

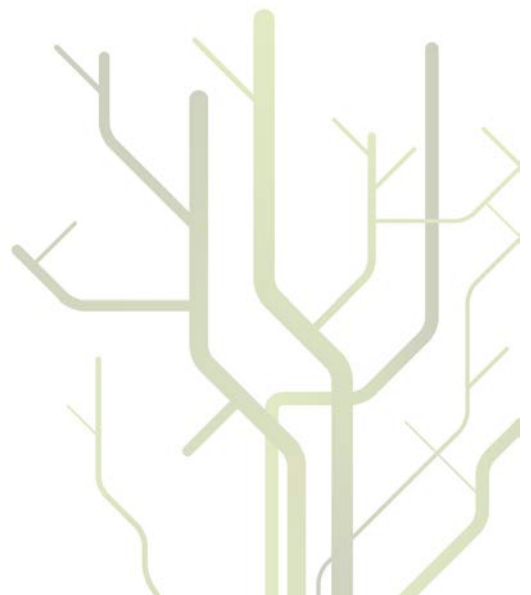
Bacterial flagellin- a novel adjuvant for vaccine strategies



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What I was told about the requirements for this PhD position was that the candidate must be motivated and social. I knew I had the social aspect down and motivation I was pretty sure I could manage; it was the scientific background that had me worried. Thank you to Roy and Atle for taking a chance on a chatty Canadian who really wanted to make a change into the field of molecular immunology. Atle, thank you for guiding me through the world of recombinant proteins and for being there to listen to my stress induced rants. Roy, I have appreciated all the assistance you have given me, from designing the studies to editing my work. Clemens, there are not enough words to say how thankful I am to you for your patience, kindness and knowledge. You taught me everything from the very beginning, were there to explain what I did not understand and to pick me up when I felt all hope was lost. I was lucky to have you as my post doc and I'll never forget all you did for me.

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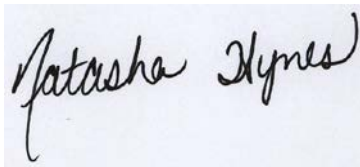
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A handwritten signature in black ink on a light blue background. The signature reads "Natasha Hynes" in a cursive script.

Natasha Hynes

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List of papers

Paper I

Natasha A. Hynes, Clemens Furnes, Børge Nilsen Fredriksen, Tori Winther, Jarl Bøgwald, Atle N. Larsen, Roy A. Dalmo. Immune response of Atlantic salmon to recombinant flagellin. *Vaccine* (2011), doi:10.1016/j.vaccine.2011.07.138.

Paper II

Natasha A. Hynes, Tiago S. Hori, Roy A. Dalmo, Matthew L. Rise. Functional genomics analysis of the immunological responses of Atlantic salmon (*Salmo salar*) spleen to injection with recombinant flagellin from *Vibrio anguillarum*. Manuscript.

Summary

Flagellin is the principal structural protein in the locomotive organ flagellum present on flagellated bacteria, and is known to be an important evolutionarily conserved PAMP. Flagellin has been shown to bind to the PRR TLR5 which induces innate immune system responses and signaling pathways. In mammals, much focus has been placed on this protein in vaccine studies for its possible function as an adjuvant. In paper I, flagellin (FlaD from *Vibrio anguillarum*) was recombinantly produced in two forms, full-length (FDL) and a truncated form (FDS) with portions of the N- and C-termini removed to prevent polymerization. Both forms of flagellin were tested alone and in combination with an antigen in a dose response study to determine the most effective dosage to produce a strong immune response. A polyclonal antibody for FDS was produced and showed good specificity in immunoblot testing. Cell culture was used to compare the NF- κ B response after stimulation with FDL versus FDS and resulted in a significantly larger response in cells stimulated with FDL. QPCR mRNA gene expression results showed a strong innate immune response with a number of genes known to be induced downstream by the TLR signaling pathway up-regulated including important TLR5S and inflammatory cytokines and chemokines (TNF α , IL-6, IL-8, IL-1 β). Due to lower production and decreased stability of FDL, FDS was selected for further injection studies in salmon.

In paper II, microarray analysis further explored the salmon's response to injection of flagellin with and without the model antigen ovalbumin. Injection with flagellin with and without ovalbumin caused reproducible gene up-regulation of inflammatory cytokines, chemokines and receptors (IL-8, TNFRSF11B, IL-1R), antimicrobial peptides (hepcidin, cathelicidin), immune genes (C/EBP, thioredoxin, C-type lectin receptor B), complement genes (complement component C7 and C7-1), peptidases (MMP-9) and genes involved in the Ras/MAPK pathway (Ras-related proteins). Down-regulation of interesting immune genes also occurred including myxovirus resistance 1, clusterin-1 and LPS-induced TNF- α factor homolog. Some genes exhibited early, delayed or extended response over the sampling time and flagellin seemed to be the key component to eliciting a response. A selection of up-regulated genes from the microarray studies were validated by QPCR. Flagellin was shown to induce the innate immune response in Atlantic salmon with further studies needed to determine its efficacy as an adjuvant in challenge studies.

Abbreviations

Abbreviation	Full name	Abbreviation	Full name
AMP	antimicrobial peptide	IRF4	interferon-regulatory factor 4
APC	antigen presenting cell	JNK	c-Jun N-terminal kinase
ATF-2	activating transcription factor 2	LRR	leucine-rich repeat
CARD	caspase activation recruitment domain	LPH	hemocyanin from <i>Limulus polyhemus</i> hemolymph
CD	cluster of differentiation	LPS	lipopolysaccharide
cDNA	Complementary DNA	MAF	macrophage activating factor
CD4 ⁺ or T _H cells	helper T cell	MAPK	mitogen-activated protein kinase
CD8 ⁺ or CTL	cytotoxic T cell	MD-2	lymphocyte antigen 96
cGRASP	consortium for Genomic Research on All Salmonids domains	MHC	major histocompatibility complex
D	domains	MMP9	matrix metalloproteinase-9
DC	dendritic cell	mRNA	Messenger ribonucleic acid
DPI	days post injection	MyD88	myeloid differentiation primary response gene
dsRNA	double-stranded ribonucleic acid	Naip5	neuronal apoptosis inhibitory protein 5
ELISA	enzyme-linked immunosorbent assay	NLRC4	NLR family CARD domain-containing protein 4
Elk-1	Ets-like gene1	NCCR	non-specific cytotoxic cell receptor
ERK	extracellular regulated kinase	NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cell
FCA	Freund's complete adjuvant	IHNV	infectious hematopoietic necrosis virus
FDL	FlaD full-length	IPNV	infectious pancreatic necrosis virus
FDS	FlaD truncated	ISA	infectious salmon anemia
FIA	Freund's incomplete adjuvant	IRAK	interleukin-1 receptor-associated kinase
Hsp	heat shock protein	IL	interleukin
IκB	NF-κB inhibitor	IKK	inhibitor of NF-κB kinase complex
IFN	interferon	Ig	immunoglobulin

Abbreviations

Abbreviation	Full name
NITR	novel immune-type receptor
NLR	nucleotide-binding domain and leucine-rich repeat-containing family
NK	natural killer cell
NOD	nucleotide-binding oligomerization domain
OVA	ovalbumin
PAMP	pathogen associated molecular pattern
PRK	protein kinase
PLG	poly-lactide-co-glycolide
PRR	pattern recognition receptors
RLA	relative luciferase assay
RPS	relative percent survival
QPCR	quantitative real time polymerase chain reaction
ssRNA	single-stranded ribonucleic acid
TAB2	TGF- β activated kinase 1/MAP3K7 binding protein 2
TAK1	TGF- β activated kinase 1
TCR	T cell receptor
T _{FH}	follicular B Helper T cells
TGF- β	transforming growth factor-beta
T _H 2	T helper 2 cells
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TLR5S	Toll-like receptor 5 soluble
TLR5M	Toll-like receptor 5 membrane-bound
TNF α	Tumor necrosis factor- α
TNFSF11B	TNF α receptor super family 11B
TRAF6	TNF receptor associated factor 6
T _{reg}	regulatory T cell
VHN	viral hemorrhagic septicemia

1. Introduction

Norway has become one of the largest producers of Atlantic salmon worldwide but it has not been without overcoming numerous disease challenges throughout its development. Many of the bacterial diseases have been effectively reduced through the use of vaccines but viral diseases remain a problem for growers. There may be a need for more efficient vaccine components (adjuvants and antigens) that are able to combat the problem of viral and intracellular bacterial diseases in salmon aquaculture [1]. With recent advances in fish immunology, there are new and exciting areas of the immune system to target including Toll-like receptor (TLR) ligands. Mammalian research has provided evidence of improved survival and antibody production with the use of flagellin, a TLR5 ligand. Fish live in an aqueous environment surrounded by numerous pathogens therefore a strong first line of defense is imperative to their survival. By focusing on flagellin, a key structural protein in the bacterial flagella, it was possible to elucidate the role that flagellin plays in stimulating the innate immune system in salmon. This not only advances the knowledge of salmonid immunology but also can assist in the formulation of more successful vaccine adjuvants.

1.1 The immune system

1.1.1 The innate immune system in mammals

The innate immune system is the first line of host defense against invading pathogens and has been found in all classes of animal life, from plants to mammals [2]. This type of defense strategy occurs almost immediately (within minutes up to a few days) and includes physical barriers, antimicrobial peptides, natural killer cells, receptors, etc. In this thesis focus has been placed on the innate immune receptors due to their role in the response to flagellin. The innate immune response relies on non-self discrimination which is the recognition of specific microbial components (lipopolysaccharide (LPS), flagellin, lipoglycans, etc) that are not found in host cells. This prevents the immune system from reacting to its own cells. These microbial components are called pathogen associated molecular patterns (PAMPs) and are often essential for the microbes survival which prevents them from being mutated over the course of evolution. Pathogen associated molecular patterns bind to pattern recognition receptors (PRRs) which are unable to undergo somatic recombination but have developed a protective adaptation to potentially harmful microbes [3, 4]. The inability to undergo somatic recombination means that these receptors are encoded in the germ-line and do not require rearrangement of genes. The distribution of receptors is non-clonal in that there are identical

receptors on all cells of the same lineage. The innate immune system does not respond faster or more powerfully to pathogens, even upon repeat exposure, meaning that there is no immunological memory.

There are numerous types of innate immune receptors in mammals. They are grouped into three main categories; secreted PRRs (mannan-binding lectin, C-reactive protein, serum amyloid protein), cell-surface receptors (macrophage mannose receptor, macrophage scavenger receptor) and intracellular PRRs (protein kinase RNA-activated (PRK), nucleotide-binding oligomerization domain (NOD)-like receptors) [4]. Toll-like receptors are a very well-known and important group of receptors in the innate immune response. Ten TLRs had been discovered in humans and mice [4, 5] but recently a novel TLR has been discovered [6]. TLR13 responds specifically to vesicular stomatitis virus in mouse cells lines but not to other known TLR ligands and appears to be an intracellular TLR. TLRs vary from each other in ligand specificities, expression patterns and the target genes that they can induce. Some are expressed on the cell-surface of important immune cells while other are expressed on the endosomal surface [4]. Due to the focal point of this work being on the role of flagellin in the innate immune response, much of the focus on receptors will be placed on TLR5 due to its established role as a flagellin receptor.

Table 1. Innate and adaptive immunity. Adapted from Janeway and Medzhitov, 2002 [4].

Property	Innate immune system	Adaptive immune system
Receptors	Fixed in genome Rearrangement is not necessary	Encoded in gene segments Rearrangement necessary
Distribution of receptors	Non-clonal All cells of a class identical	Clonal All cells of a class distinct
Recognition	Conserved molecular patterns (LPS, glycans, flagellin)	Details of molecular structure (proteins, peptides, carbohydrates)
Non-self discrimination	Perfect: selected over evolutionary time	Imperfect: selected in individual somatic cells
Action time	Immediate activation of effectors	Delayed activation or anergy
Response	Co-stimulatory molecules Cytokines (IL-1 β , IL-6) Chemokines (IL-8)	Clonal expansion or anergy IL-2 Effector cytokines: (IL-4, IFN γ)

1.1.2 The adaptive immune system in mammals

When the innate immune system is unable to free the host of a pathogen challenge, the adaptive immune system is activated. This activation takes longer to mount than the innate immune response (days to weeks), but is more specific. The innate immune system is able to instruct the adaptive about the nature of the pathogen challenge through the expression of co-

stimulatory molecules (cluster of differentiation (CD) 80, CD83) on antigen presenting cells (APCs) [4]. The adaptive immune system can also activate the innate immune response to aid in the elimination of pathogens. It has clonally distributed receptors that must undergo rearrangement of the gene segments to allow for the recognition of particular features of pathogens. This permits the host to recognize a wide variety of microbes. Immunological memory is elicited after repeat exposure to a microbe thereby mounting stronger, faster and more specific responses after re-encountering the same microbe [3, 4, 7, 8].

There are two key cell types involved in the adaptive immune response, B and T lymphocytes. B cells function in the production of antibodies which can recognize a wide variety of native macromolecules (proteins, polysaccharides, lipids and nucleic acids) and this is known as humoral immunity. After recognition of a specific antigen, the B cell receives signals from T helper 2 (T_H2) cells which allow them to produce effector cells, called plasma cells which secrete antibodies (immunoglobulins, Ig). In mammals there are five types of antibodies; IgA, IgD, IgE, IgG and IgM, each with different biological properties and handling different kinds of antigens [3]. In comparison, T cells only distinguish peptide fragments of protein antigens and only when they are presented on major histocompatibility complex (MHC) molecules of the host cell. Cytotoxic T cells ($CD8^+$ or CTL cells) recognize peptides presented on MHC-I which are present on all nucleated cells whereas helper T cells ($CD4^+$ or T_H cells) recognize peptides on MHC-II which are found on APCs (macrophages, B cells and dendritic cells). There are two main types of T helper cells; T_H1 cells which are involved in the elimination or suppression of intracellular pathogens and T_H2 cells which eliminate or neutralize extracellular bacteria, parasites and toxins and activate B cells. Regulatory T cells (T_{reg}) limit and suppress the immune system and may play a role in controlling abnormal immune responses to self-antigens. Furthermore, T_H17 cells are known to be involved in various autoimmune diseases and function in anti-microbial immunity at epithelial/mucosal barriers [3]. T_H9 and T_H21 are newly discovered T cell subsets that emerge after viral infection but little is known about their functional roles [9]. T_H9 cells which were shown to reprogram T_H2 cells towards T_H9 under the influence of transforming growth factor-beta ($TGF-\beta$), produce interleukin-9 (IL-9) [10, 11] and require interferon-regulatory factor 4 (IRF4) for its development [12]. The T follicular helper (T_{FH}) cells are a subset of $CD4^+$ T cells which play a key role in supporting protective antibody responses derived from antigen-specific B cells [13].

1.1.3 The innate immune system in fish

Fish are important in the study of the immune system because they are the earliest known class of vertebrates to possess both innate and adaptive immunity. The adaptive immune system of fish is not as highly developed as in mammals therefore fish strongly rely on the innate system for protection [14].

Antimicrobial peptides (AMPs) are small molecules with a broad spectrum of potent immune activities against bacteria, fungi, parasites and viruses [15]. Their functions include microbicidal effects, recruitment of neutrophils and fibroblasts, promotion of mast cells degranulation, enhancement of phagocytosis and decreasing fibrinolysis, as well as the prevention of tissue injury by decreasing cytokine production and stimulating apoptosis of activated/infected cells [5, 15, 16]. While a number of AMPs have been discovered in fish (hepcidin [17, 18], cathelicidins [19, 20] and lysozymes [21, 22]) there still appears to be less diversity in comparison to other classes of vertebrates [5]. This is also true for other important components of the fish innate immune system, including chemokines. In contrast, the complement system of fish appears to contain a number of isoforms and polymorphisms of some of the important complement components that are structurally and functionally diverse [5, 23]. The diversity in the fish complement system could prove to be important in an evolutionary perspective with fish expanding their recognition capabilities of the innate immune system [24]. A number of cytokines that are known in mammals have also been found in fish and have been shown to have similar functions as in mammals (Tumor necrosis factor α (TNF α), IL-1 β , interferon's (IFNs)) [5, 24].

There are some differences between mammalian and fish receptors of the innate immune system. Fish possess some novel receptors that have not been found in mammals but behave in a similar manner functionally [5, 25]. Two of these receptors are non-specific cytotoxic cell receptors (NCCR) which behave similarly to natural killer (NK) cells of mammals and novel immune-type receptors (NITR) which are members of the Ig superfamily and have no known Ig homolog in mammals. A soluble immune-type receptor (SITR) has been found in carp and resembles CD300 found in mammalian species [26]. Carp SITR has been shown to be expressed abundantly in macrophages and be secreted upon stimulation with the protozoan parasite *Trypanoplasma borreli* [27]. Currently there are more than 17 different TLRs identified in numerous fish species and they share many functional properties with their mammalian equivalents [28]. Fish possess many of the same TLRs as mammals with some exceptions. A more detailed description of TLRs is present later in the introduction.

