not require CTL responses (Guy 2007). For a full adjuvant effect, physical contact between the antigen and the aluminum salt seem essential and reasoned to be based on a need of co-uptake of adjuvant and antigen by APCs (De Gregorio et al. 2013). One of the best-known w/o emulsion adjuvant, complete Freund's adjuvant (CFA), is highly immunostimulatory, on the verge of being harmful and is thus not allowed for human use (Guy 2007). CFAs potent induction of autoimmunity is suggested to be elicited by the adjuvant's heat-killed mycobacterial addition, contributing to a delayed type hypersensitivity (DTH) through a T_H1 skewed environment (Apostólico et al. 2016). In animal husbandry, whenever another option is at hand, it should be chosen before CFA. The use of CFA as a model adjuvant in experimental animal studies, is also strictly regulated (Apostólico et al. 2016, Guy 2007). A less harmful version is incomplete Freund's adjuvant (IFA), which lack the heat-killed mycobacterial addition. Even though IFA still induce severe side effects, it has been included in clinical trials for HIV vaccines (Apostólico et al. 2016). Montanide ISA is a mineral oil adjuvant system applied in both veterinary and human medicine. These mineral oil formulations exert their adjuvanticity through a depot effect at the injection site and support phagocytosis, while the oil also contributes to concentrating and protecting added antigens against degradation (Levast et al. 2014). MF59 is an established human o/w adjuvant that is well tolerated and deploys a dose sparing effect. MF59 act by providing an 'immunocompetent environment' and is not believed to act by a depot effect (De Gregorio et

al. 2013). Saponins are well-recognized immunostimulators, although their applicability as vaccine adjuvants are hampered due to associated toxicity (Guy 2007, Skene & Sutton 2006). The toxicity of saponins is abolished when incorporated as an active component of ISCOMs (immunostimulating complexes), which are cage-like particulate formulations of cholesterol and phospholipids (Skene & Sutton 2006, Guy 2007). Saponins alone, are known to induce both T_H1 and/or T_H2 skewing, while ISCOMs promote both antibodies and cell-mediated responses (Guy 2007). The term microparticle, chaperons several types of delivery vehicles or particulates, such as virus-like particles (VPLs), virosomes and poly(lactic acid) (PLA) or poly(lactic-cogly-colic acid) (PLGA) particles of which all three can be loaded with a given antigen (Apostólico et al. 2016, Guy 2007). Virosomes are mainly carrier vehicles constructed of envelope membrane lipids and glycoproteins, while VPLs mainly consist of structural proteins. PLA/PGLAs are biodegradable polymeric micro/nanoparticles to which antigen and/or adjuvants can be encapsulated (Apostólico et al. 2016).

Combination adjuvants, such as AS04, belongs to a growing group of adjuvants that show great prospect in facilitating more targeted responses against pathogens. By combining more than one adjuvant; often in duplicate or triplicate, a synergistic mode of action (section 3.2.2) can be tailored. This may activate a variety of cells and immune mechanisms. Known combination adjuvants are based on cationic liposomes, ISCOMs, nanoemulsions, montanides and AS, to which chosen immunostimulants have been added (Levast et al. 2014). These immunostimulants range from TLR agonists, to model adjuvants like OVA (ovalbumin) and innate defense regulatory peptides (IDRs) (Gerdt 2015, Levast et al. 2014). See Fig.10 for an overview and example of individual and combination adjuvants.

ADJUVANT CATEGORY	NAME or TYPE	CLASS	MECHANISM or RECEPTOR	TYPE of IMMUNE RESPONSE	COMBINATION ADJUVANTS	MECHANISM or RECEPTOR	TYPE of IMMUNE RESPONSE
NEUTRAL LIPOSOME					AS01: MPL + QS21 + liposome	TLR4	Ab, Th1, CD8+ cells
MICROSPHERES	virosome	PF	Ag delivery	Ab, Th1, Th2			
MINERAL SALTS	ALUM	PF	Nalp3, ITAM, Ag delivery	Ab, Th2	AS04: MPL + ALUM CpG7909 + ALUM	TLR4 TLR9	Ab, Th1 Ab, Th1, CD8+ cells
CATIONIC LIPOSOMES					CAF01: TDB + cationic liposome	Ag delivery	Ab, Th1, CD8+ cells
ISCOMs					ISCOMs: saponin, phospholipid	unknown	Ab, Th1, Th2, CD8+ cells
W/O or O/W EMULSION	MF59 Montanide	PF PF	ICR, Ag uptake	Ab, Th1, Th2	Montanide ISA51 + CpG AS02: MPL + squalene + QS21 MF59 + CpG	TLR9 TLR4 TLR9	Ab, Th1, CD8+ cells Ab, Th1 Ab, Th1, CD8+ cells
PRR/TLR AGONISTS	dsRNA MPL CpG R484/R873	IM IM IM IM	TLR3 TLR4 TLR9 TLR7/8	Ab, Th1, CD8+ cells Ab, Th1 Ab, Th1, CD8+ cells Ab, Th1	IC31: cationic peptide + CpG	TLR9	Ab, Th1, Th2, CD8+ cells
SAPONINS	QS21	IM	unknown	Ab, Th1, Th2, CD8+ cells	AS15: MPL + QS21 + CpG + liposome	TLR4/9	Ab, Th1, CD8+ cells

Figure 10. Carrier and/or immunostimulatory status of individual and combination adjuvants. As indicated by the arrow, some adjuvants can be immunomodulatory and/or depot/carriers, contributing to a Th1 or Th2 environment. Alums (such as aluminum oxyhydroxide and aluminum phosphate), emulsions and ISCOMs (immunostimulating complexes) act as both immunostimulants and carriers. Unless having a specific composition, or if carrying immunostimulants, liposomes and microparticles are inert carriers. The figure presents a few examples of mammalian individual and combination adjuvants. PF, particulate formulation; IM, immunomodulatory molecule; Ag, antigen; ITAM, immunoreceptor tyrosine-based activation motif; ICR, immune cell recruitment; TDB, trehalose dibehenate. Figure adapted from (Guy 2007, Levast et al. 2014, Reed et al. 2013).

3.2.2 Adjuvanticity of TLRs – a synergistic boost

A 'new order' of adjuvants arose with the increased knowledge of PRRs and their agonists. TLRs are the best-explored PRRs regarding adjuvanticity and the class covered here. The knowledge about natural TLR-agonists spiked the development of the second-generation TLR-agonists; synthetically produced ligands. A diversity of synthetic TLR-agonists are now deployed as adjuvants in vaccine-research and other research areas, such as therapeutic cancer treatments (Kaczanowska et al. 2013, Lee & Nguyen 2015).