1.1.4 The adaptive immune system in fish

The initial emergence of both the innate and adaptive immune systems occurred in fish. There are a number of similarities between the mammalian and fish adaptive immune systems with most of the fundamental features present in fish. Some of the key similarities and differences will be highlighted here. One important difference is the immunological organs in fish which lack bone marrow, lymph nodes and Peyer's patches [29, 30]. Their immunological organs are the thymus, anterior (head) kidney, spleen and mucosa-associated lymphoid tissue.

A recent review of CTLs in teleost fish suggests the presence of CD8⁺ CTLs in fish similar to higher vertebrates (Nakanishi et al., in press [31]). This was based on previous messenger ribonucleic acid (mRNA) expression studies of T cell surface marker genes in alloantigen- or virus-specific effector cells, such as, T cell receptor α (TCR α) or β and CD8 α in ginbuna (*Carassius auratus langsdorfi*) and rainbow trout, and TCR α , β and γ in channel catfish (Nakanishi et al., in press [31]). There is also evidence of lymphocyte cell aggregation within teleostian gill epithelium in salmon [32] and expression of IL-4, IL-13 and GATA-3 in trout and salmon gills which may be indicative of a T_H2 response [33]. Macrophages appear to be the main APC in fish but dendritic-like cells (important APC in mammals) have been reported in some species [34-37]. Mammals have a diverse group of T cell subsets and in fish the expression of T cell receptors [38] has been shown in several species as well as functional studies showing the presence of different T cell types [34]. Phagocytic activity has been reported in B-lymphocytes in rainbow trout (*Oncorhynchus mykiss*) [39], Atlantic cod and Atlantic salmon (*Salmo salar*) [40]. In mammals, T_H17 cells are a subset of CD4⁺ T cells which produce the proinflammatory cytokines IL-17A-F [41]. Interleukin-17 was discovered in normal and stimulated kidney, spleen, gills and intestine of zebrafish (Kono et al., in press [42]) and five member of the IL-17 receptor family (IL-17RA-RE) were found in the teleost genome [41]. Interleukin-21 from rainbow trout up-regulated in immune-related tissues and was induced by both bacterial and viral infection which suggest an importance in host defense [43]. Interferon- γ , IL-10 and IL-22 which are signature cytokines for T_H1, T_H2 and T_H17-type responses were expressed after stimulation with IL-21 in trout [43]. To date, T cell population subsets have not been identified in fish due to a lack of functional assays and antibodies [41]. The MHC class I molecules bind foreign peptides produced by the degradation of intracellular pathogens and present them to cytotoxic T cells whereas MHC class II molecules bind and present foreign peptides derived from extracellular pathogens to helper T cells [44]. In

Atlantic salmon the MHC consists of only one expressed classical MHC class I locus (*Sasa-UBA*) and one classical class II alpha and class II beta haplotype (*Sasa-DAA/DAB*) [45]. The MHC class II has distinct class II haplotypes consisting of a combination of unique class II alpha and class II beta alleles [46]. Interestingly, Atlantic cod have not been shown to have MHC class II molecules [47] but are still able to produce an antibody response [48].

Immunoglobulins (antibodies), a key humoral component of the adaptive immune system, are present in teleosts as flexible, loosely associated tetrameric IgM, compared to the pentameric mammalian IgM [29, 30, 34]. IgM can also be present in fish serum in relatively high concentrations as natural antibodies, as was shown for Atlantic cod [49]. The B cell recognition ability may be increased due to a number of low molecular weight immunoglobulins which have been isolated from several fish species [29]. The fish immunoglobulin IgT [50] (also known as IgZ [51]) has been discovered but its functional significance was not known until a study showed the unique structure and functions of IgT in its specialized role in mucosal immunity [52]. Some fish species have a poor response in terms of specific antibody production [49] but studies have shown the ability of numerous fish species to mount a strong and specific antibody response in reply to various antigens, vaccine and bacterial challenges [30, 53-57].

1.2 Pathogen recognition receptors

1.2.1 Toll-like receptors overview

Toll-like receptors are PRR and key mediators of the innate immune system. They have been shown to recognize bacteria, fungi, protozoa and viruses and represent the host first line of defense sensing the invasion by pathogens and inducing the innate immune response [2, 4, 58]. TLRs recognize structural components of microbes called PAMPs which are not found in the host and therefore behave as non-self (examples include LPS, flagellin, viral single and double stranded ribonucleic acid (ssRNA, dsRNA), etc). Table 2 shows the known ligands of TLRs in fish and mammals. The Toll gene was initially identified in *Drosophila melanogaster* for its importance in embryogenesis in establishing the dorsal-ventral axis [59] but has also been found to play a role in the fly's immunity against fungal infections [60]. The first mammalian TLR discovered was TLR4 and it was shown to activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and the expression of inflammatory cytokines [61]. Later this TLR displayed hypersensitivity to LPS [62] and LPS was confirmed to be a ligand of TLR4 [63]. The majority of TLRs respond to PAMPs without the need of

additional adapter proteins, but some, like TLR4 require the interaction of LPS to its binding protein, lymphocyte antigen 96 (MD2) before forming a heterodimer with CD14 [64]. Not only do TLRs play a key role in activating the innate immune response, they also control multiple dendritic cell functions and activate signals that are critically involved in the initiation of the adaptive immune response [65]. A recent study [66] showed TLR2, 3, 5 and 9 ability to act as co-stimulatory receptors enhancing proliferation and/or cytokine production of T cell receptor-stimulated T lymphocytes, as well as the suppressive activity of CD4⁺ CD25^{high} T_{reg} cells by TLR2, 5 and 8. The field of TLR research is constantly revealing new discoveries which may prove to be important in developing immunotherapeutics for mammals and fish alike.

Table 2. Known key ligands of TLRs in fish and mammals. Adapted from Palti (in press [67]).

TLR	Fish species	Known ligands	
		Mammals	Fish
TLR1	Fugu, zebrafish	Lipopeptide; Pam ₃ CSk ₄	Unknown
TLR2	Fugu, zebrafish, Common carp	Lipopeptide; peptidoglycan; Pam ₃ CSk ₄	Lipopeptide; Pam ₃ CSk ₄
TLR3	Fugu, zebrafish	dsRNA; poly I:C	dsRNA, poly I:C
TLR3a/b	Common carp	N/A ^a	Unknown
TLR4	N/A	LPS	N/A
TLR4b.a/b	Zebrafish	N/A	Unknown
TLR5	Fugu, rainbow trout	Flagellin	Flagellin
TLR5b	Fugu, rainbow trout	N/A	unknown
TLR5S	Fugu, rainbow trout	N/A	Flagellin
TLR7	Fugu, zebrafish	ssRNA; R848	Unknown
TLR7a/b	Common carp	N/A	Unknown
TLR8	Fugu	ssRNA	Unknown
TLR8a/b	Zebrafish	N/A	Unknown
TLR8a1/2a	Rainbow trout	N/A	Unknown
TLR9	Fugu, zebrafish	CpG DNA	Unknown
TLR11	N/A	Profilin	N/A
TLR14	Fugu, zebrafish	N/A	Unknown
TLR19	Zebrafish	N/A	Unknown
TLR20a	Zebrafish, catfish	N/A	Unknown
TLR21	Fugu, zebrafish	N/A ^b	Unknown
TLR22	Fugu, zebrafish	N/A	dsRNA; poly I:C
TLR22a/b	Atlantic salmon	N/A	Unknown
TLR23	Fugu	N/A	Unknown

^a N/A- this gene has not been identified in this taxa group to date.

^b Recently, the chicken TLR21 was shown to recognize CpG DNA like the mammalian TLR9 [68].

1.2.1.1 Structure of TLRs

TLRs are type I integral member glycoproteins characterized by a cytoplasmic signaling domain and extracellular domains. The cytoplasmic domain is similar to the IL-1 receptor family and is therefore called the Toll/IL-1 receptor (TIR) domain. The extracellular domains are composed of leucine-rich repeats (LRR) each consisting of a β -strand and an α -helix connected by loops (Figure 1) and they are responsible for binding to PAMPs [64, 69, 70]. In human TLRs, the blocks of LRRs are usually bordered at the N and C termini by capping structures stabilized by disulfide bonds. Receptor cytoplasmic domains consist of a linker which connects the membrane to the TIR domain [71]. Currently the only TLR with a

solved crystal structure is human TLR3 [72], but the following TLR-complexes have been solved; TLR1-TLR2 heterodimer [73], TLR4-MD-2 complex with bound endotoxin antagonist eritoran [74] and mouse TLR3-ectodomains and dsRNA [75]. With no TLR5 structure solved the TLR5 ectodomain structure has been predicted to contain 20 LRRs, with five loops extending from the ascending or convex surfaces [76]. Ligand binding domains of TLR5 are described in chapter 1.4.2.

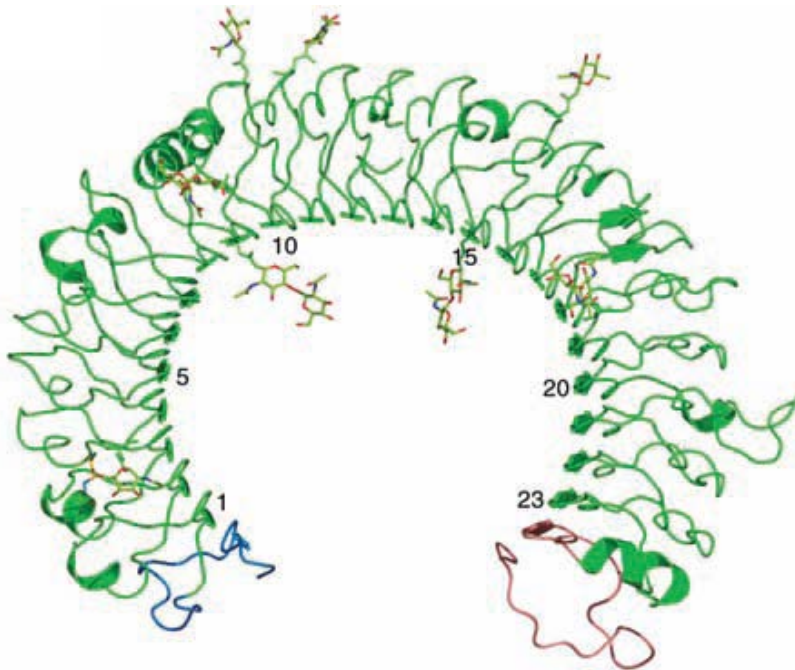


Figure 1. Overall architecture of TLR3 ectodomain in a ribbon representation. Adapted from Choe et al., 2005 [59].

The N-terminal cap region is colored blue; the 23 canonical LRRs are in green; and the C-terminal region is in pink. N-linked sugars (N-acetylglucosamines) that are observed in the electron density maps are shown in ball-and-stick representation, attached to their respective Asn residues. The disulfide bond linking LRRs 2 and 3 is drawn in orange, adjacent to the glycosylation site. Side view of TLR3 shown with the convex face pointing outwards, the concave face inwards, and the heavily glycosylated side face pointing toward the viewer. Since the crystal structure of TLR5 is not solved yet, the TLR3 is chosen to give an impression of TLR architecture.

1.2.1.2 Localization of TLRs

A wide range of innate and adaptive cell types can express TLRs including macrophages, dendritic cells (DCs), B and certain types of T cells [77]. The distribution of TLRs on various fish cells have not been easy to perform since there is a lack of specific cell determinants. The two main locations of TLR expression are the cell surface or endosomal surface (Figure 2). TLRs expressed on the cell surface include TLR4, 5, 6, 2, 1 and 10 and on the endosomal surface include TLR3, 7, 8 and 9. Interestingly, when looking phylogenetically at the extracellular domain of human TLRs, there is a clear clustering into two broad groups, the TLRs that respond to bacterial stimuli (located on cell surface) and those that recognize nucleic acid like molecules (located on endosomal surface) [67, 71]. The reason for this as

described by Werling et al. [64], is that “the positive selection was highest in the domain involved in ligand binding while regions involved in heterodimerization were shaped by purifying selection. Therefore, amino acids responsible for TLR-PAMP ligand binding are highly diverse”.

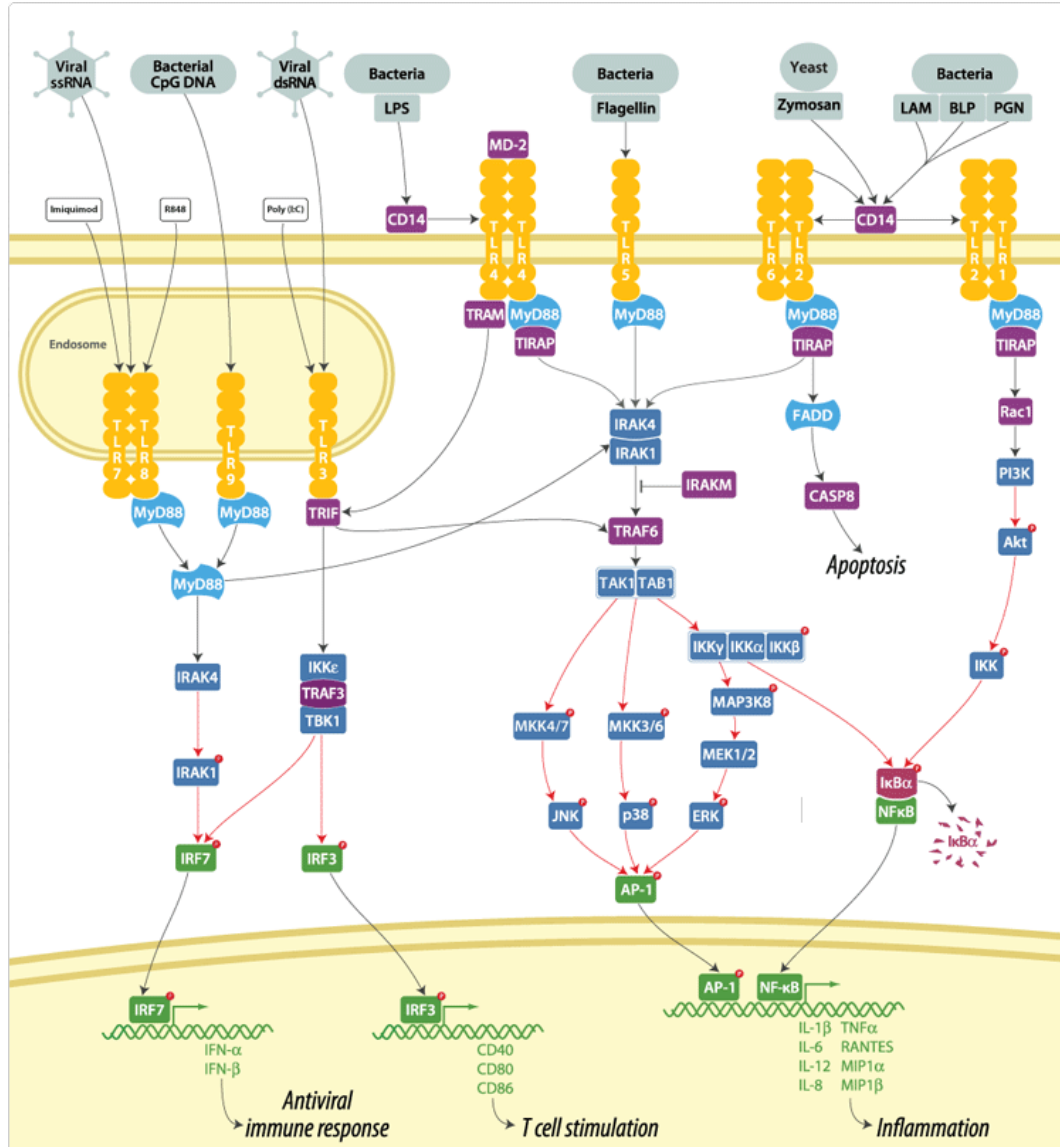


Figure 2. TLR ligands and signaling pathways. Adapted from <http://www.invitrogen.com>.

1.2.1.3 TLR signaling- MyD88-dependent pathway

TLR signaling is vital for the activation of both the innate and adaptive immune systems [65]. Initiation of TLR signaling pathways leads to the activation of the transcription factor NF- κ B and the mitogen-activated protein kinases (MAPKs), p38 and c-Jun N-terminal kinases (JNK) which then culminates in the induction of inflammatory cytokines, chemokines and co-stimulatory molecules (Figure 2). Adapter proteins such as the myeloid differentiation primary response gene (MyD88) are intracellular proteins that contain a C-terminal TIR

domain used for binding to the TIR domain of liganded TLRs and a N-terminal death domain which engages the death domain of downstream target interleukin-1 receptor-associated kinase (IRAK) [4, 77]. There are five adapter proteins discovered but I will focus on MyD88 due to its role in binding to TLR5 and because in general, it is known to bind to all TLRs except TLR3 [77]. Even though different ligands bind to a range of TLRs, they all engage the same adapter protein, but can activate distinct albeit overlapping genes. Some TLRs are completely dependent on one adapter protein and signaling pathway (TLR2 and TLR9), however, TLR4 is able to induce more than one type of adapter protein [4].

Once the TLR binds to MyD88, the IRAK family is stimulated (IRAK1, IRAK2, IRAK4 and IRAK-M) [58, 77-79]. After phosphorylation, the IRAKs dissociate from MyD88 and interact with TNF receptor associated factor 6 (TRAF6) which then associates with TGF- β activated kinase 1 (TAK1), TGF- β activated kinase 1/MAP3K7 binding protein 2 (TAB2) or NF- κ B inducing kinase (NIK). After a few intermediate steps, inhibitor of NF- κ B kinase complex (IKK) is activated and then phosphorylates the NF- κ B inhibitor (I κ B), inducing its degradation and the release of NF- κ B which controls the expression of various cytokines, chemokines, acute phase proteins and cell adhesion molecules for example, IL-1 β , IL-6, IL-8, CD80, CD86. The activation of TAK1 results in the activation of the MAPK pathways, p38 and JNK. JNK activates a number of important transcription factors including c-Jun, activating transcription factor 2 (ATF-2), Ets Like gene1 (Elk-1) and the nuclear factor of activated T cells whereas the p38 triggers Elk-1 and ATF-2. The MAPK pathways also culminate in the expression of various important inflammatory cytokines and chemokines.

1.2.1.4 TLR's in fish

Since the discovery of the first teleost TLR in goldfish stimulated macrophages [80], studies have been undertaken to elucidate TLRs role in the innate and adaptive immune systems of fish. The TLRs in fish share a number of similarities with their mammalian counterparts. Six major TLR families are present in all vertebrate taxa and they are shown as a phylogenic tree comparison of mammals and fish (Figure 3). These similarities may be due to strong selective pressure from pathogenic microbes which aided in preserving TLR recognition and signaling throughout vertebrate evolution [81]. Seventeen TLRs have been identified in more than a dozen teleost species (TLR1, 2, 3, 4, 5, 5S, 7, 8, 9, 13, 14, 18, 19, 20, 21, 22, 23) [5, 28]. There are a few key differences such as the detection of novel TLRs found only in fish such as, TLR5S, 13, 20-23. To date, TLR6 and TLR 10 have not been found in any fish species. Fish TLR3 has been shown to detect both viral and molecular patterns, in

contrast to the mammalian counterpart that detects only viral. TLR4 may have been lost in some fish species as it has yet to be identified in many species, or may function in the down-regulation of TLR activity in others. Some argue that TLR4 has been lost in the genome of most fishes as TLR4 genes found in zebrafish do not recognize the mammalian agonist LPS and are likely paralogous, not orthologous to mammalian TLR4 (Palti, in press [67]). Sepulcre et al. [82] showed that LPS signaled via a TLR4- and MyD88-independent manner in fish and that the zebrafish (*Danio rerio*) TLR4 orthologs negatively regulated the MyD88-dependent signaling pathway which may help the fish resistance to endotoxic shock. Atlantic cod TLR's are unique due to the absence of several TLR's that recognize bacterial surface antigens (TLR1, TLR2 and TLR5) [47]. They do possess TLR14 and TLR18 which are teleost-specific members of the TLR1 family and expanded TLR's that recognize nucleic acid (TLR7, TLR8, TLR9 and TLR22). This gives cod the highest number of TLR's in teleost to date and points to an increased role in the cod immune system on nucleic-acid detection to recognize pathogens.

Genome and gene duplications have contributed to the genomic diversity in fish (Palti, in press [67]). The distinct features and large diversity of teleost TLRs may have derived from their diverse evolutionary history and distinct environments in which they live. There are a number of duplicate multi-copy TLR genes identified including; TLR3 and 7 in carp, TLR4b, 5, 8 and 20 in zebrafish, TLR8a in rainbow trout and TLR22 in rainbow trout and Atlantic salmon (Palti, in press [67]). To date, direct ligand specificity has only been shown for TLR2, TLR3, TLR5M, TLR5S and TLR22 (Palti, in press [67]). Little functional analysis has been done on TLR in fish, with the main body of work stemming from gene expression studies.

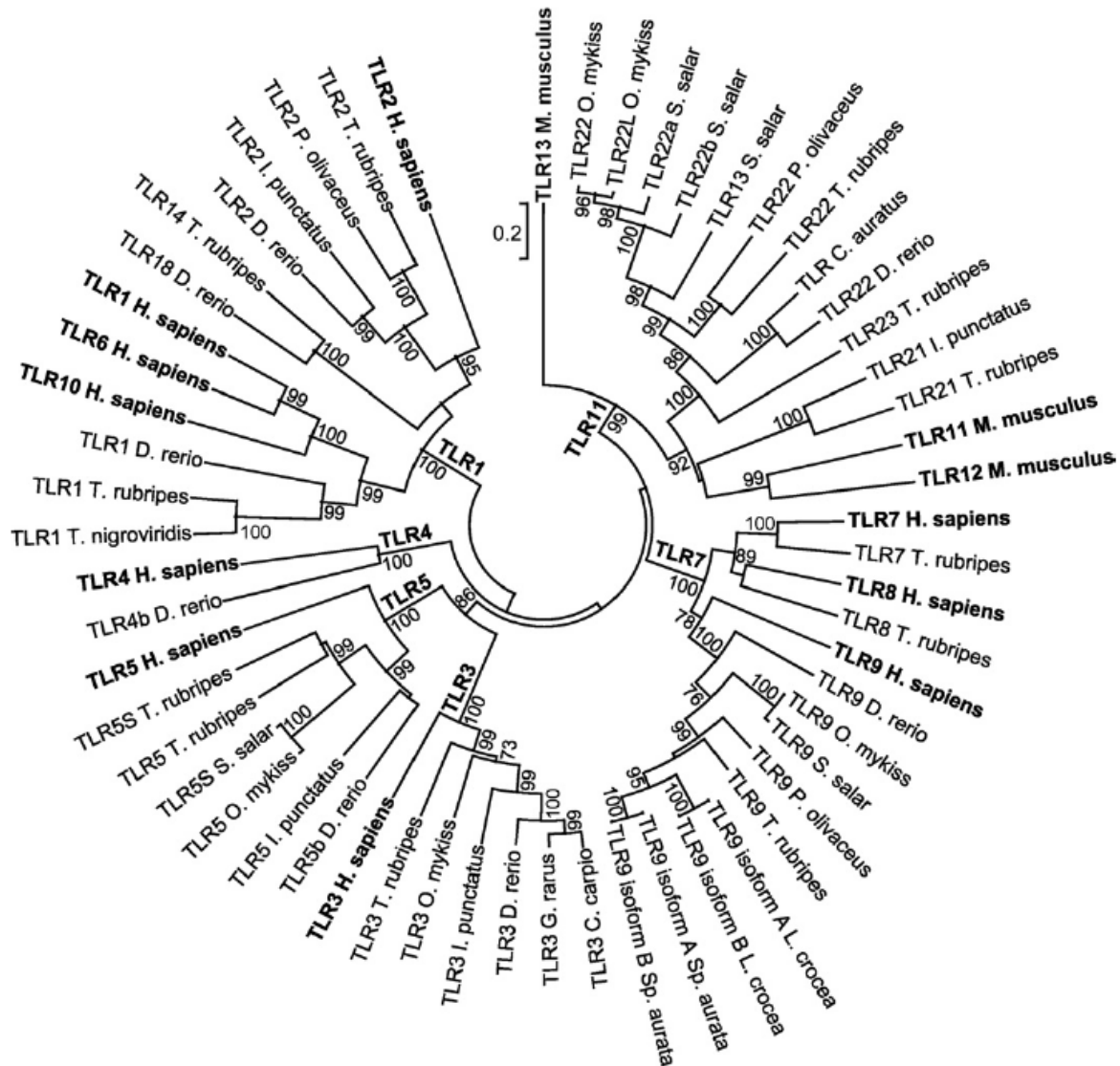


Figure 3. Circular phylogenetic tree of all known full-length fish TLR amino acid sequences and the orthologue mammalian TLR amino acid sequences. From Rebl et al., 2010 [25].

MEGA v3.1 [83] reconstructed a phylogenetic tree according to the Neighbor Joining method using the Poisson correction distance model. Only bootstrap values above 70% are indicated. The sequences were derived from *Carassius auratus* (TLR, accession number: AAO53555), *Cyprinus carpio* (TLR3, ABL11473), *Danio rerio* (TLR1, NP_001124065; TLR2, NP_997977; TLR3, NP_001013287; TLR4B, NP_997978; TLR5B, NP_001124067; TLR9, NP_001124066; TLR18, NP_001082819; TLR22, NP_001122147), *Gobiocypris rarus* (TLR3, ABL11471), *Ictalurus furcatus* (TLR3, ABH10661), *Ictalurus punctatus* (TLR2, ABD17347; TLR3, ABD93872; TLR5, ABF74618; TLR21, ABF74623); *Larimichthys crocea* (TLR9A, ACF60624; TLR9B, ACF60625), *Oncorhynchus mykiss* (TLR3, AAX68425; TLR5, BAC65467; TLR9, NP_001123463; TLR22, TLRI, CAF31506; TLR22L, TLRII, CAI48084), *Paralichthys olivaceus* (TLR2, BAD01044; TLR9, BAE80690; TLR22, BAD01045), *Salmo salar* (TLR5S, Toll-like leucine-rich repeat protein, AAV35178; TLR9, NP_001117125; TLR13, NP_001133860; TLR22A, CAJ80696; TLR22B, CAR62394), *Sparus aurata* (TLR9A, AAW81698; TLR9B, AAW81699), *Takifugu rubripes* (TLR1, AAW69368; TLR2, AAW69370; TLR3, AAW69373; TLR5, AAW69374; TLR5S, AAW69378; TLR7, AAW69375; TLR8, AAW69376; TLR9, AAW69377; TLR14, AAW69369; TLR21, AAW69371; TLR22, AAW69372; TLR23, AAW70378), *Tetraodon nigroviridis* (TLR1, ABO15772); *Mus musculus* (TLR11, AAS37672; TLR12, NP_991392; TLR13, NP_991389), and *Homo sapiens* (TLR1, NP_003254; TLR2, NP_003255; TLR3, NP_003256; TLR4, NP_612564; TLR5, NP_003259; TLR6, NP_006059; TLR7, NP_057646; TLR8, NP_619542; TLR9, NP_059138; TLR10, NP_001017388). Names from mammalian species are printed in bold face letters.

1.2.1.5 TLR5 in fish- membrane bound and soluble

TLR5 binds to flagellin which elicits recognition and response by the innate immune system [67, 84-86]. The types of TLRs found in fish are membrane-bound and soluble. Humans have the membrane-bound form of TLR5 but are lacking the soluble ortholog that has been found in fish such as pufferfish (*Fugu rubripes*) [87], rainbow trout [88], Atlantic salmon [89] and Japanese flounder (*Paralichthys olivaceus*) [90]. The membrane-bound form of TLR5 (TLR5M) consist of an extracellular domain containing leucine-rich repeats (LRRs), a transmembrane region and a cytoplasmic signaling domain, called the TIR domain whereas the soluble form of TLR5 (TLR5S) contains only the extracellular LRR domain [87, 88]. Rainbow trout TLR5M was shown to be ubiquitously expressed (muscle, testis, gill, stomach, intestine, kidney, heart, spleen, brain and liver) whereas TLR5S was predominantly expressed in the liver [88]. The organ expression distribution appears to vary between fish species as TLR5S in catfish was mainly expressed in the liver but was also present in the head kidney, spleen, gills, skin, ovary and brain [91]. A microarray study of Atlantic salmon infected with *Aeromonas salmonicida* showed up-regulation in the liver of an EST corresponding to the extracellular region of TLRs and most similar to TLR5 of rainbow trout and the TLR5S of pufferfish (*Takifugu rubripes*) [92]. Interestingly, both soluble and membrane-bound forms of TLR5 are absent in Atlantic cod [47].

Tsujita proposed that flagellin first induces the basal activation of NF- κ B via TLR5M, facilitating the production of TLR5S which then amplifies TLR5M cellular responses in a positive feedback loop which would enhance the innate immune response in fish [88]. When the TIR domain of human TLR5 is connected to the C-terminal end of TLR5S from rainbow trout (rtTLR5S) they signal the presence of flagellin which suggest that rtTLR5S recognizes flagellin in the fluid phase. Therefore the flagellin recognition properties of TLR5S are conserved along with the NF- κ B system between mammals and fish [86].

1.2.2 Nod-like receptors (NLR)

1.2.2.1 Overview

A new type of intracellular PRR has been discovered and is an exciting area of research for the innate immune system. The nucleotide-binding domain and leucine-rich repeat-containing family (NLR) are cytoplasmic receptors that are involved in regulating NF- κ B signaling, IL-1 β production and cell death [93]. In humans 22 NLR proteins have been identified and 33 in mice. The structure of NLR is tripartite containing a variable N-terminal

protein-protein interaction domain, a central NOD and a central leucine-rich repeat that detects PAMPs [93, 94]. In mammals NLRs are primarily expressed in immune cells (lymphocytes, APCs such as macrophages and DCs) but they can also be expressed in non-immune cells (such as epithelial and mesothelial cells) [93].

1.2.2.2 Naip5 and NLRC4

Recent studies in mice and humans describe two NLRs, NLR family caspase activation recruitment domain (CARD) domain-containing protein 4 (NLRC4) and neuronal apoptosis inhibitory protein 5 (Naip5), that are able to bind to cytosolic flagellin and contribute to the activation of the inflammasome and an inflammatory response, including caspase-1 and NF- κ B activation [95]. The Naip5 receptor specifically recognizes 35 amino acids at the carboxyl terminus of flagellin to trigger inflammasome activation [96]. The NLRs involved in flagellin recognition have not yet been identified in fish. Little research has been done on NLRs in teleost fish but NLRs have been identified in zebrafish, [97], channel catfish (*Ictalurus punctatus*, [98]) and grass carp (*Ctenopharyngodon idella*, [99]) and could prove to be important in the flagellin dependent immune response in salmonids.

1.4 Flagellin

1.4.1 Overview and Structure

Flagellin is the structural protein that forms the major portion of the flagellar filaments that contribute to virulence through chemotaxis, adhesion to and invasion of host surfaces [100, 101]. The bacterial flagellum is a locomotive organ that enables bacteria to swim by rotating a filament that is powered by a proton-driven rotary motor [102]. The flagellum is made up of three main parts; basal body (the motor), torsion hook and the helical hollow filament. Flagellar filaments are composed of 11 protofilaments which wrap around each other to form the filament. The filament is a tubular structure that is composed of up to 30 000 units of flagellin [102] and each flagellin protein ranges in size from 28-80 kDa [100]. There are four main globular domains (D) of the flagellin monomer (Figure 4). The D0 regions are conserved N- and C-termini regions that form packed α -helical structures that flank the central region and are positioned in the filament core along with the D1 region. The D1 region is also composed of α -helical structures, is highly conserved and known to be involved in TLR5 signaling. The D2 and D3 regions are hypervariable (in residue composition and size) and are composed of a β -sheet folded structure on the filament outer surface [100, 102, 103].

The first structure of flagellin that was crystallized was the F41 fragment (missing 52 N- and 44 C-terminal residues) of *Salmonella typhimurium* [104]. Due to flagellin's strong tendency to polymerize, it has been difficult to crystallize which was why the N- and C-terminal residues were removed. More recently, a cell-surface flagellin from *Sphingomonas* sp. was crystallized bound to a polysaccharide and results showed that residues 20-40 and 353-363 were responsible for alginate binding [105]. Both structures were solved at 2.0-Å resolution and had similar α -helical D1 domains but differences in the β -domain.