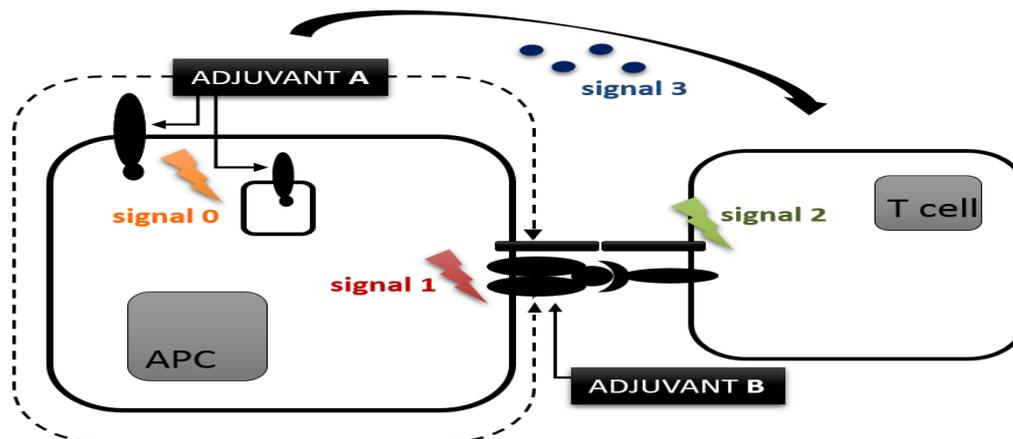


Figure 11. Directing adjuvanticity towards T helper responses. In theory, adjuvants can, alone or combined, act on either signal (zero to three) to initiate a T_h -response. TLR agonists fall under A type adjuvants, since they act directly on signal 0 and indirectly on signal 2 and 3, by activation of APCs and the triggering of cytokine secretion. Selected TLR agonists may also directly trigger signal 0 on regulatory T cells and B cells expressing the corresponding receptor. Liposomes, microspheres and some emulsion adjuvants fall under B type adjuvants, since they target APCs and/or favor antigen capture. However, to only trigger signal 1 is not sufficient for a full immune response. Co-delivery of a second immunostimulatory signal, such as through signal 0 may facilitate a full immune response. Figure adapted from Guy (2007) and Gerdtts (2015).

In general, based on mammalian literature, most adjuvants act by causing some kind of tissue injury that leads to immune cell recruitment and subsequent immune system recognition of danger signals (Calabro et al. 2011, Goto & Akama 1982). The immune cell recruitments are characterized by macrophages, neutrophils and DCs. Engagement of PRRs on these types of cells and presentation of antigens through specific receptors activate innate and adaptive immunity that shape and direct the response towards, i.e. humoral or cellular immunity (Awate et al. 2013, Hoebe et al. 2004). Discrimination based on how different adjuvants, including TLRs, act and why the interaction between APCs and T cells are important for adjuvanticity is viewed in Fig. 11 and further explained here. T_h cell activation require antigen presentation through MHC to T cells (*signal 1*). *Signal 2* and *signal 3* provide co-stimulatory signals that direct the T_h response further, for example towards cellular (T_h1), humoral (T_h2) immunity or cytotoxic (CTLs) (Gerdtts 2015). Without co-stimulation by specific receptor-ligand

interaction (*signal 2*; such as CD40-CD40L, CD80/86-CD28/CTLA4), the APC will enter an energy state. *Signal 0* is an additional cue, more recently discerned and mandatory for the induction of APCs. *Signal 0* is mainly induced by PAMP-recognition by a specific PRR (Bianchi 2007, Steinman & Hemmi 2006).

TLR-ligands holds the advantage of acting direct or indirect (*signal 0*) on all T_h cell activation signals (Guy 2007). Further, some PRR-agonists act directly on a specific subset of immune cells such as B cells or T cells (Boeglin et al. 2011, Kaczanowska et al. 2013). Indeed, not all cell types express TLRs, and which cell type that express a given TLR, vary both within phylum and between species. As such, human TLRs are generally only expressed on monocytes, mature macrophages and DCs, and specifically; human plasmacytoid DCs (pDCs) express TLR7 and TLR9 (Hennessy et al. 2010). As recently reviewed by Kremlitzka and co-authors (2016), a set of murine and human TLRs are differentially expressed during B cell development, such as TLR 2/6, 7 and 9 that are highly expressed on human memory B cells, while present in lower levels on naïve B cells and plasma cells. Further, TLRs provide an intrinsic system that exert control on many levels within the organism, especially by cross-talk that reduce reaction against self, while still alerting when foreign danger signals are recognized (Tan et al. 2014).

A synergistic boost can, as mentioned earlier (3.2.1), be evident by combining several adjuvants. The combination of two or more TLR-agonists that act through different receptors with different downstream signaling pathways, have displayed synergistic effects and proven to be highly efficient adjuvant combos (Mäkelä et al. 2009, Liu & Ling Ding 2016). Commonly, a TLR3 (TRIF pathway) or TLR4 (both TRIF and MyD88-dependent pathway) agonist is combined with a TLR7, 8 or 9 agonist (MyD88-dependent pathway). Studies across species, describe synergistic responses against various viral infections (Arsenault et al. 2013, Duggan et al. 2011, Mäkelä et al. 2011, Strandskog et al. 2008). Although unwittingly at the time of development, the empirical vaccine YF-17D elicits a full immune response by synergistic activation of several TLRs; TLR2, 7, 8 and 9 (Querec et al. 2006). Which may also hold true for many whole virus fish vaccines, since they by themselves are a mix of several PAMPs. Table 4 provide an overview of TLRs in teleost and mammals based on cell localization and their agonists, while section 3.2.3 focuses on the two TLR-agonists investigated in this thesis, CpG and poly I:C.

Table 4. Mammalian and teleost TLRs; cell localization and their individual agonists.

		PRESENT IN		PAMPs		IDENTIFIED IN	
		Teleost	Mammals	Teleost	Mammals	Teleost species ^b	
TLR	SURFACE	1	●	●	N/I	Triacyl lipopeptides	Japanese flounder, Japanese pufferfish, Orange spotted grouper, Rainbow trout, Zebrafish
		2	●	●	PG, LA, Pam ₃ CSK ₄ ^a		Channel catfish, Common carp, Japanese flounder, Japanese pufferfish, Orange spotted grouper, Zebrafish
		4	○	●	N/I	LPS	Grass carp, Rare minnow, Zebrafish
		5M	●	●	Flagellin	Flagellin	Japanese flounder, Japanese pufferfish, Rainbow trout, Zebrafish
		5S	●	–	Flagellin	N/A	Atlantic salmon, Channel catfish, Japanese flounder, Japanese pufferfish, Rainbow trout
		6	–	●	N/I	LA	N/I
		10	–	●	N/I	N/I	N/I
	ENDOSOMAL	3	●	●	dsRNA, poly I:C ^a		Atlantic cod, Channel catfish, Common carp, Grass carp, Japanese flounder, Japanese pufferfish, Large yellow croaker, Rare minnow, Zebrafish
		7	●	●	N/I	ssRNA, IQ	Atlantic cod, Common carp, Grass carp, Japanese flounder, Japanese pufferfish, Rainbow trout, Zebrafish
		8	●	●	N/I	ssRNA, IQ	Atlantic cod, Atlantic salmon, Japanese flounder, Japanese pufferfish, Rainbow trout, Zebrafish
9		●	●	Bacterial CpG DNA		Atlantic cod, Atlantic salmon, Common carp, Gilthead seabream, Large yellow croaker, Japanese flounder, Japanese pufferfish, Rainbow trout, Zebrafish	

PA, peptidoglycan; LA, Lipoteichoic acid; IQ, imidazoquinoline; N/I, not identified; ● Indicate presence. ○ TLR4 is only identified in order Cypriniformes, and does not recognize LPS such as mammalian counterparts do. ^aseveral other PAMPs identified in mammals ^bcredits for scientific findings stated in (Aoki et al. 2013) and table adapted from same publication.

3.2.3 Now I know my CpGs and promiscuous poly I:C's

3.2.3.1 CpGs are divided into four classes based on structure and immunomodulatory trait

The natural ligand for mammalian TLR9, chicken TLR21 and teleost TLR9 and 21 is unmethylated bacterial DNA that contains repeats of cytosine (C) and guanine (G) deoxynucleotides with a phosphodiester link. Hence, dubbed CpG motifs. Indeed, CpG motifs

naturally exist in vertebrates as well. However, the presence of such CpGs are suppressed and only present in contexts not related to immune responses. Further, the major difference is that the majority of vertebrate CpGs (~80%) are methylated, while bacterial CpGs are not and the frequency of C and G in bacteria is >4-fold higher compared to vertebrates (Krieg 2001). Modifications to synthetic CpG motifs facilitates uptake to target cells and phosphothiorate (PTO)-modified CpGs are more readily taken up compared to phosphodiester (PO)-modified CpGs (Dalpke et al. 2002). Further, PTO-modifications protect the CpG oligodeoxynucleotide (ODN) from nuclease degradation, while synthetic alterations, such as addition of a poly G tail, either to the 3' or 5' end or both, enhance cellular uptake (Dalpke et al. 2002). CpG ODNs can seemingly be infinite modified in consideration to ODN length, number and location of CpG motifs, as well as in the flanking regions between the CpG repeats. Thus, synthetic CpG ODNs have been grouped into class A (or D), B (or K), C and P (Fig. 12) based on their specific structure and immunomodulatory traits in higher vertebrates (Shirota & Klinman 2014, Vollmer & Krieg 2009). CpG class A ODNs induce pDCs to mature and secrete IFN type I, but exert no effect on B cells (Shirota & Klinman 2014). Structurally, A class CpGs are defined by 5'- and 3' end G-runs and to have a single CpG motif, flanked by PO palindromic sequences. The class A backbone must be chimeric and the stimulatory effect is lost if the full length of the backbone is PTO-modified (Shirota & Klinman 2014, Vollmer & Krieg 2009). CpG class B ODNs are potent stimulators of B cell responses, inducers of pDC differentiation and monocyte maturation in higher vertebrates. B class CpGs consists of one or more CpG motifs and there are different optimal CpG class B motifs in for example humans (5'-GTCGTT-3') and mice (5'-GACGTT-3') (Vollmer & Krieg 2009). Class B CpGs commonly have a PTO-backbone, where PTO-modifications are more immunostimulatory than PO-modifications, and chemical modification of the backbone may enhance immunostimulatory effects (Vollmer & Krieg 2009, Shirota & Klinman 2014). CpG class C ODNs display traits from both the A and B class CpGs; stimulating B cells to secrete interleukins and pDCs to produce type I IFN. Class C ODNs holds a PTO-backbone (like class B CpGs) and palindromic CpG motifs (like class A CpGs) (Shirota & Klinman 2014). CpG class P ODNs induce the most potent IFN type I production of all CpG classes and contains double palindromic sequences providing P class CpGs with the ability to create hairpins (Shirota & Klinman 2014). Across all CpG classes, the individual CpG immunostimulatory effect depend on the length, base content and eventual chemical modification (Vollmer & Krieg 2009).

CpG ODN Classes

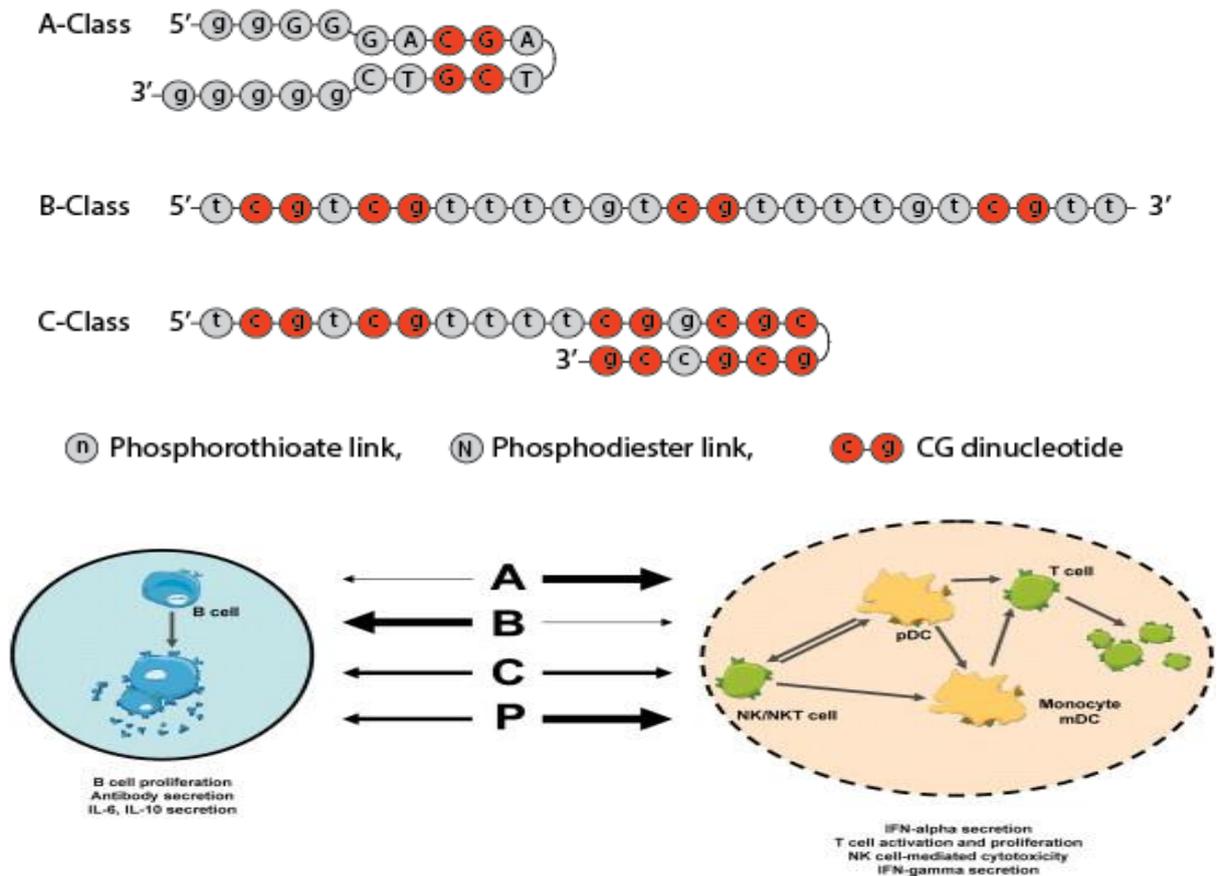


Figure 12. CpG motifs and their effects on the innate and adaptive immune system. Class A, B, C and P CpGs contain different CpG sequence motifs (B class) and palindromic sequences (A, C and P class) and activate TLR9-expressing human B cells or plasmacytoid dendritic cells (pDCs) with diverse outcomes. Class B CpGs induce strong B cell activation, while only moderate IFN-alpha secretion from pDCs. Class A ODNs stimulate high amounts of IFN-alpha, albeit possess minor B cell stimulatory capabilities. Class C CpGs combine the immune effects of Class A and B CpG ODN, only to a lower degree. Class P CpG ODNs resemble Class C CpGs by their B cell stimulation and pDC activation, however, P class CpGs are similar to Class A ODNs in the strength of the IFN-alpha response. Figures from (Vollmer & Krieg 2009, InvivoGen 2016).