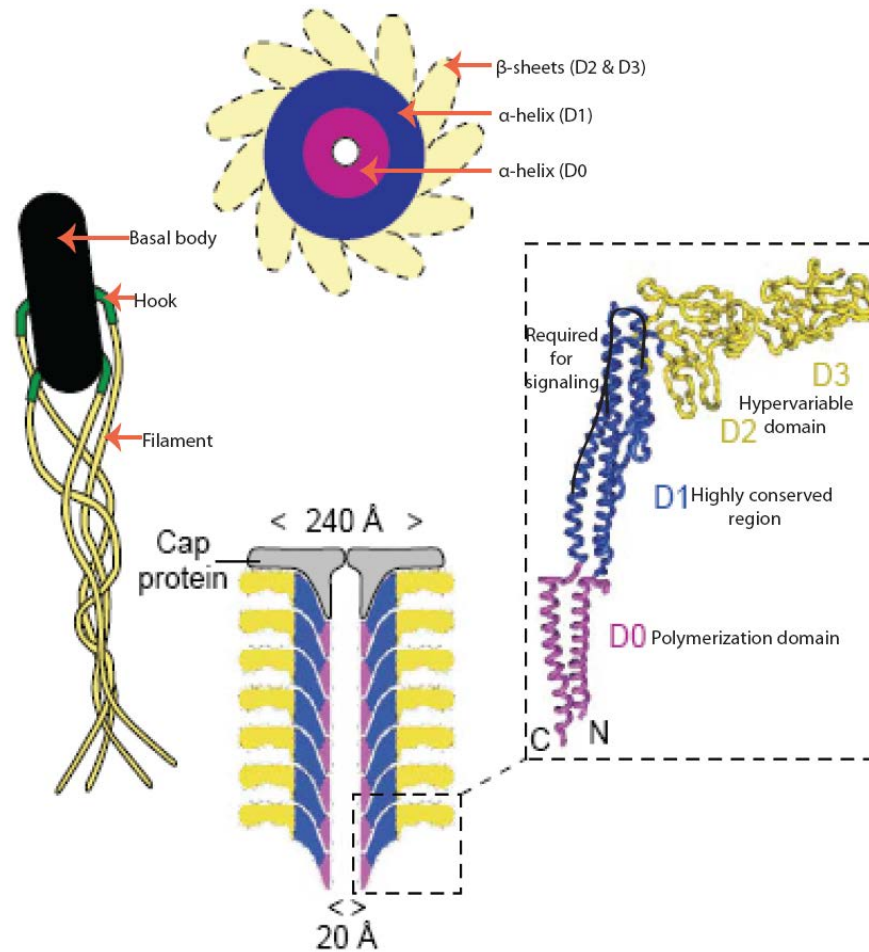


Figure 4. Structure and organization of flagellum and flagellin * Adapted from: Ramos et al., 2004 [100], Honko and Mizel, 2005 [106].

Flagella structures protruding from bacteria comprise a hook (dark green) and a filament, referred to as a flagellum (yellow). Schematic transversal and longitudinal views of the flagellum and ribbon diagram of the $C\alpha$ backbone of flagellin from *Salmonella enterica* serovar Typhimurium. The flagellin domains are colour coded as follows: the terminal α -helix chains (D0, purple), the central α -helix chains (D1, blue), and the hypervariable region with β -sheets (D2 and D3, yellow). The concentric circles on the end-on view show the organization of domains within the flagellum. The α -helix regions (purple and blue), necessary for filament architecture and motility functions, are embedded in the flagellum inner core. Flagellin monomer is the molecular pattern that is detected by innate receptors. In the monomer, flagellin D0 terminal chains are totally disordered, whereas the D1, D2 and D3 domains remain compactly folded. Both α -helix chains of D1 domain (blue rectangle) are required for TLR5 signaling in mammals, suggesting that a flagellin conformation is detected by TLR5.

1.4.2 Binding to TLR5

The recognition and binding of flagellin to TLR5 is known to occur in many species and produce both innate and adaptive immune responses. In 2003, two studies reported the location of the binding site on flagellin that recognizes TLR5. Jacchieri et al. [107] used a search for complementary hydrophathy between the sequences of TLR5 and flagellin to predict the binding site location to the 88-97 chain fragment of flagellin (*S. enterica*) which is located in the α helix. Using deletional, insertional and alanine-scanning mutagenesis, Smith et al. [103] mapped the location of the TLR5 recognition site on flagellin to a cluster of 13 amino acid residues in the D1 domain that participate in intermolecular interactions within flagellar protofilaments and that are required for bacterial motility. Filamentous flagellin reduced TLR5 stimulatory activity by 96% compared to that of the monomeric form. This could be due to the D1 domain being buried in polymerized filamentous flagellin and exposed in monomeric flagellin, which suggest that TLR5 recognizes flagellin monomers that are released on depolymerization of flagellin polymers [103]. Flagellin was also shown to co-precipitate with TLR5, indicating a close physical interaction between the protein and receptor [103]. The location of the TLR5 binding site was examined using truncation studies that demonstrate that the flagellin-binding site in TLR5 is located between residues 386 and 407, placing it in LRR14 [108]. The sequence of LRR14, which is 32 residues in length, reveals a six-residue insertion after position 15. Based upon the truncation data, it is probable that this insertion contributes to flagellin binding [76].

1.4.3 Immune response to flagellin

Flagellin is the only known TLR PAMP that is purely protein, which allows for research into its specific immune responses and for its manipulation for use in adjuvant research. Mammalian TLR5 has been shown to recognize both gram-positive and gram-negative bacterial flagellin and activate the NF- κ B pathway and TNF- α production [109]. In MyD88 positive mice stimulated with flagellin, the animals were shown to produce IL-6 but MyD88 knock-out mice did not respond to flagellin stimulation [109]. This provides evidence of the innate immune response to flagellin and the signaling pathways involved. Numerous studies in mammals have tested the adjuvant abilities of flagellin, as fusion proteins (flagellin bound to an antigen) or flagellin and the antigen used separately (reviewed in [84]). Flagellin is able to promote important innate immune processes which are vital to the development of an adaptive response. These processes include induction of proinflammatory cytokines and

chemokines by lymphoid and non-lymphoid cells, the generalized recruitment of T and B lymphocytes to secondary lymphoid sites, DC activation and direct activation of T lymphocytes [84]. In mice, co-administration of monomeric flagellin expressed in *Escherichia coli* or polymeric flagellin isolated from *Salmonella* with inactivated influenza virus resulted in production of specific antibodies titers, that induced full protection against highly lethal challenge by homologous virus (in comparison to partial protection from unadjuvanted inactivated virus) and proinflammatory cytokine production (IL-4, MIP-2, TNF α , IL-6 and IFN γ) [110]. A fusion protein vaccine containing type A and B flagellin proteins linked to the outer membrane proteins OprF and OprI from *Pseudomonas aeruginosa* were successful in clearing mice of non-mucoid *P. aeruginosa* and elicited robust antigen and flagellin specific IgG responses in mice and African green monkeys [111, 112].

The use of flagellin for vaccines or immunostimulants has not been widely studied in fish. A study in salmon tested recombinant subunit vaccines produced from *Piscirickettsia salmonis* for protection from rickettsial septicaemia [113]. Three different combinations of recombinant proteins (including different flagellin proteins) were emulsified with Freund's incomplete adjuvant (FIA) and tested for efficacy. The highest relative survival (95%) was in the vaccine that included the flagellar protein FlgG and two heat shock proteins (Hsp60 and Hsp 70) [113]. The results suggest that not all flagellin proteins produce the same protective immune response and that the combination of proteins in the vaccine formulation may be important. McGee et al. [114] showed that like the flagellin protein FlaA, FlaD and FlaE also showed evidence of being involved in the virulence of *Vibrio anguillarum*. It is possible that the roles the protein plays in the bacterium may give clues to the immune response produced when injected into host species. A study in red snapper (*Lutjanus sanguineus*) vaccinated with the recombinant protein FlaC from *Vibrio alginolyticus* showed the production of specific antibodies and high resistance to infection by *V. alginolyticus* [115]. Jiao et al. have studied the immunoprotectivity of the FliC from *Edwardsiella tarda* in both recombinant protein and as a DNA vaccine constructs injected into Japanese flounder [116, 117]. They found that the most promising vaccine candidate was a chimeric DNA vaccine of the antigen Eta6 or Et18 fused in-frame to FliC. These constructs produced significantly higher levels of protection than just the antigen alone as well as serum specific antibodies and genes involved in innate and adaptive immunity (IL-1 β , IFN, Mx, CD8x, MHC Ia, MHC IIa, IgM).

1.5 Vaccines in Aquaculture

1.5.1 Overview

Vaccination is the administration of antigenic material to stimulate an adaptive immune response. Vaccines can prevent or ameliorate the effects of infection by a pathogen. The use of vaccines in aquaculture has caused the decreased use antibiotics and losses associated with disease [1, 118]. This has allowed for a three-fold increase in fish production from 1985 to 2003 in the Norwegian aquaculture sector [1]. It is estimated that 10% of all cultured aquatic animals are lost due to infectious diseases which amounts to a global loss of 8-10 billion USD annually [119]. While a number of effective bacteria vaccines are available for many fish species, there is still a need for more efficacious viral and parasite vaccines. Recent advances in fish genomics and proteomics have opened new avenues for research into novel vaccines.

1.5.2 Administration

Fish can be vaccinated by injection, immersion or orally. The easiest method to use is oral vaccination whereby the antigen is included in the feed. However this method has had poor and inconsistent results due to antigen degradation in the gut and requires more advanced delivery methods such as poly-(lactic-co-glycolic) (PLG) formulations, liposomes or alginate beads [1, 120]. Immersion of the fish in a diluted vaccine solution is easy for small fish and provides good protection for live, attenuated vaccines, but less for inactivated vaccines. This method is easy but can be cost prohibitive due to the large amount of vaccine needed. The highest level of protection comes from injection vaccination whereby the vaccine is injected into the body cavity of the fish. This method requires a smaller amount of vaccine therefore can be cost effective and each fish is injected with the same amount of vaccine. The challenge with this method is that it is a labor-intensive process, stressful for the fish and the fish must be over a certain size [1, 119, 120].

1.5.3 Vaccine types

Currently, the most used type of vaccine in the aquaculture industry is the inactivated vaccine. PHARMAQ are worldwide producers of fish vaccines and have developed a multi-valent inactivated vaccine that is effective against furunculosis, vibriosis, coldwater vibriosis, winter sore and IPN¹. This multi-valent vaccine had annual sales for salmon reported in July

¹ <http://www.pharmaq.no/Products/Ject/index.html>. Information retrieved on June 1, 2011.

2010 of 236 million doses and this is expected to be much higher for 2011². PHARMAQ also have other inactivated vaccines available for the following diseases; pancreatic disease, infectious salmon anemia (ISA), salmonid rickettsial septicaemia, pasteurellosis and flavo bacteriosis². This type of vaccine is advantageous because it is cost-effective, easy to produce and has shown to be very effective in a number of bacterial diseases. A study testing the efficacy of oil-adjuvanted inactivated vaccines from *V. anguillarum* and *A. salmonicida* in Atlantic cod showed high levels of protection against homologous challenge with 94 and 77% relative percent survival (RPS) respectively [121].

Live attenuated vaccines are created by reducing the virulence of a pathogen, altering it so that it becomes harmless or less virulent [122]. This type of vaccine has potential for use in aquaculture but there are a number of safety concerns present, such as persistence in the fish and the environment, reversion to virulence and risk of spreading to non-target animals [120]. Attenuated vaccines have been successful for some bacterial and viral diseases (ex, infectious hematopoietic necrosis virus, IHNV) but they still have problems with safety issues and government restrictions [1], therefore none are currently commercially available in Norway.

DNA vaccines contain a genetically engineered, small, circular piece of DNA (plasmid) that is injected into a host to produce one or two specific protein antigens from a pathogen in order to elicit an immune response [123]. Nucleic acid vaccines have many characteristics that make them superior to classical vaccines. Among these characteristics are vaccine stability at ambient environmental temperatures, thus removing the necessity for a cold chain for vaccine storage, lack of any living organism in the formulation, removal of the possibility of reversion of that organism to pathogenic status and the lack of tissue damaging adjuvants [124]. This treatment has been shown to be effective against a number of viruses including channel catfish virus [124], viral hemorrhagic septicemia (VHS) [125] and IHNV [126], infectious pancreatic necrosis virus (IPNV) [127] and the bacterium *Renibacterium salmoninarium* [128]. DNA vaccine use is limited due to governmental regulations and safety risks, such as, the integration into chromosomal DNA, pathological processes at the site of infection, distribution to internal organs and longevity of retention of foreign DNA in these organs [119, 123, 129].

Recombinant subunit vaccines contain specific recombinantly produced protein antigens and are therefore advantageous from a safety point of view as they cannot invade the

² http://www.pharmaq.no/Statistics/Vaccines_Norway_2011-04_EN.pdf. Information retrieved on June 1, 2011.

host genome or replicate in the environment. They can be difficult to produce as is the case for a number of viral membrane antigens and generating the correct monomeric conformation can be a challenge. There have been problems associated with effective protection from subunit vaccines against IHNV most likely due to incorrect folding of the expressed proteins, but promising results in combating IPNV [129, 130]. There are no commercial DNA or subunit vaccines currently available in Norway but research is underway to develop more effective and safe formulations.

1.5.4 Use of microarray in fish vaccine studies

The profiling of immune responses during fish vaccination is in its infancy and there are only a few numbers of scientific attempts that have addressed direct molecular correlates of protection other than antibody responses and up-regulation of certain transcripts. To find molecular signatures that may be acknowledged as correlates of protection one should not only analyze single genes but also assess transcriptomics [131]. There may be molecules that have no apparent immunological function but probably is quite involved in disease protection [131]. In recent years, cDNA microarrays have been developed for species such as, zebrafish, salmonids, catfish, sea bream, halibut and Atlantic cod. They can be used for investigations on cell biology, reproduction, nutrition, physiology, stress, effects of environmental pollution and immunology [132, 133]. Transcriptomics is emerging as a powerful tool in the development of effective vaccines in the aquaculture industry. Both immune-related genes and genes not known to be involved in the immune response can simultaneously be measured to provide a broad view of the response seen in fish. The use of microarrays for this purpose has not been done in a large number of studies. To my knowledge, there is only one study that has addressed the “correlates of protection” issue in fish. A study that investigated low and high resistant salmon that were vaccinated and challenged by *A. salmonicida* concluded that the efficiency of vaccination against furunculosis depends largely on the ability of host to neutralize the negative impacts of immune responses combined with efficient clearance and prevention of tissue damages [134]. They were able to use microarray and QPCR to determine the mechanisms involved in resistance to the disease and the specific genes that had marked expression differences between high and low resistance that can be considered as positive and negative correlates of vaccine protection against furunculosis. A review paper by Aoki et al [135] outlines microarray studies that were successful in measuring differences in responses of viral and bacterial vaccinated fish and highlights the importance of this technique for the discovery of immune relevant genes.

1.5.5 Adjuvants

The term adjuvant stems from the latin word “adjuvare” meaning to help and its role in vaccine development is dependent on its ability to stimulate or prime the immune system [136]. This allows for a more efficient and effective response to the antigen. Many of the vaccines currently available for salmon are inactivated vaccines which are preferred due to safety but they tend to lack immunogenicity and therefore require the use of adjuvants [119]. Generally, adjuvants function in several ways including; (1) increasing the immunogenicity of weak antigens, (2) enhancing the speed and duration of the immune response (depot effect), (3) modulating antibody avidity, specificity, isotype or subclass distribution, (4) stimulating CTL, (5) promoting the induction of mucosal immunity, (6) enhancing immune responses in immunologically immature or senescent individuals, (7) decreasing the dose of antigen in the vaccine to reduce costs, (8) helping to overcome antigen competition in combination vaccines, (9) improve antigen presentation and (10) reduce need for booster immunizations [136-138]. Many of the vaccines used for fish use oil-based adjuvants but in humans, oil adjuvants cannot be used due to severe side-effects therefore aluminum-based adjuvants are used which despite poor adjuvanticity, are safe and produce a T_H2 biased response [139].

Promising results using flagellin as an adjuvant have been shown in previous research in mice [110, 111, 140-142], monkeys [112] and Atlantic salmon [113] which demonstrated effective immune responses and increased survival in challenge studies during and after flagellin treatment. Flagellin is also an attractive candidate for use in human vaccines as was shown in a recombinant hemagglutinin influenza-flagellin fusion vaccine in elderly patients [143]. It is effective at very low doses, does not promote IgE responses, prior immunity to flagellin does not impair its adjuvant activity, antigen sequences can be inserted at the amino or C terminus or within the hypervariable region of the protein without any loss of flagellin signaling via TLR5, has no detectable toxicity in rabbits when given intranasally or intramuscularly and it can easily be made in fairly large amounts under good manufacturing practices conditions [84].

1.5.5.1 Modes of action

Despite many years of research into the mode of action of adjuvants, little is currently known. Janeway has referred to adjuvants as “immunology’s dirty little secret” due to the inability of antigens to induce a robust immune response in the absence of an adjuvant [144]. Understanding how adjuvants function is key to producing more specific vaccine components that can more accurately direct the immune response and increase efficacy. With the

propensity for adverse side-effects from oil-based adjuvants, focus has been placed on TLR agonists as new candidates for adjuvants. The activation of TLRs directs an increased number of innate immune cells at the infection site, along with production of proinflammatory cytokines and chemokines, resulting in the induction of antigen-specific adaptive immune responses [65].

There are three types of adjuvants which can induce three signals either alone or in combination and they are described by Barr et al. [139] and Guy [136] and shown in Figure 5. Signal 0 is induced mostly through the recognition of PAMPs by PRRs and increases antigen uptake and presentation by APCs. Signal 1 is activated by specific recognition of peptides presented by MHC II molecules to the TCR and improves antigen presentation, but must be accompanied by a co-stimulatory signal to prevent anergy. Signal 2 is the co-stimulatory signal with the receptor-ligand interaction occurring between APCs and T-cell antigens. Type A adjuvants can act on signal 0 and indirectly on signal 2 to activate APCs and induce secretion of cytokines. Type B adjuvants target APCs or favor antigen capture and they act solely on signal 1. Adjuvants that directly stimulate signal 2 are type C and are specific ligands of co-stimulatory molecules. TLRs are special in being able to stimulate all three modes of action for adjuvants and are therefore of high interest for adjuvant research.