3.2.3.2 Poly I:C is a potent T_H1 adjuvant

Double stranded RNA is the natural ligand of TLR3, while poly I:C is a synthetic equivalent that exhibit potent adjuvanticity. Poly I:C may also, depending of the length of the poly I:C sequence, act as a ligand for RIG-I (recognizes shorter dsRNAs) and MDA5 (recognizes longer dsRNAs) (Takeuchi & Akira 2010). A cooperative activation of TLR3 and MDA5 has been suggested for adjuvanticity (Kumar et al. 2008). In addition, human TLR3 expression is present on a wide range of cells, such as monocyte-derived macrophages, myeloid DCs, Langerhans cells, synovial and dermal fibroblasts, keratinocytes, muscle cells and endothelial cells; however, TLR3 is not expressed on pDCs (Hafner et al. 2013). Poly I:C may prove a more

beneficial adjuvant compared to other TLR agonists, since it is not restricted to act on a highly specific cell subset, although this increases a possibility of an inappropriate overstimulation of innate immunity (Hafner et al. 2013). Poly I:C has proven itself as a potent Th1 adjuvant in higher vertebrates by inducing type I IFNs and inflammatory cyto/chemokines, by a capability to activate specific sets of DCs, CD4+ T cells and also upregulation of MHC I and subsequent activation of CTLs (Jin et al. 2010, Hafner et al. 2013, Matsumoto & Seya 2008).

3.2.3.3 CpGs and poly I:C are well-explored as adjuvants in human vaccines

As human vaccine adjuvant candidates, poly I:C and CpG have mainly been formulated separately with an antigen and/or other adjuvants as combination vaccines (Levast et al. 2014, Reed et al. 2013). Due to degradation of poly I:C by nucleases in humans, derivatives has been designed, such as polyICLC and Poly I:C_{12U} (or Ampligen). Co-administration of Ampligen with an inactivated influenza vaccine resulted in complete protection against hetero- and homologous influenza viruses (Ichinohe et al. 2007). Ampligen is in phase I/II clinical trials to evaluate immunogenicity combined with the intranasal flu vaccine, FluMist, and in phase II and III clinical studies against cancer and HIV, respectively (Dowling & Mansell 2016). HEPLISAV, a promising CpG adjuvanted recombinant hepatitis B vaccine, reduces immunizations and elicits high seroprotective Ab titers and is now in phase III clinical trials (Shirota & Klinman 2014). CpG is included in many clinical trials (Reed et al. 2013, Shirota & Klinman 2014) and two-component adjuvant complexes such as IC31, have been developed. IC31 contains an ODN sequence without a CpG motif, still functioning as a TLR9 agonist, with the purpose to reduce eventual systemic side effects of the CpG (Olafsdottir et al. 2009).

3.2.3.4 CpGs and poly I:C have mainly been applied as immunostimulants in teleost

Class B CpGs have been explored as an immunostimulant across teleost species and elicit activation of macrophages, leukocyte proliferation and stimulates cytokine production *in vitro* in salmonids and carp (Carrington et al. 2004, Jørgensen, Johansen, et al. 2001, Jørgensen, Zou, et al. 2001, Meng et al. 2003, Tassakka & Sakai 2003, 2004). *In vivo*, CpGs, mainly B class, have protected salmonids and flounder against bacterial and viral challenges (Carrington & Secombes 2007, Jørgensen et al. 2003, Zhou et al. 2014). *In vitro* immunostimulatory effects of class A, B and C CpG ODNs have been characterized in Atlantic salmon (Strandskog et al. 2007). Further, the combination of two CpG classes, A and B, have induced synergistic responses in the same species (Strandskog et al. 2008). In salmonids and fugu, poly I:C has

induced type I IFN expression (Matsuo et al 2008, Svingerud et al 2012, Collet 2004) and the agonist has inhibited infection against a range of fish viruses (Jensen et al. 2002, Kim et al. 2009, Lockhart et al. 2004, Plant et al. 2005, Takami et al. 2010).

In fish, exploration of CpG and poly I:C as vaccine adjuvants, are in its infancy. CpG ODNs have been inserted to, or injected with DNA vaccines. Incorporation of CpG motifs into the plasmid backbone greatly enhanced immunogenicity of a VHSV DNA vaccine (Martinez-Alonso et al. 2011), while injection of CpG with a DNA vaccine against nervous necrosis virus in grouper had a less prominent effect (Chen et al. 2015). CpG ODNs have shown promising results also in other DNA vaccine studies (Liu et al. 2010b, Zhou et al. 2014) and i.p. injection of CpG motifs, alone or in plasmids have induced protective responses against bacterial diseases (Byadgi et al. 2014, Liu et al. 2010a). To immunize fish with a live pathogenic virus and subsequently administer poly I:C, i.e. 'poly I:C immunization' have displayed higher vaccine efficacies against several viral diseases (Kim et al. 2016, Oh et al. 2012, Kim et al. 2009).

Aims of thesis

The battle against viral diseases in the aquaculture industry is much more heated than many of the cold fjords where the major losses are evident. In higher vertebrates, the immunostimulatory properties of TLR-ligands and their relevance as vaccine adjuvants are well documented. To explore and adapt that knowledge within aquaculture, focusing on development of improved viral vaccines, is important for maintaining a sustainable finfish farming industry.

Main objective

Continue to investigate the immunostimulatory ability of the selected TLR-ligand combo (CpG 2006 ODNs and poly I:C) and its prospect as an adjuvant in fish viral vaccines. Determine if, and how the combo activates adaptive humoral immune responses.

Sub-objectives

- ❖ Test the *in vivo* immunoprotective potential of the TLR-ligand combo alone, or together with an inactivated whole SAV antigen formulation during vaccination and challenge studies in Atlantic salmon.
- ❖ Functionally validate immunoprotective difference between the TLR-ligand combo and other known adjuvants.
- ❖ *In vitro* characterization of MHCII⁺ leukocyte subpopulations, the effects of CpG treatment and identification of APCs.
- ❖ Optimizing isolation and purification of Atlantic salmon B cells from the immunological organs HK, spleen and peripheral blood lymphocytes.
- ❖ Investigate *in vitro* whether TLR-stimulation directly affect B cell responses by measuring mRNA transcripts and functional responses.

Summary of papers

Paper I: Immuno-protective activity of a salmonid alphavirus vaccine: Comparison of the immune responses induced by inactivated whole virus antigen formulations based on CpG class B oligonucleotides and poly I:C alone or combined with an oil adjuvant.