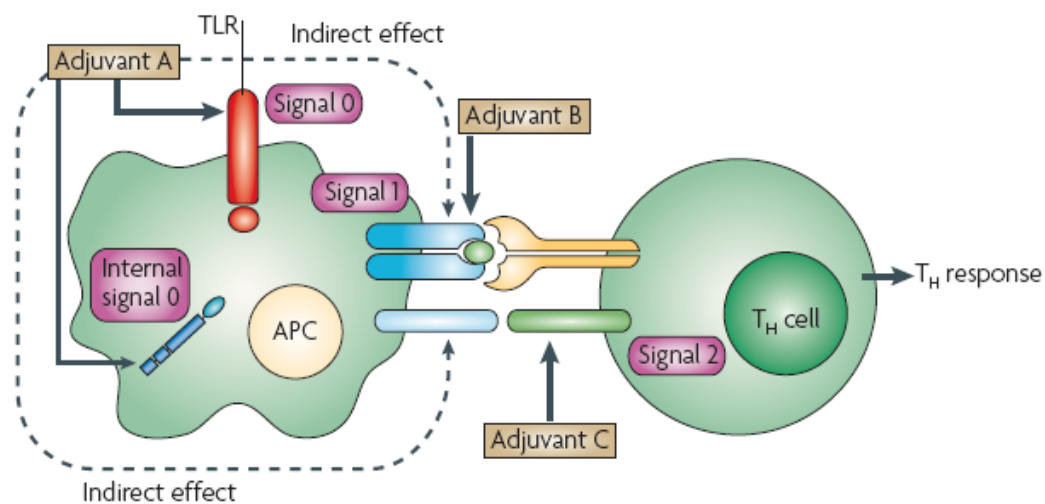


Figure 5. Where do adjuvants act? From Guy [120].

The initiation of T helper cell responses requires three signals; signal 0, signal 1 and signal 2. In theory adjuvants can act alone or in combination on each of these three signals and an adjuvant acting on each signal is shown. Some specific adjuvants, such as TLR agonists, can be considered as type A adjuvants: they act on signal 0, and indirectly on signal 2, by activating APCs and triggering the secretion of cytokines. TLR agonists can act on signal 1 by favoring efficient presentation of the co-administered antigen. Some TLR agonists also directly trigger signal 0 on regulatory T cells and B cells expressing some of the corresponding receptors. Adjuvants targeting APCs or favoring Ag capture can be viewed as type B adjuvants acting on signal 1, as their effect is eventually mediated by enhanced Ag presentation to T cells. Targeting signal 1 is not sufficient and an immunostimulatory signal should be co-delivered for an optimal response. Some specific ligands of co-stimulatory molecules can directly enhance signal 2, acting as type C adjuvants; such compounds must however be used with caution.

1.5.5.2 Side-effects

Problems associated with adverse adjuvants effects are well known in vaccine design and have been shown to occur in numerous fish species following vaccination with oil-based vaccines. Midtlyng and Lillehaug [145] showed that salmon injected into the intraperitoneum with oil-adjuvanted vaccines had retarded growth which was associated with increased severity of abdominal lesions. A series of studies by Mutoloki et al. of oil-adjuvanted vaccines have further elucidated the adverse reactions seen in salmon, rainbow trout and Atlantic cod. Briefly, oil adjuvants must be injected with antigen to produce a strong inflammatory reaction [146] and persistent antigens at the injection site perpetuate the inflammatory reaction, leading to adverse side-effects, including granulomas and intra-abdominal lesions [55, 147-149]. A study involving the comparison of aluminum and FIA in Japanese flounder showed that while the FIA resulted in higher percent of survival, more severe intra-abdominal adhesions were also produced [150]. The consistent development of side-effects in bony fish after administration of oil-adjuvanted vaccines highlights the need for more sophisticated adjuvants in the aquaculture industry. Autoimmune responses have also been attributed to the use of oil-adjuvants or/and formulation [151-153].

2. Aims of Study

Many vaccines currently on the market include oil-based adjuvants that are known to cause adverse side-effects in fish. There is a need for new adjuvants which can provide effective immune system stimulation with minimal negative effects. The general purpose of this study was to produce recombinant flagellin for examination of the innate immune response after immunization of Atlantic salmon.

The major aims were:

1. To clone, express and purify recombinant flagellin from *Vibrio anguillarum*.
2. To produce a polyclonal flagellin antibody.
3. To assess *in vitro* NF- κ B responses of COS-7 cells after stimulation with flagellin.
4. To examine the immune response of Atlantic salmon after injection with flagellin by ELISA, QPCR and microarray.
5. To examine the immune response of Atlantic salmon after injection with a combination of flagellin and a model antigen (LPH or OVA).

3. Abstract of papers

Paper I. Immune response of Atlantic salmon to recombinant flagellin

Many viral vaccines used in aquaculture are unable to stimulate an appropriate level of immunity to withstand infection. By targeting specific components of the immune system it may be possible to trigger stronger, more effective responses to antigens. Flagellin has the ability to stimulate both the soluble and membrane-bound forms of toll-like receptor 5 (TLR5) in salmon leading to a proinflammatory response and activation of both the innate and adaptive immune system. In this study flagellin (FlaD from *Vibrio anguillarum*) was recombinantly produced in two forms, full-length (FDL) and a truncated form (FDS) with portions of the N- and C-termini removed to prevent polymerization. FDS was used to produce an antibody that was able to bind both forms of flagellin in immunoblot analysis. In cell culture using COS-7 cells, FDL was shown to stimulate the NF- κ B pathway more effectively than FDS. Both forms of flagellin were used as an adjuvant with the antigen LPH (Hemocyanin from *Limulus polyphemus* hemolymph) in an immunization dose response study. FDS and FDL stimulated the innate immune system of salmon inducing proinflammatory effects on days 2, 4 and 7 and the gene expression of important cytokines such as TNF α , IL-6, IL-8, and IL-1 β were significantly up-regulated ($p < 0.05$) in the spleen. TLR5S was more highly up-regulated than TLR5M indicating that the soluble form of TLR5 may play an important role in the innate immune response in salmon. ELISA analysis showed that the use of flagellin as an adjuvant with LPH was not able to significantly induce flagellin or LPH antibodies. This study shows that flagellin has the potential to be a highly effective adjuvant for salmon immunization, but further research is needed.

Paper II. Functional Genomics Analysis of the Immunological Responses of Atlantic salmon (*Salmo salar*) Spleen to Injection with Recombinant Flagellin from *Vibrio anguillarum*

The use of vaccines in salmon aquaculture has been successful in controlling many diseases in the industry and has aided in the decreased use of antibiotics. There are still challenges in creating vaccines against diseases caused by intracellular pathogens therefore a need exists for new vaccine components and adjuvants. Flagellin has been focused on in mammalian research because of its important role in binding to the innate immune receptor TLR5 and has produced promising results in vaccine studies. This study was conducted to examine the effect of intraperitoneal injection of recombinant flagellin on Atlantic salmon spleen transcript expression using DNA microarray hybridizations and QPCR. Four injections were compared: FlaD (purified recombinant FlaD protein from *Vibrio anguillarum*), FlaD+OVA (combination of FlaD and ovalbumin protein), OVA (ovalbumin alone) and saline control. The design of the microarray experiment used a pooled RNA approach to identify transcripts that were reproducibly differentially expressed in FlaD, OVA or FlaD+OVA compared with saline injected controls 2 days post injection (2DPI) or 7DPI. These time points allowed an expression analysis of innate defence genes. Some genes exhibited early, delayed or extended response over the sampling time and flagellin seemed to be the key component to eliciting a response. Microarray results showed that flagellin injection caused significant up-regulation of inflammatory cytokines, chemokines and receptors (IL-8, TNFRSF11B, IL-1R), antimicrobial peptides (hepcidin, cathelicidin), complement genes (complement component C7 and C7-1), immune genes (C/EBP, thioredoxin, C-type lectin receptor B), peptidases (MMP-9) and genes involved in the Ras/MAPK pathway (Ras-related protein, Ras protein activator) in salmon. Down-regulation occurred in some important immune genes (LITAF, Mx1 and clusterin-1). A selection of up-regulated genes from the microarray studies was also validated by QPCR using individual samples to assess biological variability. Flagellin was shown to induce the innate immune response in Atlantic salmon.

4. Discussion

The search for effective adjuvants for the aquaculture industry is an important area of research in vaccine design. Improved adjuvants could aid in the immune response to increase survival while minimizing adverse side-effects. Flagellin is a pathogen-associated molecular pattern (PAMP) that is known to stimulate toll-like receptor 5 (TLR5) to initiate an innate immune response [67, 84-86]. Much of the recent focus has been placed on flagellin in vaccine studies with many promising results [110-113, 140-142]. This thesis was the first to inject purified soluble recombinant flagellin protein into Atlantic salmon and study the immune response by microarray and QPCR analysis.

In paper I, two forms of soluble recombinant flagellin protein were produced; a truncated form (FDS) to avoid polymerization and a full-length (FDL) form. Both were used in a luciferase reporter gene assay to evaluate binding and activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) response with FDL showing a higher response than FDS. As FDS was easier to produce recombinantly, it was selected for antibody production in rabbit and the antibody was shown to bind to both forms of flagellin in immunoblot analysis. A dose response study was performed with Atlantic salmon injected intraperitoneally with two forms and doses of flagellin and hemocyanin from *Limulus polyphemus* hemolymph (LPH) as the antigen. Spleen samples were analyzed for mRNA gene expression using quantitative real time polymerase chain reaction (QPCR) with focus on innate immune genes. Enzyme-linked immunosorbent assay (ELISA) was performed to measure the anti-flagellin and anti-LPH antibody response, with low antibody levels measured in all groups tested. Several important innate immune genes were significantly up-regulated by flagellin and the inclusion of the antigen LPH extended the response of some cytokines.

Based on the results from paper I, the truncated form of flagellin (FDS) was used in paper II (FlaD). FlaD was injected intraperitoneally into salmon and spleen samples taken 2 days post injection (DPI) and 7DPI. With 27,917 Atlantic salmon and 4,065 rainbow trout cDNA features, the consortium for Genomic Research on All Salmonids Project (cGRASP) 32K salmonid complementary DNA (cDNA) microarray [154] allowed for further exploration of a variety of genes. Flagellin alone and in combination with the model antigen ovalbumin (OVA) were injected into the peritoneum of salmon and this resulted in numerous immune response genes reproducibly up-regulated. Some genes exhibited early, delayed or extended response over the sampling time and flagellin seemed to be the key component to eliciting a

response. Microarray results showed that flagellin caused significant up-regulation of inflammatory cytokines, chemokines and receptors, antimicrobial peptides, complement genes, peptidases and genes involved in the Ras/MAPK pathways. Down-regulation occurred in some important immune genes, such as Mx1, clusterin 1 and LITAF. Microarray gene lists were validated using QPCR of selected genes from the microarray results.

The results of the present study are discussed thoroughly in papers I-II. Selected topics will be discussed further here. It should be noted that due to difference in the abbreviations used between papers, in this discussion samples days will be referred to as days post injection (DPI) and flagellin proteins are truncated (FDS) and full-length (FDL) as in paper I and as FlaD in paper II.

4.1 Recombinant production of flagellin protein

In flagellated bacteria, flagellin is an important structural protein in the locomotive organ flagellum. It is important in subunit vaccine research due to its role in binding to the pathogen recognition receptor (PRR) TLR5, which causes an increased immune response in mammals and fish [67, 84-86]. Producing a purified form of flagellin from fish pathogens is key to investigating the immune response in salmon. The flagellum is composed of multiple flagellin proteins (e.g. FlaA, FlaE, FlaD), which vary in size, function and number of flagellin proteins between species. Two fish pathogens were used in this study; *V. anguillarum* (causing classical vibriosis) and *Aliivibrio salmonicida* (renamed from *Vibrio salmonicida*) (causing cold water vibriosis). All of the flagellin proteins (FlaA-E from *V. anguillarum* and FlaA-F from *A. salmonicida*) began the trial in both full-length and truncated forms from each species of bacteria. Truncated forms of the flagellin proteins with portions of the N- (1-47) and C- (337-377) termini removed, were chosen for expression based on the first successful crystallization of flagellin [104] which used a truncated form of the *Salmonella typhimurium* flagellin protein to avoid polymerization of the protein aiding in the production of crystals. There were proteins from both pathogens and of both lengths that expressed successfully in *Escherichia coli* BL21 (DE3) cells harboring pET151/D TOPO flagellin constructs in expression trials but FlaD from *V. anguillarum* was chosen for injection trials due to its role in virulence and adhesion [114] and because both the full-length and truncated forms of FlaD expressed in soluble forms.

There were some challenges associated with protein expression trials. When expressed at any temperature above 15°C, the proteins were produced in insoluble form so the temperature used for greatest production in soluble form was 13°C. *A. salmonicida* flagellin genes were more difficult to clone than genes from *V. anguillarum*. The *A. salmonicida* genes (full-length *flaE*, *flaB* and truncated *flab*) either did not express or expressed in low amounts in insoluble form, and were therefore not used in the study. Any protein used for vaccines in aquaculture should be relatively easy and inexpensive to produce. In comparison, seven *V. anguillarum* flagellin genes (truncated *flaB*, truncated *flaA*, full-length *flaC*, truncated and full-length *flaE* and truncated and full-length *flaD*) from both full-length and truncated forms had successful cloning and all could be seen using SDS-PAGE analysis after expression. The full-length FlaD protein (FDL) produced at lower amounts than the truncated form and problems were seen with stability during storage. In an unpublished degradation trial, both forms were tested for stability at 4°C and -20°C over a period of 6 weeks using SDS-PAGE

analysis. Results showed that FDS was highly stable at both temperatures over the 6-week trial whereas FDL degraded at both storage temperatures. Also, when FDL was concentrated over 1 mg/ml polymerization or precipitation into the insoluble form occurred.

Having a recombinantly produced and highly purified flagellin protein from *V. anguillarum* provides a tool for various fish immunological studies. Having the crystal structure of this important PAMP would aid in understanding how flagellin binds to TLR5 and the conformational changes that can occur. Highly purified (>95% estimated by SDS-PAGE analysis) recombinant FDS was submitted to The Norwegian Structural Biology Centre (NorStruct) for crystallization trials. Numerous buffers (varying in solution components, pH, salt concentration, etc) were tried for crystal production but no usable crystals were produced for crystallization trials (unpublished results). Due to the ease of producing recombinant FDS, it was selected for polyclonal antibody production in rabbit and is referred to as pAb FDS. In immunoblot analysis, pAb FDS was shown to work effectively as an antibody, binding specifically to purified and unpurified FDS and FDL while not binding to other proteins tested. Further work should be performed to test pAb FDS in immunohistochemistry as well as accessing its ability to recognize other flagellin proteins (from *V. anguillarum* and other bacterial strains) in immunoblot analysis.

4.2 Flagellin-antigen interactions

In both papers, the experiments examined the immune response in the spleen after injection into Atlantic salmon of recombinant flagellin mixed in solution with a protein antigen; LPH in paper I and OVA in paper II. Hemocyanin from the horseshoe crab *Limulus polyphemus* is among the largest respiratory proteins found in nature ((3.5 MDa) [155]) and has been shown to induce an antibody response when injected into the peritoneum of salmon when conjugated to the hapten 4-hydroxy-3-iodo-5-nitrophenyl-acetic acid (NIP) [156], trinitrophenyl-acetic acid (TNP), fluorescein-5-iso-tricyanate (FITC) or alone [57, 157]. Ovalbumin (45 kDa [158]) is the major protein found in avian egg white and has been used as an antigen in numerous vaccine studies where it has been shown to induce antibody production and inflammatory cytokines in mice [159-163]. Ovalbumin is hydrophobic in solution which leads to protein aggregation [164] and protein aggregates have been shown to enhance immune responses over the monomeric form of the protein [165]. Since OVA has been widely used as a model antigen in vaccine trials of mammalian species [159-163] and due to the lack of antibody response with LPH in paper I, OVA was chosen as the antigen in paper II. In the paper I ELISA results, there were no significant antibody responses in LPH or

flagellin+LPH injected salmon. This may be due to the tendency of antibody responses in fish studies to vary and to not always infer to immune response or survival in challenge studies [29]. Antigen competition [57, 157] could have played a role in the lack of antibody response however this experiment was not designed to test for antigen competition therefore further work would be needed into this matter in the future.

In the paper I QPCR results, LPH did appear to play a role in extending the immune response in salmon injected with a high dose of flagellin+LPH even though no antibody response was detected. This was evident with Toll-like receptor 5 membrane-bound (TLR5M), interleukin-8 (IL-8), interferon α (IFN α) and TLR5 soluble (TLR5S) significant up-regulation of gene expression on 14DPI compared with the flagellin only injection and with 7DPI gene responses seen. The reason for the extended response is uncertain but could be due to the high concentration of flagellin effectively stimulating the innate immune system, causing the recruitment and further stimulation with LPH of immune-responsive cells. In the paper II QCR results, there were no significant mRNA gene expression differences between salmon injected with FlaD or FlaD+OVA for many of the genes that were reproducibly up-regulated (TLR5S, IL-8, interleukin-1 receptor (IL-1R), tumor necrosis factor receptor family super family 11B (TNFSF11B), hepcidin (HEP1), or matrix metalloproteinase 9 (MMP9) on 2DPI or 7DPI. There were also no reproducible differences in expression of a number of down-regulated genes in microarray analysis such as lipopolysaccharide-induced tumor necrosis factor-alpha factor homolog (LITAF), myxovirus resistance 1 (Mx1), clusterin-1, transcription factor 21). There were many genes in common between FlaD and FlaD+OVA on 2DPI (34), 7DPI (75) and for all groups and times (22) pointing to more of a time importance for gene expression rather than the inclusion of OVA. A number of genes that were up-regulated only by one injection group at one time point (eg. 69 genes up-regulated in FlaD 2DPI only) and these could provide clues as to the flagellin and OVA differences but they require further investigation. Flagellin appears to be the dominant factor producing the immune response in salmon with OVA not enhancing or negatively impacting the response. The inclusion of LPH as the model antigen resulted in the prolongment of the gene expression up-regulation of a number of innate immune genes.

4.3 Innate Immune response

4.3.1 *In vitro* response

Cell culture trials were implemented to examine if the recombinant flagellin proteins bind to TLR5M, activating the NF- κ B response in a COS-7 cell line (from green monkeys) as measured by luciferase assay. FDS and FDL proteins were tested at increasing concentrations in this *in vitro* cell culture assay with or without the human anti-TLR5 antibody (pAb huTLR5). This antibody blocks the membrane-bound form of TLR5 found in mammals and therefore should reduce the NF- κ B response as flagellin is known to bind to TLR5 causing the downstream activation of the NF- κ B response in the innate immune system [28, 78, 166]. The relative luciferase assay (RLA) seen in the *in vitro* cell culture experiments showed that FDS was less effective at stimulating the NF- κ B response as FDL. This indicated that the N-(1-44) and C-(337-377) terminal residues may play some role in the binding of flagellin to TLR5. In the presence of a high concentration of FDL the addition of pAb huTLR5 caused the reduction of NF- κ B response which suggests that most of the activation occurred via TLR5. The same result did not occur in FDS stimulated cells which showed a much lower NF- κ B response and a lack of reduction of NF- κ B response after pAb huTLR5 addition. FDL appears to activate the TLR5 pathway more effectively than FDS in this assay. The response of the cells blocked with pAb huTLR5 was never completely suppressed, which indicated that there could be another pathway activated to initiate the NF- κ B pathway. Another possibility is that huTLR5 antibodies did not possess affinity enough against monkey COS-7 TLR5M to abrogate the TLR5M binding completely. Based on these results, we were interested in comparing the salmon's response to FDL and FDS injection *in vivo* as was done in paper I.

4.3.2 *In vivo* response

TLR5

Teleost fish are unique in having two forms of TLR5, soluble (TLR5S) and membrane bound (TLR5M). This unique feature could prove to be an important part of the innate immune response in fish. The response that we expected to see when flagellin is injected in Atlantic salmon is based on flagellin binding to TLR5. TLR5's role in binding to flagellin initiates the myeloid differentiation primary response gene (MyD88)-dependent signaling cascade and culminates with the production of inflammatory cytokines and chemokines [28, 78, 85, 167]. In paper I QPCR results, TLR5M was significantly up-regulated 7DPI in the highest dose flagellin+LPH groups and 14DPI high down-regulation occurred in most groups,

whereas TLR5S was up-regulated in all groups 4DPI and only in the high dose flagellin+LPH groups 7DPI. The significance of the high down-regulation 14DPI is not known, but could prove to be important in the overall reduction of the innate immune response. In microarray results from paper II, TLR5M (represented as a feature on the 32K microarray) was not shown to reproducibly up-regulate. In a study with rainbow trout (*Oncorhynchus mykiss*) infected with *Yersinia ruckeri* (a facultative intracellular bacterium [168]), expression of TLR5M in the liver was low, ranging from 1.0-2.8 fold change and was never significantly different from the phosphate buffered saline (PBS) injected controls. However, there was significant up-regulation of a number of important inflammatory cytokines (IL-1 β , IL-6, TNF α , etc) which are known to be stimulated downstream of the NF- κ B pathway [169] and were also seen in the paper I results. Paper II QPCR results showed that TLR5S mRNA gene expression was significantly up-regulated on 2DPI and 7DPI in the spleens of FlaD and FlaD+OVA injected fish. Tsukada et al. [86] speculated that the stimulation of TLR5M by flagellin causes the induction of TLR5S expression in liver which can then bind to circulating flagellin and transport it back to TLR5M in the liver amplifying the danger signal in a positive feedback loop [86]. It would be interesting to follow flagellin after injection to see which organs flagellin enters and measure both forms of TLR5 in a time dependent manner to get a better understanding of which form is functioning and to see if a positive feedback loop functions in salmon.

Ras/MAPK pathway

In mammalian species flagellin has been shown to stimulate the Ras/MAPK signaling pathways. The Ras genes family is involved in intracellular signaling networks and respond to diverse extracellular stimuli [170]. They represent an upstream signaling pathway that occur prior to the expression of the various inflammatory and apoptotic cytokines and chemokines of the immune system [170]. It can be difficult to measure the intermediate steps from receptor recognition of flagellin to cytokine production using QPCR or microarray analysis as there are often small fold changes in the various kinases and Ras genes throughout the signaling pathways that are constantly changing forms or being phosphorylated and de-phosphorylated in a regulatory fashion [171, 172]. In paper II, two Ras genes up-regulated 2DPI in FlaD+OVA injected salmon (Ras protein activator like 2 and Ras-related protein Rab-35) followed by a number of important inflammatory cytokines and chemokines. Purified flagellin activated the MAPK, stress-activated protein kinase and IKK signaling pathways leading to inflammatory gene expression in mouse embryo fibroblasts [85]. In mammals there

are more focused research into the interplay between the TLR and MAPK signaling pathways [173] and this is an interesting area that has begun in fish research [174-176].

Inflammation

Stimulation of TLR5M and TLR5S with flagellin, initiates signaling pathways (TLR and Ras/MAPK pathways) which culminate in the expression of inflammatory cytokines that elicit various proinflammatory and chemotactic effects to aid in shaping both the innate and adaptive immune response. A number of important inflammatory cytokines, chemokines and receptors were significantly up-regulated in both studies in salmon injected with recombinant flagellin. In paper II, microarray results (confirmed by QPCR) for FlaD and FlaD+OVA injected salmon showed significant mRNA gene expression up-regulation of TNFRSF11B. TNFRSF11B is a member of the superfamily of TNF receptors which are important in a number of cellular signaling pathways involving inflammation, apoptosis, lymphocyte homeostasis and tissue development (Wiens and Glenney, in press [177]). In mammals, TNF ligands are known to bind to more than one receptor (Wiens and Glenney, in press [177]). Although TNFRSF11B has not been shown to bind to TNF α , it seems to be important in the immune response of fish and in paper I, expression of TNF α was very high on 2DPI and 4DPI for all injection groups tested that included flagellin. TNF α is an important macrophage-activation factor (MAF) produced by leukocytes and has been shown to induce a typical activated macrophage response evidenced by increases in respiratory burst activity, phagocytosis and nitric oxide production in rainbow trout [178], turbot [179], seabream [180, 181] and catfish [182].

TNF α and IL-8 are important T helper (T_H) cytokines which can induce the production of acute-phase proteins or chemokines, incite feverish reactions and up-regulate expression of adhesion molecules on postcapillary venules, thereby triggering the recruitment of inflammatory cells, including neutrophils and monocytes, into cells [79]. IL-8 produces biological effects on neutrophils including: increased cytosolic calcium levels and respiratory burst, a change in neutrophil shape and chemotaxis [24]. In paper II, IL-8 reproducibly up-regulated 2DPI in response to FlaD injection (microarray and QPCR results). In paper I, IL-8 and IL-1 β were weakly up-regulated in response to low dosage flagellin, but it appears that a high flagellin dose and antigen inclusion is necessary for elevated responses as was seen for IL-8 7DPI and IL-1 β 4DPI. IL-1 β is well documented for its function in mediating responses to microbial invasion, inflammation, immunological reactions and tissue injury [183] and in mice has been shown to activate the humoral immune response [184]. In paper II, there was

significant up-regulation of the IL-1R in the FlaD and FlaD+OVA groups 2DPI (QPCR and microarray) and significant but lower up-regulation 7DPI (QPCR only). This receptor is a homologue of the mammalian type II IL-1R, which is mainly expressed in neutrophils, B cells and monocytes in comparison to the type I receptor which mainly expresses on T cells and fibroblast [185]. In mammals, the IL-1R type I mediates all known biological responses to IL-1 whereas IL-1R type II is unable to deliver biological signals due to a lack of a signal transducing cytosolic domain and therefore it acts as a molecular trap for IL-1, negatively regulating IL-1 activity [185]. Due to the presence of a cytoplasmic tail in the salmonid IL-1R the function of this receptor may be different than in the mammalian homologue. However, IL-1R could be involved in the negative regulation of IL-1 β but research is needed to examine this possible role.

In general, Atlantic salmon appear to be very sensitive to low concentrations of recombinant flagellin as was seen with few significant differences in gene expression between salmon treated with high and low doses of FDL and FDS (TLR5S, TNF α , IL-1 β , and IL-8). African green monkeys intramuscularly immunized with very low doses (1, 3, 10 or 30 μ g) of an Oprf₃₁₁₋₃₄₁-Oprl-A- and B-flagellin vaccine also showed a robust antigen-specific IgG response [112]. In order to prolong an immune response to flagellin, there appears to be a need for both a higher concentration of flagellin and the inclusion of an antigen (TLR5S, TLR5M, IL-6 and IL-8 up-regulation on day 7). There may be interplay that occurs among the innate immune response genes (IL-1 β , TNF α , IL-8, etc) but further focus is needed on how they interact to gain a more thorough understanding of the immune system of salmon.

Other innate immune genes

It is thought that fish rely heavily on innate immune responses when defending against pathogens due to limited immunological memory [7, 23, 24, 34]. There were a number of immune genes not commonly known to respond to recombinant flagellin that reproducibly up-regulated in response to injection in salmon in paper II. There was up-regulation of antimicrobial peptides (AMP), which have broad antimicrobial activity against bacteria, fungi, parasites and viruses [16]. Cathelicidin and hepcidin exhibited highly up-regulated mRNA expression in microarray analysis, with hepcidin results confirmed by QPCR analysis. Hepcidin appeared to be an early immune gene, only significantly up-regulating 2DPI whereas cathelicidin maintained gene expression on 2DPI and 7DPI. Hepcidin is a regulator of iron homeostasis controlling iron absorption and macrophage iron release and also can permeate membranes of invading micro-organisms [186]. In mammals, cathelicidins play a

multifunctional role in antibacterial defenses, angiogenesis, chemotaxis and recruiting cells to the adaptive immunity to the site of infection [187]. Cathelicidins are expressed in immune related tissues (kidney and spleen) or in tissue with direct contact with the environment (gills and skin) in *Salmo trutta fario* [19] and similarly in rainbow trout (gills, head kidney, and spleen for rainbow trout cathelicidin 1 [20]), which suggest involvement of these peptides in the innate immune system of salmonids.

The complement system is important in innate immunity with roles in chemotaxis, opsonisation and destruction of invading pathogens [188]. Activation of this system can even enhance B-cell proliferation which links the innate and adaptive immune systems. In teleost fish, the complement system is well developed and includes many active subtypes, which may point to a more complex role in fish than in mammals [188]. In paper II, a number of complement genes were up-regulated including, complement component C7-1, C7 and complement factor D precursor. Complement component C7 is a single-chain glycoprotein that is involved in the cytolytic phase of complement activation through a sequence of polymerization reactions with other terminal components [189]. C7-1 from trout and C7 from flounder are highly similar and are known to play a crucial role in the hydrophilic-amphiphilic transition of the membrane attack complex thereby playing an essential role in the elimination of invading pathogens [189]. Complement factor D precursor is a member of the chymotrypsin family of serine proteases and plays an essential role in host-defense as the rate-limiting enzyme in the alternative pathway of complement activation [190]. The combination of complement genes up-regulated in paper II, point to a role of the complement system in responding to the PAMP flagellin.

C-type lectin receptor B was found to be up-regulated 2DPI in FlaD and FlaD+OVA groups and the 7DPI in FlaD+OVA group in microarray analysis. The family of C-type lectins and receptors are known to be important in innate immunity with roles in pathogen recognition and immune response [191]. They contain putative binding sites for the CCAAT/enhancer binding protein (C/EBP) transcription factors in their regulatory regions which may mediate their response to bacteria and LPS in salmon [191]. Interestingly, multiple C/EBP genes up-regulated in response to FlaD and FlaD+OVA 2DPI and 7DPI which may provide evidence of the linkage between these genes in the salmon's response to flagellin. C/EBP transcription factors play vital roles in many cellular processes, including differentiation, energy metabolism and inflammation [192]. In human cell culture experiments, C/EBP was shown to activate the promoter driving transcription of the TNF receptor 1 (TNFR1) and a C/EBP binding site was identified in the TNFR1 promoter [192]. Two-DPI

and 7DPI in both groups TNFRSF11B was up-regulated and while its role is not known in relation to C-type lectin or C/EBP, the possibility that all three genes work together in a flagellin stimulated immune response in salmon could be interesting for future studies.

Role of down-regulation

When studying the immune response it is important to not only monitor the expression of up-regulated genes, but also to pay attention to the role that down-regulated genes play in the response. Down-regulated genes expression can be paramount in understanding the overall immune response, from initiation to a return to recovery state. In paper I, important cytokines (TNF α , IL-6, IL-1 β , IL-8) known to be produced downstream of the TLR signaling were up-regulated in response to flagellin injection and then down-regulation occurred by 7DPI or 14DPI. This shows the salmon's ability to reduce the inflammatory response which can be stressful and require increased energy usage. Down-regulation of IL-12 occurred on most days for all formulations of flagellin and LPH which was unexpected because IL-12 is an inflammatory cytokine known to be expressed by the MyD88 dependent signaling and NF- κ B pathways. IL-12 is secreted by the T_H1 cells along with IFN γ to protect the host against viral infections and infections by intracellular pathogens [193]. It could be possible that because the salmon was responding to an extracellular bacterial protein, then the genes responsible for intracellular pathogens defense were down-regulated. This possibility was supported by results in paper II, whereby the myxovirus resistance 1 (Mx1) gene down-regulated in FlaD and FlaD+OVA groups 7DPI. The family of Mx proteins are antiviral proteins with intrinsic GTPase activity and function in endocytosis, intracellular vesicle transport and mitochondrial distribution (reviewed in [194]).

4.4 Future prospects for vaccine design in fish

4.4.1 Systems vaccinology

The production of new fish vaccines in aquaculture have predominantly been based on relatively rudimentary methods. The majority of vaccines in use were developed empirically whereby inactivated pathogens were injected into fish and the survival measured [1]. This has produced some highly effective vaccines against bacterial diseases but variable results are seen for viral diseases and there are no successful vaccines against parasite infection. For salmonids, the majority of vaccines on the market are injectable multivalent oil-emulsions which are known to produce a number of serious side effects such as granulomas and intra-abdominal lesions [55, 147-149]. The future of vaccine design for fish must look to new

methodologies for further understanding the immune response and for selecting more strategic components for vaccine components.

In mammalian research, systems vaccinology is a new approach in vaccine development that encompasses a high-throughput multi-systems approach for biological measurement to determine molecular signatures that correlate with protective immune responses [195, 196]. A study in humans vaccinated with the well-established and effective yellow fever vaccine YF-17D followed the immune response up to one year to determine predictive gene signatures [131]. Using such high-throughput technologies as gene expression profiling (microarray and QPCR), assay for neutralizing antibodies, multiplex analysis of cytokines and chemokines, and multi-parameter flow cytometry, combined with computational modeling, Querec et al. [131] were able to determine multiple gene signatures. For example, the expression of the B cell growth factor tumor necrosis factor receptor superfamily, member 17 (TNFRSF17) predicted the neutralizing antibody response with up to 100% accuracy. This model study demonstrated that “vaccine-induced innate immune responses can be used to predict the magnitude of the subsequent adaptive immune response and uncover new correlates of vaccine efficacy” [131]. Systems vaccinology can also be used for identifying vaccine responders versus non-responders, identifying new correlates of protection or mechanisms of vaccine action and predicting vaccine-induced adverse reaction [131, 195].