Hanna L. Thim¹, Dimitar B. Iliev¹, Karen E. Christie, Stephanè Villoing, Marian F. McLoughlin, Guro Strandskog, Jorunn B. Jørgensen

Vaccine (2012) ¹authors contributed equally

The TLR-ligand combo CpG/poly I:C was formulated with a SAV inactivated whole virus antigen (high or suboptimal dose) in Paper I to test if the combo could enhance immune responses and/or protection against SAV3 upon i.p. injection challenge. A clear impact of the adjuvant combo was demonstrated by the magnitude of the humoral immune response measured by neutralizing antibodies. However, it was impossible to state a putative adjuvant effect by comparing the induced protection by the CpG/poly I:C adjuvanted treatments against the SAV High Ag alone treatment. This, since neither any nsP1 transcripts were detected at 1 week post challenge in the SAV High Ag alone treatment, nor had any SAV specific heart lesions manifested at 3wpc for that same treatment. The TLR-ligand adjuvanted SAV High Ag sera gave 100% seroprevalence both pre and post challenge compared to the SAV High Ag alone with 50% and 93% seroprevalence, respectively. As a control, Montanide ISA 673A, a licensed aquaculture oil-adjuvant was formulated in combination with the TLR-ligand adjuvanted SAV High Ag vaccination. However, this formulation gave significantly lower seroprevalence with 13% pre challenge and 50% post challenge. While the SAV High Ag alone treatment did not commence an early immune gene expression, the CpG/poly I:C adjuvanted formulations induced both type I and type II IFNs. This study demonstrate an onset of both innate and humoral adaptive responses after CpG/poly I:C adjuvanted vaccination.

Paper II: Vaccine adjuvants in fish make a difference: Comparing three adjuvants (Montanide ISA763A Oil, CpG/poly I:C combo and VHSV glycoprotein) alone or in combination formulated with an inactivated whole salmonid alphavirus antigen

Hanna L. Thim, Stéphane Villoing, Marian F. McLoughlin, Karen E. Christie, Søren Grove, Petter Frost, Jorunn B. Jørgensen

Vaccines (2014)

In paper II, a known potent genetic adjuvant, VHSV G DNA, was included to test the hypothesis if more than two PRR agonists can induce even stronger, more durable protective immune responses. Protection, nAbs, and immune related transcript levels were analyzed after cohabitant challenge with SAV3. Again, the hypothesized effect of the TLR-ligand adjuvant combo on protection against SAV could not be distinguished due to the full protection mediated by the SAV Ag alone vaccination. Several immune responses, putatively involved in protection, were enhanced by CpG/poly I:C adjuvanted formulations and confirmed our earlier studies; such as the strongly enhanced IFN type I and type II responses at 12 and 48 hours post vaccination, and the potent nAb responses both pre and post challenge. There were no additive effects displayed by introducing the genetic adjuvant. The importance of complement activation for clearance of SAV was demonstrated through analyses of heat inactivated nAb responses. This paper demonstrates the inductive boost CpG/poly I:C exerts on protective immune responses against a viral infection. Hence, the combo demonstrates a solid potential as an adjuvant in viral fish vaccines.

Paper III: Homing of antigen-presenting cells in head kidney and spleen – salmon head kidney hosts diverse APC types

Dimitar B. Iliev, Hanna L. Thim, Leidy Lagos, Randi Olsen, Jorunn B. Jørgensen

Frontiers in Immunology (2013)

Characterizing salmon APCs ability to take up soluble antigens and migrate toward secondary lymphoid organs was one specific scope of Paper III. *In vivo*, the uptake of i.p. injected fluorescence-labeled CpG class B ODNs together with the model antigen ovalbumin (OVA), predominately accumulated in head kidney (HK) MHCII-positive (MHCII⁺) leukocytes compared to spleen. *In vitro*, the adherent HK leukocytes processed and accumulated OVA within their endosomes, indicating that those cells were professional APCs. Upon *in vitro* CpG stimulation, three different MHCII⁺ HK leukocyte subpopulations were identified. One of these MHCII⁺ populations was suggested to be maturing APCs, since they endocytosed high amounts of OVA and expressed the highest CD86 and TNF mRNA levels, while CCR6 was downregulated. Further, the HK harbored a suggested B cell population; MHCII⁺/IgM⁺ cells that had a typical lymphocyte morphology with round nuclei, low cytoplasm versus nucleus ratio. Compared to the other MHCII⁺ cell types, the IgM⁺ cells expressed relatively low levels of pro-inflammatory cytokines, while both the basal and the CpG-induced IFN γ expression was very high in these

cells. In addition, mRNA transcript levels of PAX5 complemented those of membrane bound IgM (mIgM) in unstimulated cells. After CpG treatment, PAX5 and mIgM were downregulated, while soluble IgM (sIgM) was upregulated, indicating that CpG treatment induce differentiation of antibody-secreting cells (ASCs).

Paper IV: Profiling B cell populations in Atlantic salmon – Toll-like Receptor expression and responsiveness to CpG stimulation

Shiferaw Jenberie¹, Hanna L Thim¹, Ingvill Jensen, Jorunn B Jørgensen

Manuscript under preparation ¹authors contributed equally

Paper I-II demonstrate that the TLR-agonist combo CpG/poly I:C induce longer-lived antibody responses in fish after vaccination and Paper III suggests that CpG treatment of IgM⁺/MHCII⁺ HK leukocytes induces those cells to differentiate toward antibody-secreting cells (ASCs). In Paper IV, the frequencies of IgM and IgT positive cells of total B cells in Atlantic salmon systemic lymphoid tissues were analyzed by flow cytometry. Magnetic-activated cell sorting (MACS) of IgM⁺ B cells from HK, PB and spleen allowed further profiling of the pure IgM⁺ B cells. Basal nucleic acid-sensing TLR expression levels were determined that showed that TLR3/22, TLR9 and TLR8a1 had relatively high constitutive transcript levels, while the fish and chicken specific TLR21 was the least expressed TLR across the tissues. In addition, the data showed that CpG, alone or in combination with conditioned media, upregulated transcript levels of secreted and membrane bound IgM and modulates the secretion of IgM in sorted IgM⁺ B cells from systemic lymphoid tissues. This work provides a base for further characterization of specific B cell responses in Atlantic salmon that within a future scope may aid the development of more efficient prophylactic strategies against viral diseases.

Discussion of results

The link between innate and adaptive immunity is well described in higher vertebrates such as humans and mice. In lower vertebrates, as for instance the teleost; one of the oldest living organisms with both innate and adaptive immunity present, much less is known of how this link is functionally executed. With the steadily growing importance of aquaculture as a food source, dissecting how innate immunity can be manipulated to shape desired adaptive effector mechanisms is crucial for development of efficient viral vaccines.

In vivo adjuvanted vaccine effects and homing of APCs

CpG/poly I:C adjuvanted formulations potently enhance protection related immune responses – skewing towards a T_H1 -resembling environment?

Of today's common fish vaccine adjuvants, detrimental side effects are evident and well documented in Atlantic salmon. To identify potent adjuvants with reduced, or optimally, no side effects would increase both production value and fish welfare. We have examined the combined prophylactic attributes of two TLR-ligands; CpG and poly I:C, that are considered as safe adjuvants in human vaccine development (Duthie et al. 2011, Shirota & Klinman 2014).

In mammals, class B CpGs stimulate both B cells and pDCs, while indirectly activating a magnitude of other immune cells through TLR9 activated cytokine production (Krieg 2002). Combined with an antigen, class B CpGs have proven themselves as potent adjuvants against viral diseases due to a strong bias towards a T_H1 -based response. This effect is augmented by improved antigen-presenting by DCs, T_H1 cytokine secretion that induces CD40L-independent T cell help and in addition, by direct activation of B cells to secrete antibodies (Krieg 2002, Krieg & Davis 2001). Poly I:C polarize towards a T_H1 response through activation of TLR3. In turn, TLR3 ligation may be required for RIG-I (a cytosolic PRR) activation, which stand as a complementary pathway for detecting and responding to intracellular viral particles (Duthie et al. 2011, Palm & Medzhitov 2009).

Prior to what is presented here, our group showed that injection of the CpG/poly I:C combo to Atlantic salmon, gave synergistic upregulation of important immune genes (Strandskog et al. 2008) and provided unspecific protection against a SAV3 infection (Strandskog et al. 2011). In paper I and II; the adjuvant combo was prepared together with an inactivated whole SAV1 Ag formulation and investigated as a prospective vaccine candidate. Treatments formulated with CpG/poly I:C displayed significantly pronounced immune gene transcripts post

vaccination in HK and spleen. In addition, serum nAb responses were elevated several magnitudes compared to the SAV Ag alone formulations both pre and post challenge. At transcript level, CpG and poly I:C enhanced inflammatory mediators (CXCL10), induced IFN α 1 and ISGs (Mx and Vig-1) and displayed a prominent IFN γ upregulation as early as 12h_{pv} (hours post vaccination; paper II), which was prominent also at 5d_{pv} (days post vaccination; paper I). These findings infer that the immune responses this adjuvant combo significantly potentiate are skewed towards, what in higher vertebrates would be defined as, a T_h1 response. The slight, yet significant, protection in the SAV Low Ag Oil CpG/poly I:C formulation evident in Paper I, suggests that an adaptive response is generated. The SAV Low Ag Oil dose was not enough to confer full protection compared to the High Ag Oil dose. Hence, the induced immune transcripts present in SAV Low Ag Oil CpG/poly I:C treated fish, indicate that the subsequent protection may have been adjuvant induced. CpG and poly I:C are, in mammals, known to activate immature professional APCs, such as DCs, predestining their terminal differentiation/maturation (Hartmann et al. 1999). Activated APCs secrete large amounts of type I IFNs that in turn may activate expansion of CTLs. Hypothetically, this could indicate a presence of a cellular, T_h1-resembling environment in the SAV Low Ag CpG/poly I:C group, contributing to the elimination of virus-infected cells in infected individuals. Whether this in fact holds true, requires further investigation. Of note, the prominent nAb levels, from both paper I and II in CpG/poly I:C adjuvanted SAV treatments, certainly communicate that CpG itself may act as a secondary signal that can, alone or together with the antigen, directly elevate antibody secretion and contribute to an enhanced T_h1-resembling environment.

vhsG does not contribute to an enhanced adjuvant effect against the model virus IHN_V/VHS_V cross-protection has been documented for IPNV and nodavirus (Somerset et al. 2003, LaPatra et al. 2001). In Paper II, the VHS_V G-protein (vhsG), was included as a genetic adjuvant to discern an eventual additive effect against SAV and in addition, if it could further potentiate the adjuvant effect of the TLR-ligand combo. In a recent study (Pereiro et al. 2014), turbot were i.m. injected with a vhsG DNA vaccine (pM_{CV}1.4-G₈₆₀) and HK tissue samples, harvested at 8, 24 and 72 h_{pv}, were subjected to microarray. Their study correlates with our gene transcript analyses that displayed elevated early transcript upregulation of B and T cell markers in vhsG treated groups. In the turbot study, T cell related genes (especially CD8 α) were the main immune factors slightly induced by the DNA vaccination at 8 and 24 h_{pv}.

Further, IFN and MHC related gene markers were in the turbot study elevated first at 72 hpv, which could explain the absence of antiviral induced genes in vhsG-injected fish in our study. The efficacy evident in the turbot study was suggested to depend on humoral responses (nAbs) (Pereiro et al. 2014, Pereiro et al. 2012). Hence, a negative effect from the polyvalent vaccination, as discussed in Paper II is plausible, yet warrants further investigation. A slight, vhsG additive effect was seen for the SAV Ag vhsG treated individuals compared to SAV Ag alone, thus indicating an additive effect.

Dual role of Montanide ISA763A oil on protection against the model virus

In the first two papers, the oil adjuvant Montanide ISA763A was included as a control adjuvant. Oil adjuvants mainly release their antigens in extracellular compartments and consequently elicit humoral responses. Further, depending on the emulsion's formulation, w/o, o/w or w/o/w, the release of the antigenic components provides a longer- or shorter-term depot effect. In both presented challenge experiments, most oil adjuvanted (w/o) SAV Ag treatments displayed lower efficacy compared to without oil (RPPsc. between 17.32-34.12% for SAV Low and 92.41-100% for SAV High in Paper I, and 58.3-85.7% in Paper II). The same trend was seen in Paper I for the oil-formulated TLR-ligand combo compared to TLR-ligands alone at 1wpc (11.93% to 43.17% respectively). While a depot effect was noted for the same treatments at 3wpc (17.32% to 4.36%, respectively). Hence, the oil adjuvant displayed variable efficacy. In fish, the general onset of a humoral response may be slow, and paradoxical, while the oil depot is intended to lengthen the response; the initiation of the response might be impeded. The importance of an unspecific trigger through PRRs to prompt the commencement of humoral mediated responses may determine if a pathogen manages to establish an infection or not. Hence, innate mediators, such as TLR-ligand adjuvants, have important roles as immune enhancers, initiating adaptive ques faster (Cerutti et al. 2011, Bendelac et al. 2001). To have included a SAV Low CpG/poly I:C treatment group without oil in the experimental set-up could have answered if the weaker TLR-ligand combo alone would have been potent enough to provide a faster onset of the antiviral T_h1 environment compared to oil; still with lesser reduction in efficacy over time.

Homing of CpG and OVA engulfing APCs in Atlantic salmon

The HK functions both as a primary and secondary lymphoid organ in fish. In Paper III, the data suggests that putative salmon APCs can internalize soluble antigens (fluorescent-CpG and/or

OVA) in the periphery and migrate to the spleen and HK. APCs in HK that had taken up the soluble, fluorescent antigen were present for the two weeks measured. However, to inject OVA together with CpGs (50µg, same dose as in the challenge experiments in Paper I-II), did not enhance OVA accumulation in HK leukocytes. Additionally, these cells displayed features typical for immature APCs. The CpG dose of 50µg combined with an equal dose of poly I:C, have been used by our group in previous *in vivo* studies (Strandskog et al. 2008), as well as in Paper I and II. If an additional cue, such as from another PRR, is needed for further maturation of APCs, is discussed under the *in vitro* section below.

In vitro CpG effects on sorted HKLs and IgM positive B cells

To understand the mechanisms behind the significantly enhanced immune responses elicited by the TLR-ligand combo (Paper I and II), as well as to further characterize the prospective APCs (Paper III), *in vitro* studies were executed. In paper III, CpG treated HK leukocytes were initially sorted into four populations based on their ability to endocytose OVA and their surface IgM and MHCII expression (see Table 5). Thereafter, the populations were further characterized by microscopy and by basal transcript levels of a variety of immune cytokines and markers, including the regulation of these factors post CpG treatment (2µM dose). In paper IV, lymphocytes from isolated HK, PB and spleen leukocyte populations were positively selected based on their expression of surface IgM. These IgM⁺ B cell populations were further characterized based on transcript levels of TLRs and B cell related markers upon CpG stimulation. One of the main intentions was to investigate if CpG exert direct effects on B cells that may be related to its potency as a vaccine adjuvant.