While fish research is currently lacking some of the key tools needed for adapting the systems biology approach to vaccine design, there are still lessons that can be learned from this method. Mammalian research uses a combination of research methods from genomics, transcriptomics, proteomics, metabolomics and genetics with advanced computational methods [196]. With the advent of the more high-throughput techniques in fish research such as microarray and high-throughput DNA sequencing, the opportunity for adopting the systems vaccinology approach may be possible in future years. Vaccine development should focus on using a combination of methods for designing more efficacious vaccines, combining experimental, theoretical and molecular based approaches.

4.4.2 What next?

The production of a highly purified, soluble, recombinant flagellin protein provides an array of opportunities for future research studies. As these studies were the first to inject highly purified recombinant soluble flagellin into salmon, only the surface was scratched in terms of the roles flagellin plays in stimulating the immune response in salmon. Using the pAb FDS in immunohistochemistry studies could follow the organ distribution of flagellin after intraperitoneal injection in a time dependent manner, as well as investigating the location of flagellin intracellularly by electron microscopy.

Many of the bacterial diseases in salmon aquaculture have been controlled through the use of inactivated vaccines. Viral diseases continue to be a problem in the industry and may require a new, more sophisticated approach to vaccine design. The use of subunit vaccines may prove to be successful in controlling viral diseases. We have shown flagellin injection to stimulate important genes in the innate immune response of salmon and the next step would be to test the adjuvanticity of flagellin in a challenge trial with a viral disease. It would be interesting to mix the vaccine antigen and flagellin adjuvant with mineral oil, as mineral oil has been shown to produce a depot effect [197], an inflammatory response [146, 147] and could possibly prevent the degradation of protein subunits [198]. Due to the side-effects from mineral oil use in vaccines [55, 149, 199, 200], it would be interesting to also include a flagellin and antigen vaccine without mineral oil to compare the responses and efficacy as a vaccine.

Studies in mice [110, 111, 142, 201, 202] and monkeys [112] have shown improved immunogenicity of vaccines when fusion proteins containing flagellin attached to an antigen are used and show potencies at lower dosages than when flagellin and antigen are used separately. One hypothesis proposed that the fusion protein allows flagellin to deliver the antigen in a form that facilitates antigen processing but does not contribute to the response via TLR5 signaling [84]. An appropriate antigen could be expressed with the flagellin adjuvant protein creating a fusion protein for viral challenge trials in salmon.

5. Main conclusions

- Soluble form of highly purified recombinant flagellins from *Vibrio anguillarum* were produced in *Escherichia coli* BL21 (DE3) cells harboring pET151/D TOPO flagellin constructs in two lengths (full-length (FDL) and truncated (FDS)).
- Both forms of flagellin (FDS and FDL) stimulate the immune system similarly but FDL expresses at lower amounts and with greater difficulty than FDS, as well as being less stable over time and at different storage temperatures therefore FDS is a stronger vaccine adjuvant candidate.
- Recombinant FDL was shown to stimulate the NF- κ B response more efficiently than the FDS *in vitro* in a luciferase assay.
- Polyclonal flagellin antibody against FDS was produced in rabbit and shown to be functional in immunoblot analysis.
- Innate immune response measured in Atlantic salmon after injection with recombinant flagellin as assessed by transcriptome analysis (QPCR and microarray).
- Flagellin is a strong adjuvant candidate for further vaccine studies.

6. References

- [1] Sommerset I, Krossoy B, Biering E, Frost P. Vaccines for fish in aquaculture. *Expert Rev Vaccines* 2005 Feb;4(1):89-101.
- [2] Kaisho T, Akira S. Toll-like receptor function and signaling. *J Allergy Clin Immunol* 2006;117(5):979-87.
- [3] Abbas AK, Lichtman AH, editors. Basic immunology: functions and disorders of the immune system. Second edition ed. Philadelphia, PA: Saunders Elsevier, 2006.
- [4] Janeway CA, Medzhitov R. Innate immune recognition. *Annu Rev Immunol* 2002;20:197-216.
- [5] Plouffe DA, Hanington PC, Walsh JG, Wilson EC, Belosevic M. Comparison of select innate immune mechanisms of fish and mammals. *Xenotransplantation* 2005 Jul;12(4):266-77.
- [6] Shi ZC, Cai ZY, Sanchez A, Zhang TT, Wen S, Wang J, et al. A novel Toll-like receptor that recognizes Vesicular Stomatitis Virus. *J Biol Chem* 2011 Feb;286(6):4517-24.
- [7] Du Pasquier L. The immune system of invertebrates and vertebrates. *Comp Biochem Physiol B-Biochem Mol Biol* 2001 May;129(1):1-15.
- [8] Janeway CA. The immune-system evolved to discriminate infectious nonself from noninfectious self. *Immunol Today* 1992 Jan;13(1):11-6.
- [9] Whitmire JK. Induction and function of virus-specific CD4+ T cell responses. *Virology* 2011;411(2):216-28.
- [10] Dardalhon V, Awasthi A, Kwon H, Galileos G, Gao W, Sobel RA, et al. IL-4 inhibits TGF-beta-induced Foxp3(+) T cells and, together with TGF-beta, generates IL-9(+) IL-10(+) Foxp3(-) effector T cells. *Nat Immunol* 2008 Dec;9(12):1347-55.
- [11] Veldhoen M, Uyttenhove C, van Snick J, Helmbj H, Westendorf A, Buer J, et al. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol* 2008 Dec;9(12):1341-6.
- [12] Staudt V, Bothur E, Klein M, Lingnau K, Reuter S, Grebe N, et al. Interferon-Regulatory Factor 4 Is Essential for the Developmental Program of T Helper 9 Cells. *Immunity* 2010;33(2):192-202.
- [13] Deenick EK, Ma CS, Brink R, Tangye SG. Regulation of T follicular helper cell formation and function by antigen presenting cells. *Curr Opin Immunol* 2011;23(1):111-8.
- [14] Tort L, Balasch JC, Mackenzie S. Fish immune system. A crossroads between innate and adaptive responses. *Inmunología* 2003;22(3):277-86.
- [15] Rajanbabu V, Chen JY. Applications of antimicrobial peptides from fish and perspectives for the future. *Peptides* 2011 Feb;32(2):415-20.
- [16] Noga EJ, Ullal AJ, Corrales J, Fernandes JMO. Application of antimicrobial polypeptide host defenses to aquaculture: Exploitation of downregulation and upregulation responses. *Comp Biochem Physiol D-Genomics Proteomics* 2011 Mar;6(1):44-54.
- [17] Douglas SE, Gallant JW, Liebscher RS, Dacanay A, Tsoi SCM. Identification and expression analysis of hepcidin-like antimicrobial peptides in bony fish. *Devel Comp Immunol* 2003 Jun-Jul;27(6-7):589-601.
- [18] Shike H, Shimizu C, Lauth X, Burns JC. Organization and expression analysis of the zebrafish hepcidin gene, an antimicrobial peptide gene conserved among vertebrates. *Devel Comp Immunol* 2004 Jun;28(7-8):747-54.
- [19] Scocchi M, Pallavicini A, Salgaro R, Bociek K, Gennaro R. The salmonid cathelicidins: A gene family with highly varied C-terminal antimicrobial domains. *Comp Biochem Physiol B-Biochem Mol Biol* 2009 Apr;152(4):376-81.

- [20] Chang CI, Zhang YA, Zou J, Nie P, Secombes CJ. Two cathelicidin genes are present in both rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). *Antimicrob Agents and Chemother* 2006 Jan;50(1):185-95.
- [21] Ye X, Zhang LL, Tian YY, Tan AP, Bai JJ, Li SJ. Identification and expression analysis of the g-type and c-type lysozymes in grass carp *Ctenopharyngodon idellus*. *Devel Comp Immunol* 2010 May;34(5):501-9.
- [22] Larsen AN, Solstad T, Svineng G, Seppola M, Jorgensen TO. Molecular characterisation of a goose-type lysozyme gene in Atlantic cod (*Gadus morhua* L.). *Fish Shellfish Immunol* 2009 Jan;26(1):122-32.
- [23] Watts M, Munday BL, Burke CM. Immune responses of teleost fish. *Aust Vet J* 2001 Aug;79(8):570-4.
- [24] Whyte SK. The innate immune response of finfish - A review of current knowledge. *Fish Shellfish Immunol* 2007 Dec;23(6):1127-51.
- [25] Rubio-Godoy M. Teleost fish immunology. Review. *Rev Mex Cienc Pecu* 2010 Jan-Mar;1(1):43-57.
- [26] Takatsu H, Hase K, Ohmae M, Ohshima S, Hashimoto K, Taniura N, et al. CD300 antigen like family member G: A novel Ig receptor like protein exclusively expressed on capillary endothelium. *Biochem Bioph Res Co* 2006;348(1):183-91.
- [27] Ribeiro CMS, Bird S, Raes G, Ghassabeh GH, Schijns V, Pontes M, et al. A novel soluble immune-type receptor (SITR) in teleost fish: Carp SITR is involved in the nitric oxide-mediated response to a protozoan parasite. *PLoS One* 2011 Jan;6(1).
- [28] Rebl A, Goldammer T, Seyfert HM. Toll-like receptor signaling in bony fish. *Vet Immunol Immunopathol* 2010 Apr;134(3-4):139-50.
- [29] Morrison RN, Nowak BF. The antibody response of teleost fish. *Semin Avian Exot Pet Med* 2002 Jan;11(1):46-54.
- [30] Solem ST, Stenvik J. Antibody repertoire development in teleosts - a review with emphasis on salmonids and *Gadus morhua* L. *Devel Comp Immunol* 2006;30(1-2):57-76.
- [31] Nakanishi T, Toda H, Shibasaki Y, Somamoto T. Cytotoxic T cells in teleost fish. *Dev Comp Immunol*; in press, corrected proof (doi: 10.1016/j.dci.2011.03.033).
- [32] Haugarvoll E, Bjerkas I, Nowak BF, Hordvik I, Koppang EO. Identification and characterization of a novel intraepithelial lymphoid tissue in the gills of Atlantic salmon. *J Anat* 2008 Aug;213(2):202-9.
- [33] Takizawa F, Koppang EO, Ohtani M, Nakanishi T, Hashimoto K, Fischer U, et al. Constitutive high expression of interleukin-4/13A and GATA-3 in gill and skin of salmonid fishes suggests that these tissues form Th2-skewed immune environments. *Mol Immunol* 2011;48(12-13):1360-8.
- [34] Magnadottir B. Immunological Control of Fish Diseases. *Mar Biotechnol* 2010 Aug;12(4):361-79.
- [35] Pettersen EF, Ingerslev H-C, Stavang V, Egenberg M, Wergeland HI. A highly phagocytic cell line TO from Atlantic salmon is CD83 positive and M-CSFR negative, indicating a dendritic-like cell type. *Fish Shellfish Immunol* 2008;25(6):809-19.
- [36] Ganassin R, Bols N. Development of long-term rainbow trout spleen cultures that are haemopoietic and produce dendritic cells. *Fish Shellfish Immunol* 1996;6(1):17-34.
- [37] Iliev DB, Jorgensen SM, Rode M, Krasnov A, Harneshaug I, Jorgensen JB. CpG-induced secretion of MHCII beta and exosomes from salmon (*Salmo salar*) APCs. *Devel Comp Immunol* 2010 Jan;34(1):29-41.
- [38] Nakanishi T, Fischer U, Dijkstra JM, Hasegawa S, Somamoto T, Okamoto N, et al. Cytotoxic T cell function in fish. *Devel Comp Immunol* 2002 Mar;26(2):131-9.

- [39] Li J, Barreda DR, Zhang YA, Boshra H, Gelman AE, LaPatra S, et al. B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. *Nat Immunol* 2006 Oct;7(10):1116-24.
- [40] Overland HS, Pettersen EF, Ronneseth A, Wergeland HI. Phagocytosis by B-cells and neutrophils in Atlantic salmon (*Salmo salar* L.) and Atlantic cod (*Gadus morhua* L.). *Fish Shellfish Immunol* 2010 Jan;28(1):193-204.
- [41] Kono T, Korenaga H, Sakai M. Genomics of fish IL-17 ligand and receptors: A review. *Fish Shellfish Immunol*; In Press, Corrected Proof (doi: 10.1016/j.fsi.2010.11.028).
- [42] Gunimaladevi I, Savan R, Sakai M. Identification, cloning and characterization of interleukin-17 and its family from zebrafish. *Fish Shellfish Immunol* 2006 Oct;21(4):393-403.
- [43] Wang TH, Diaz-Rosales P, Costa MM, Campbell S, Snow M, Collet B, et al. Functional characterization of a nonmammalian IL-21: Rainbow trout *Oncorhynchus mykiss* IL-21 upregulates the expression of the Th cell signature cytokines IFN-gamma, IL-10, and IL-22. *J Immunol* 2011 Jan;186(2):708-21.
- [44] Kjøglum S, Larsen S, Bakke HG, Grimholt U. How specific MHC class I and class II combinations affect disease resistance against infectious salmon anaemia in Atlantic salmon (*Salmo salar*). *Fish Shellfish Immunol* 2006 Oct;21(4):431-41.
- [45] Grimholt U, Drablos F, Jørgensen SM, Hoyheim B, Stet RJM. The major histocompatibility class I locus in Atlantic salmon (*Salmo salar* L.): polymorphism, linkage analysis and protein modelling. *Immunogenetics* 2002 Nov;54(8):570-81.
- [46] Stet RJM, de Vries B, Mudde K, Hermsen T, van Heerwaarden J, Shum BP, et al. Unique haplotypes of co-segregating major histocompatibility class II A and class II B alleles in Atlantic salmon (*Salmo salar*) give rise to diverse class II genotypes. *Immunogenetics* 2002 Aug;54(5):320-31.
- [47] Star B, Nederbragt AJ, Jentoft S, Grimholt U, Malmstrom M, Gregers TF, et al. The genome sequence of Atlantic cod reveals a unique immune system. *Nature* 2011;477(7363):207-10.
- [48] Pilstrom L, Warr GW, Stromberg S. Why is the antibody response of Atlantic cod so poor? The search for a genetic explanation. *Fish Sci* 2005 Oct;71(5):961-71.
- [49] Magnadottir B, Gudmundsdottir S, Gudmundsdottir BK, Helgason S. Natural antibodies of cod (*Gadus morhua* L.): Specificity, activity and affinity. *Comp Biochem Physiol B-Biochem Mol Biol* 2009 Nov;154(3):309-16.
- [50] Hansen JD, Landis ED, Phillips RB. Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. *Proc Natl Acad Sci U S A* 2005 May;102(19):6919-24.
- [51] Danilova N, Bussmann J, Jekosch K, Steiner LA. The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z. *Nat Immunol* 2005 Mar;6(3):295-302.
- [52] Zhang YA, Salinas I, Li J, Parra D, Bjork S, Xu Z, et al. IgT, a primitive immunoglobulin class specialized in mucosal immunity. *Nature Immunology* 2010 Sep;11(9):827-U82.
- [53] Fjalestad KT, Larsen HJS, Roed KH. Antibody response in Atlantic salmon (*Salmo salar*) against *Vibrio anguillarum* and *Vibrio salmonicida* O-antigens: Heritabilities, genetic correlations and correlations with survival. *Aquaculture* 1996 Oct;145(1-4):77-89.
- [54] Eggset G, Mikkelsen H, Killie JEA. Immunocompetence and duration of immunity against *Vibrio salmonicida* and *Aeromonas salmonicida* after vaccination of Atlantic salmon (*Salmo salar* L.) at low and high temperatures. *Fish Shellfish Immunol* 1997 May;7(4):247-60.
- [55] Mutoloki S, Alexandersen S, Gravningen K, Evensen O. Time-course study of injection site inflammatory reactions following intraperitoneal injection of Atlantic cod