Table 5. Traits of four suggested cell types of *in vitro* CpG stimulated and subsequently sorted HKLs

POPULATION	1	2	3	4
TRAIT				
Size	Variable	Large	Small	Large
Granularity	Low	High	Weakly	Highly
MHCII	-	+	+	+
IgM	-	-	+	-
OVA uptake	-	+	-	-
Cell characteristic	Lymphocyte like	Maturing APC	Lymphocyte like/B cells	Granulocytes

MHCII, major histocompatibility complex II; OVA, ovalbumin; APC, antigen presenting cell. + for possessing trait. – for not possessing trait.

Immature HKL APCs may need a second signal for full maturation

The heterogeneity of the HK leukocyte populations is clearly exemplified by our findings. The ability of APCs to migrate from the periphery to secondary lymphoid organs and act as APCs

is suggested in Paper III. The basal transcript expression of CD83, an APC marker, was most pronounced for the APC like cells (population 2) along with CD86 and B7-H1 suggesting them to be maturing APCs, related to macrophage or DC-like cells. Hypothetically, that the proposed APCs in Paper III was not fully matured, may depend on the need of stimulation through several receptors, such as another PRR, paracrine stimulation of maturation inducing cytokines or CD40/CD40L interaction (Mäkelä et al. 2009, Napolitani et al. 2005, Gautier et al. 2005, Lagos et al. 2012). These factors were likely not present in sufficient amounts in the sorted *in vitro* cultures in Paper III. In mice, poly I:C treatment alone has provided higher efficacy against smallpox, when administered post exposure to the pathogen, than CpG treatment alone. This possibly through poly I:C downstream signaling cascades involving several PRRs (Israely et al. 2014). In contrast, TLR9 expression is restricted to a limited number of cell types (Hemmi et al. 2000).

APC markers were upregulated on B cell like subpopulations in CpG stimulated HKLs
In Paper III, transcripts of both B7-H1 and CD40, molecules important for antigen presentation, were upregulated by CpG stimulation in the IgM lymphocyte like population. Fig. 13 show that CpG treated IgM sorted cells from HK, PB and spleen potently upregulate CD83, another APC marker. Interestingly, CD83 displayed a further enhanced upregulation in sorted cells when CpG was combined with a pulse stimulated adherent HKL supernatant (PAS). Further, CD40 (Fig. 13) was also upregulated after CpG treatment in all three tissues and to a lesser extent when combined with PAS. Fish B cells are known to be phagocytic (Sunyer 2012) and the findings by Korytář et al (2013) show that the peritoneum of unstimulated trout is dominated by IgM positive cells. By initiating a vaccination study that includes the CpG/poly I:C combo; the concept of lymphocytes in the peritoneum being able to engulf vaccine antigens and migrate to secondary lymphoid tissues for further presentation and differentiation, could be explored. Such a study could contribute to further understanding of teleost adaptive immune mechanisms.

Effects of CpGs on IFN γ in leukocyte and B cell subsets

IFN γ is a hallmark T_h1 cytokine in mammals, mainly secreted by T_h1 cells, NK cells and CTLs. IFN γ executed effects are; induced Ab secretion by PC, T_h1 differentiation, promoted leukocyte migration and increased MHCI expression on normal cells, which may lead to further elimination of virus-infected cells by CTLs (Savan et al. 2009, Schoenborn & Wilson 2007). In

salmon, IFN γ has been demonstrated to exert antiviral activity directly against SAV (Sun et al. 2011). Significantly elevated IFN γ transcript levels were repeatedly present in Paper I and II for the CpG/poly I:C treated groups after vaccination. Further, Paper III presents CpG induced IFN γ transcripts in all four sorted cell populations, where both the basal and GpG-induced levels were most prominent in population 3; IgM⁺/MHCII⁺ lymphocytes, followed by population 1, which likely contains IgT positive cells, T cells, NK-like cells and lymphoid progenitor cells. However, which specific leukocyte subset(s) within population 1 that are responsible for the elevated IFN γ levels, warrants further investigation. The highly upregulated IFN γ levels in population three upon CpG stimulation suggests that subsets of salmon B cells are capable of producing high levels of IFN γ . Recently, a unique innate B cell subset (CD11^{hi}FcRIII^{hi}CD19⁺) was generated after pathogen or TLR-ligand challenge (Bao et al. 2014). These specific B cells secreted high amounts of IFN γ in a CD40/CD40L-dependent manner. Supported by the findings in mice, we suggest that salmon B cells, directly activated by CpGs, can secrete this central cytokine to further prime the host's adaptive and antiviral response, which presently, is under further investigation.

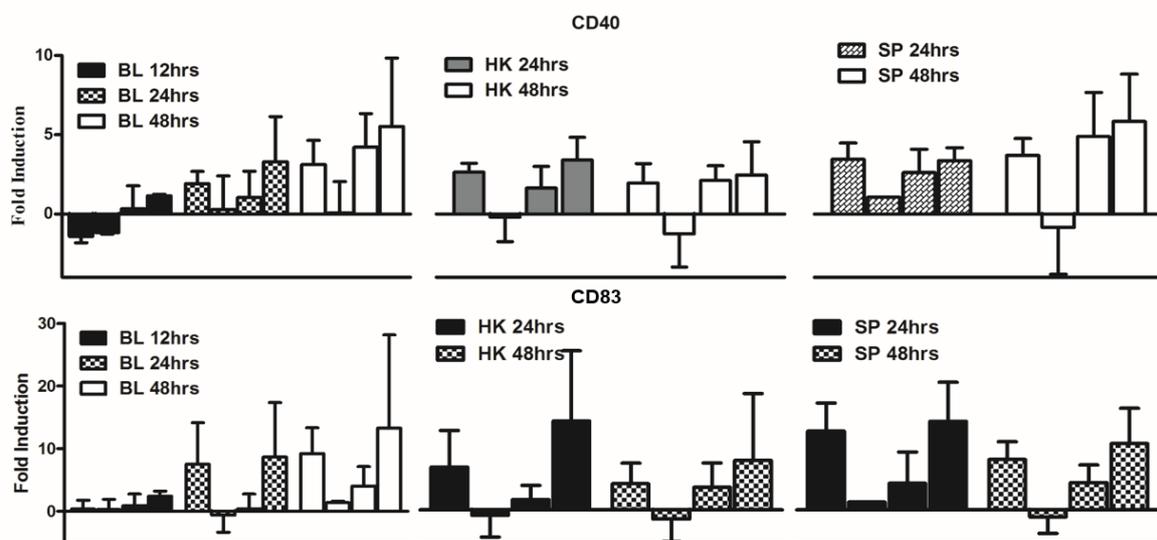


Figure 13. Relative immune genes expression profile of MACS sorted B cells. IgM sorted B cells from Atlantic salmon peripheral blood (PBL), head kidney (HKL) and spleen (SPL) leukocytes were analyzed by qRT-PCR for expression of the antigen-presentation markers, CD40 and CD83. Gene expression data were normalized against EF1a β for each time point control and fold inductions were calculated by the Pfaffl method. Data were obtained from at least three individuals ($n \geq 3$) and error bars indicates standard deviation. Data belong to the same material that is described in detail and presented in Paper IV.

Outline of inferable immune responses activated upon TLR-ligand stimulation

The immune system is complex, containing many players and means of positive and negative regulation. Manipulation of the immune system by vaccination enables targeting of several ‘immune-central posts’ that aspiringly may induce the crucial ‘full’ immune response and ultimately, provide sufficient protection against future infections. A brief, inferable, summary of how the tested CpG/poly I:C adjuvanted antigen formulations may have targeted important immune posts *in vivo*, further inferred by the *in vitro* studies is outlined in Fig. 14.

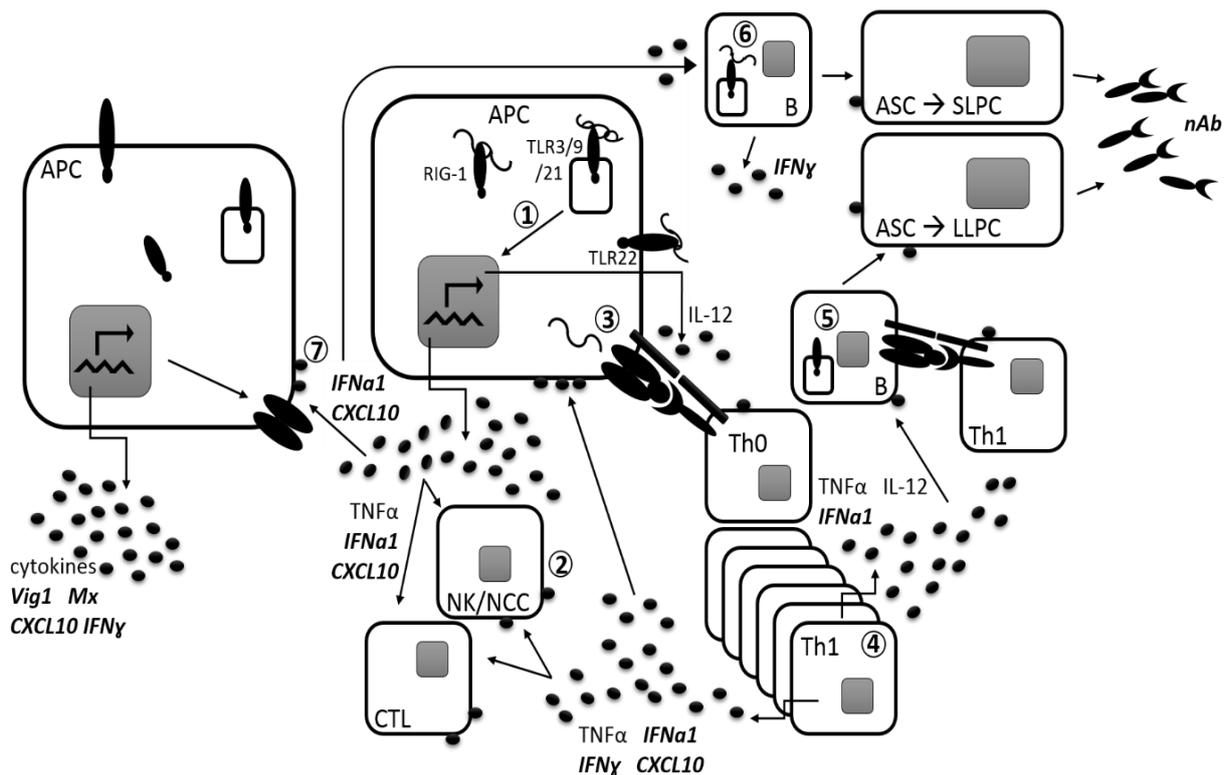


Figure 14. Schematic outline of inferred synthetic nucleic acid and viral antigen immune responses in Atlantic salmon. ① Binding of a specific ligand to a surface or intracellular PRR present on an APC (Ag presenting cell) initiates a signaling cascade that subsequently releases antiviral and inflammatory cytokines to the proximate environment. ② The cytokines attract cells to the inflammation/infection area, as well as activate cell-mediated responses such as CTLs (cytotoxic T lymphocytes) and NCCs (non-specific cytotoxic cells). ③ APCs process foreign molecules and presents antigenic peptides through MHCII, which upon recognition by TcR and co-receptors differentiate immature T helper cells to induce a specific T helper cytokine environment, such as Th1. ④ In turn, activated T helper cells secrete cytokines that act on nearby APCs, CTLs or NCCs. ⑤ Putative T helper cells will specifically activate B cells to proliferate and differentiate to ASC (Ab secreting cells) and upon the correct cues further differentiate to LLPCs (long-lived plasma cells) for anamnestic purposes. ⑥ Since fish lack germinal centers and isotype switch, the classical T helper-response can be questioned. Instead, polyclonal activation of the entire B cell pool through CpG specific activation of B cells may occur. CpG activated B cells merely seem to differentiate to SSPCs (short-lived PCs), thou with parallel activation by Ag, anamnestic responses are probable. Further, IFNs are in mammals known to activate B cells directly. ⑦ Activated APCs secrete cytokines to the environment affecting neighboring cells to initiate a crucial antiviral/IFN response, leading to secretion of ISGs (IFN stimulated genes). Further, paracrine stimulation of immature APCs upregulates immune related factors such as MHCII. Factors in bold *italic* were upregulated upon CpG/poly I:C stimulation in Paper I-II. However, where these factors may have exerted their function is purely hypothetical, and not functionally investigated. Figure based on Paper I-IV and the general introduction.

Future perspectives

- ❖ Identify and develop functional markers for B cell characterization in Atlantic salmon
- ❖ Establish solid *in vitro* models for profiling of salmon B cell subpopulations
- ❖ Establish protocols for a proliferation assay and an ELISPOT assay
- ❖ Optimize an (viral) antigen-model to study CpG and/or poly I:C induced effects in relation to B cell responses *in vivo*

The roles B cells play in shaping adaptive responses in regards to long-term memory and mechanisms that regulate naïve B cell proliferation in Atlantic salmon are scarcely explored. Solid protocols for both *in vitro* and *in vivo* applications, along with suitable functional markers, are required to achieve such profiling. CpG and poly I:C, alone or in combination, are here suggested as promising 'model-agonists' within the frames of the proposed protocols when they are established. As 'model-agonists' they may aid in increased understanding of B cell immunology and the development of improved viral vaccine adjuvant concepts in fish. However, if such adjuvant concepts would be applicable commercially is indeed also a question of cost.

Main conclusions

- ❖ CpG and poly I:C formulated with a SAV Ag formulation prominently enhance protection-related immune responses in Atlantic salmon against i.p. and co-habitant virus challenge
- ❖ APCs predominately accumulate in the HK after uptake of soluble Ag in the periphery
- ❖ The frequency of IgM and IgT positive cells in systemic lymphoid tissues of Atlantic salmon resembles those reported for rainbow trout
- ❖ CpG stimulation of IgM⁺/MHCII⁺ cells downregulate early B cell markers, while upregulation of secreted IgM indicate differentiation to ASCs.
- ❖ TLR3, TLR8a1, TLR9, TLR21 and TLR22 are expressed in pure salmon IgM⁺ B cells
- ❖ PRR stimulation through a class B CpG is enough to induce IgM secretion in pure IgM⁺ B cell cultures derived from HK and spleen

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