- (*Gadus morhua* L.) with oil-adjuvanted vaccines. *Fish Shellfish Immunol* 2008 Apr;24(4):386-93.
- [56] Steine NO, Melingen GO, Wergeland HI. Antibodies against *Vibrio salmonicida* lipopolysaccharide (LPS) and whole bacteria in sera from Atlantic salmon (*Salmo salar* L.) vaccinated during the smolting and early post-smolt period. *Fish Shellfish Immunol* 2001 Jan;11(1):39-52.
- [57] Killie JEA, Jorgensen TO. Immunoregulation in fish .1. Intermolecular-induced suppression of antibody responses to haptened protein antigens studied in Atlantic salmon (*Salmo salar* L). *Devel Comp Immunol* 1994 Mar-Apr;18(2):123-36.
- [58] Akira S. Toll-like receptor signaling. *J Biol Chem* 2003 Oct;278(40):38105-8.
- [59] Wu LP, Anderson KV. Related signaling networks in *Drosophila* that control dorsoventral patterning in the embryo and the immune response. *Cold Spring Harbor Symp Quant Biol* 1997;62:97-103.
- [60] Lemaitre B, Nicolas E, Michaut L, Reichhart J-M, Hoffmann JA. The dorsoventral regulatory gene cassette *spätzle/Toll/cactus* controls the potent antifungal Response in *Drosophila* adults. *Cell* 1996;86(6):973-83.
- [61] Medzhitov R, PrestonHurlburt P, Janeway CA. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 1997 Jul;388(6640):394-7.
- [62] Poltorak A, He XL, Smirnova I, Liu MY, Van Huffel C, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: Mutations in Tlr4 gene. *Science* 1998 Dec;282(5396):2085-8.
- [63] Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, et al. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: Evidence for TLR4 as the Lps gene product. *J Immunol* 1999 Apr;162(7):3749-52.
- [64] Werling D, Jann OC, Offord V, Glass EJ, Coffey TJ. Variation matters: TLR structure and species-specific pathogen recognition. *Trends Immunol* 2009 Mar;30(3):124-30.
- [65] Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 2004 Oct;5(10):987-95.
- [66] Oberg H-H, Juricke M, Kabelitz D, Wesch D. Regulation of T cell activation by TLR ligands. *Euro J Cell Biol* 2011 June-July;90(6-7):582-92.
- [67] Palti Y. Toll-like receptors in bony fish: From genomics to function. *Developmental & Comparative Immunology*; In Press, Corrected Proof (doi: 10.1016/j.dci.2011.03.006).
- [68] Kestera AM, de Zoete MR, Bouwman LI, van Putten JPM. Chicken TLR21 is an innate CpG DNA receptor distinct from mammalian TLR9. *J Immunol* 2010 Jul;185(1):460-7.
- [69] Jin MS, Lee JO. Structures of TLR-ligand complexes. *Curr Opin Immunol* 2008 Aug;20(4):414-9.
- [70] Botos I, Segal DM, Davies DR. The structural biology of Toll-like receptors. *Structure* 2011 Apr;19(4):447-59.
- [71] Gay NJ, Gangloff M. Structure of Toll-like Receptors. In: Bauer S, Gunther H, editors. *Toll-like receptors (TLRs) and Innate Immunity*. Berlin Heidelberg: Springer-Verlag, 2008: 181-200.
- [72] Choe J, Kelker MS, Wilson IA. Crystal structure of human Toll-like receptor 3 (TLR3) ectodomain. *Science* 2005 Jul;309(5734):581-5.
- [73] Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik SG, et al. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. *Cell* 2007 Sep;130(6):1071-82.
- [74] Kim HM, Park BS, Kim JI, Kim SE, Lee J, Oh SC, et al. Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist eritoran. *Cell* 2007 Sep;130(5):906-17.

- [75] Liu L, Botos I, Wang Y, Leonard JN, Shiloach J, Segal DM, et al. Structural basis of toll-like receptor 3 signaling with double-stranded RNA. *Science* 2008 Apr;320(5874):379-81.
- [76] Bell JK, Mullen GED, Leifer CA, Mazzoni A, Davies DR, Segal DM. Leucine-rich repeats and pathogen recognition in Toll-like receptors. *Trends Immunol* 2003;24(10):528-33.
- [77] Brikos C, O'Neil LAJ. Signalling of Toll-like Receptors. In: Bauer S, Gunther H, editors. *Toll-like Receptors and Innate Immunity*. Berlin Heidelberg: Springer-Verlag, 2008: 21-50.
- [78] Kawai T, Akira S. TLR signaling. *Semin Immunol* 2007 Feb;19(1):24-32.
- [79] Winkler P, Ghadimi D, Schrezenmeir J, Kraehenbuhl JP. Molecular and cellular basis of microflora-host interactions. *J Nutr* 2007 Mar;137(3):756S-72S.
- [80] Stafford JL, Ellestad KK, Magor KE, Belosevic M, Magor BG. A toll-like receptor (TLR) gene that is up-regulated in activated goldfish macrophages. *Dev Comp Immunol* 2003 Sep;27(8):685-98.
- [81] Purcell MK, Smith KD, Aderem A, Hood L, Winton JR, Roach JC. Conservation of Toll-like receptor signaling pathways in teleost fish. *Comp Biochem Physiol D-Genomics Proteomics* 2006 Mar;1(1):77-88.
- [82] Sepulcre MP, Alcaraz-Perez F, Lopez-Munoz A, Roca FJ, Meseguer J, Cayuela ML, et al. Evolution of Lipopolysaccharide (LPS) Recognition and Signaling: Fish TLR4 Does Not Recognize LPS and Negatively Regulates NF-kappa B Activation. *J Immunol* 2009 Feb;182(4):1836-45.
- [83] Kumar S, Tamura K, Nei M. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* 2004 Jun;5(2):150-63.
- [84] Mizel SB, Bates JT. Flagellin as an adjuvant: Cellular mechanisms and potential. *J Immunol* 2010 Nov;185(10):5677-82.
- [85] Tallant T, Deb A, Kar N, Lupica J, de Veer MJ, DiDonato JA. Flagellin acting via TLR5 is the major-activator of key signaling pathways leading to NF-kappa B and proinflammatory gene program activation in intestinal epithelial cells. *BMC Microbiol* 2004 Aug;4:33.
- [86] Tsukada H, Fukui A, Tsujita T, Matsumoto M, Iida T, Seya T. Fish soluble Toll-like receptor 5 (TLR5S) is an acute-phase protein with integral flagellin-recognition activity. *Int J Mol Med* 2005 Mar;15(3):519-25.
- [87] Oshiumi H, Tsujita T, Shida K, Matsumoto M, Ikeo K, Seya T. Prediction of the prototype of the human Toll-like receptor gene family from the pufferfish, *Fugu rubripes*, genome. *Immunogenetics* 2003 Feb;54(11):791-800.
- [88] Tsujita T, Tsukada H, Nakao M, Oshiumi H, Matsumoto M, Seya T. Sensing bacterial flagellin by membrane and soluble orthologs of toll-like receptor 5 in rainbow trout (*Onchorhynchus mikiss*). *J Biol Chem* 2004 Nov;279(47):48588-97.
- [89] Tsoi S, Park KC, Kay HH, O'Brien TJ, Podor E, Sun GL, et al. Identification of a transcript encoding a soluble form of toll-like receptor 5 (TLR5) in Atlantic salmon during *Aeromonas salmonicida* infection. *Vet Immunol Immunopathol* 2006 Jan;109(1-2):183-7.
- [90] Hwang SD, Asahi T, Kondo H, Hirono I, Aoki T. Molecular cloning and expression study on Toll-like receptor 5 paralogs in Japanese flounder, *Paralichthys olivaceus*. *Fish Shellfish Immunol* 2010 Oct;29(4):630-8.
- [91] Baoprasertkul P, Xu P, Peatman E, Kucuktas H, Liu Z. Divergent toll-like receptors in catfish (*Ictalurus punctatus*): TLR5S, TLR20, TLR21. *Fish Shellfish Immunol* 2007 Dec;23(6):1218-30.
- [92] Ewart KV, Belanger JC, Williams J, Karakach T, Penny S, Tsoi SCM, et al. Identification of genes differentially expressed in Atlantic salmon (*Salmo salar*) in response to infection by *Aeromonas salmonicida* using cDNA microarray technology. *Devel Comp Immunol* 2005;29(4):333-47.

- [93] Chen G, Shaw MH, Kim YG, Nunez G. NOD-Like receptors: Role in innate immunity and inflammatory disease. *Annu Rev Pathol-Mech Dis* 2009;4:365-98.
- [94] Kufer TA, Sansonetti PJ. NLR functions beyond pathogen recognition. *Nat Immunol* 2011 Feb;12(2):121-8.
- [95] Franchi L, Eigenbrod T, Munoz-Planillo R, Nunez G. The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat Immunol* 2009 Mar;10(3):241-7.
- [96] Lightfield KL, Persson J, Brubaker SW, Witte CE, von Moltke J, Dunipace EA, et al. Critical function for Naip5 in inflammasome activation by a conserved carboxy-terminal domain of flagellin. *Nat Immunol* 2008 Oct;9(10):1171-8.
- [97] Laing KJ, Purcell MK, Winton JR, Hansen JD. A genomic view of the NOD-like receptor family in teleost fish: identification of a novel NLR subfamily in zebrafish. *BMC Evol Biol* 2008 Feb;8.
- [98] Sha ZX, Abernathy JW, Wang SL, Li P, Kucuktas H, Liu H, et al. NOD-like subfamily of the nucleotide-binding domain and leucine-rich repeat containing family receptors and their expression in channel catfish. *Dev Comp Immunol* 2009 Sep;33(9):991-9.
- [99] Chen WQ, Xu QQ, Chang MX, Nie P, Peng KM. Molecular characterization and expression analysis of nuclear oligomerization domain proteins NOD1 and NOD2 in grass carp *Ctenopharyngodon idella*. *Fish Shellfish Immunol* 2010 Jan;28(1):18-29.
- [100] Ramos HC, Rumbo M, Sirard JC. Bacterial flagellins: mediators of pathogenicity and host immune responses in mucosa. *Trends Microbiol* 2004 Nov;12(11):509-17.
- [101] Uematsu S, Akira S. Toll-Like Receptors (TLRs) and Their Ligands. In: Bauer S, Gunther H, editors. *Toll-Like Receptors (TLRs) and Innate Immunity*. Berlin Heidelberg: Springer-Verlag, 2008: 1-20.
- [102] Beatson SA, Minamino T, Pallen MJ. Variation in bacterial flagellins: from sequence to structure. *Trends Microbiol* 2006 Apr;14(4):151-5.
- [103] Smith KD, Andersen-Nissen E, Hayashi F, Strobe K, Bergman MA, Barrett SLR, et al. Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. *Nat Immunol* 2003 Dec;4(12):1247-53.
- [104] Samatey FA, Imada K, Vonderviszt F, Shirakihara Y, Namba K. Crystallization of the F41 fragment of flagellin and data collection from extremely thin crystals. *J Struct Biol* 2000 Nov;132(2):106-11.
- [105] Maruyama Y, Momma M, Mikami B, Hashimoto W, Murata K. Crystal structure of a novel bacterial cell-surface flagellin binding to a polysaccharide. *Biochemistry* 2008 Feb;47(5):1393-402.
- [106] Honko AN, Mizel SB. Effects of flagellin on innate and adaptive immunity. *Immunol Res* 2005;33(1):83-101.
- [107] Jacchieri SG, Torquato R, Brentani RR. Structural study of binding of flagellin by Toll-like receptor 5. *J Bacteriol* 2003 Jul;185(14):4243-7.
- [108] Mizel SB, West AP, Hantgan RR. Identification of a sequence in human toll-like receptor 5 required for the binding of Gram-negative flagellin. *J Biol Chem* 2003 Jun;278(26):23624-9.
- [109] Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 2001 Apr;410(6832):1099-103.
- [110] Skountzou I, Martin MD, Wang BZ, Ye L, Koutsonanos D, Weldon W, et al. Salmonella flagellins are potent adjuvants for intranasally administered whole inactivated influenza vaccine. *Vaccine* 2010 May;28(24):4103-12.

- [111] Weimer ET, Lu HP, Kock ND, Wozniak DJ, Mizel SB. A fusion protein vaccine containing OprF epitope 8, OprI, and Type A and B flagellins promotes enhanced clearance of nonmucoid *Pseudomonas aeruginosa*. *Infect Immun* 2009 Jun;77(6):2356-66.
- [112] Weimer ET, Ervin SE, Wozniak DJ, Mizel SB. Immunization of young African green monkeys with OprF epitope 8-OprI-type A- and B-flagellin fusion proteins promotes the production of protective antibodies against nonmucoid *Pseudomonas aeruginosa*. *Vaccine* 2009 Nov;27(48):6762-9.
- [113] Wilhelm V, Miquel A, Burzio LO, Roseblatt M, Engel E, Valenzuela S, et al. A vaccine against the salmonid pathogen *Piscirickettsia salmonis* based on recombinant proteins. *Vaccine* 2006 Jun;24(23):5083-91.
- [114] McGee K, Horstedt P, Milton DL. Identification and characterization of additional flagellin genes from *Vibrio anguillarum*. *J Bacteriol* 1996 Sep;178(17):5188-98.
- [115] Liang HY, Xia LQ, Wu ZH, Jian JC, Lu YS. Expression, characterization and immunogenicity of flagellin FlaC from *Vibrio alginolyticus* strain HY9901. *Fish Shellfish Immunol* 2010 Aug;29(2):343-8.
- [116] Jiao XD, Zhang M, Hu YH, Sun L. Construction and evaluation of DNA vaccines encoding *Edwardsiella tarda* antigens. *Vaccine* 2009 Aug;27(38):5195-202.
- [117] Jiao XD, Hu YH, Sun L. Dissection and localization of the immunostimulating domain of *Edwardsiella tarda* FliC. *Vaccine* 2010 Aug;28(34):5635-40.
- [118] Subasinghe R. Disease control in aquaculture and the responsible use of veterinary drugs and vaccines: The issues, prospects and challenges. *Options Mediterraneennes* 2009;86:5-11.
- [119] Evensen O. Development in fish vaccinology with focus on delivery methodologies, adjuvants and formulations. *Options Mediterraneennes* 2009;A/no.86:177-86.
- [120] Toranzo AE, Romalde JL, Magarinos B, Barja JL. Present and future of aquaculture vaccines against fish bacterial diseases. *Options Mediterraneennes* 2009;A/no. 86:155-75.
- [121] Mikkelsen H, Schroder MB, Lund V. Vibriosis and atypical furunculosis vaccines; efficacy, specificity and side effects in Atlantic cod, *Gadus morhua* L. *Aquaculture* 2004 Dec;242(1-4):81-91.
- [122] Ada G. Overview of Vaccines and vaccination. *Mol Biotechnol* 2005 Mar;29(3):255-71.
- [123] Tonheim TC, Bogwald J, Dalmo RA. What happens to the DNA vaccine in fish? A review of current knowledge. *Fish Shellfish Immunol* 2008 Jul;25(1-2):1-18.
- [124] Nusbaum KE, Smith BF, DeInnocentes P, Bird RC. Protective immunity induced by DNA vaccination of channel catfish with early and late transcripts of the channel catfish herpesvirus (IHV-1). *Vet Immunol Immunopathol* 2002 Jan;84(3-4):151-68.
- [125] Lorenzen N, Lorenzen E, Einer-Jensen K, Heppell J, Davis HL. Genetic vaccination of rainbow trout against viral haemorrhagic septicaemia virus: small amounts of plasmid DNA protect against a heterologous serotype. *Virus Res* 1999 Sep;63(1-2):19-25.
- [126] Corbeil S, LaPatra SE, Anderson ED, Kurath G. Nanogram quantities of a DNA vaccine protect rainbow trout fry against heterologous strains of infectious hematopoietic necrosis virus. *Vaccine* 2000 Jun;18(25):2817-24.
- [127] Cuesta A, Chaves-Pozo E, de las Heras AI, Saint-Jean SR, Pérez-Prieto S, Tafalla C. An active DNA vaccine against infectious pancreatic necrosis virus (IPNV) with a different mode of action than fish rhabdovirus DNA vaccines. *Vaccine* 2010;28(19):3291-300.
- [128] Gomez-Chiarri M, Brown LL, Levine RP. Protection against *Renibacterium salmoninarum* infection by DNA-based immunization. *Aquaculture Biotechnology Symposium Proceedings* 1996:155-7.
- [129] Myhr AI, Dalmo RA. Introduction of genetic engineering in aquaculture: Ecological and ethical implications for science and governance. *Aquaculture* 2005 Dec;250(3-4):542-54.

- [130] Clark TG, Cassidy-Hanley D. Recombinant subunit vaccines: Potentials and constraints. In: Midtlyng PJ, editor. Progress in Fish Vaccinology. Basel, Karger: Developmental Biology, 2005: 153-63.
- [131] Querec TD, Akondy RS, Lee EK, Cao WP, Nakaya HI, Teuwen D, et al. Systems biology approach predicts immunogenicity of the yellow fever vaccine in humans. Nat Immunol 2009 Jan;10(1):116-25.
- [132] Goetz FW, MacKenzie S. Functional genomics with microarrays in fish biology and fisheries. Fish Fish 2008 Dec;9(4):378-95.
- [133] Zhang J, Chu W, Fu G. DNA microarray technology and its application in fish biology and aquaculture. Frontiers of Biology in China 2009;4(3):305-13.
- [134] Skugor S, Jorgensen SM, Gjerde B, Krasnov A. Hepatic gene expression profiling reveals protective responses in Atlantic salmon vaccinated against furunculosis. BMC Genomics 2009 Oct;10.
- [135] Aoki T, Hirono I, Kondo H, Hikima J, Jung TS. Microarray technology is an effective tool for identifying genes related to the aquacultural improvement of Japanese flounder, *Paralichthys olivaceus*. Comp Biochem Physiol D-Genomics Proteomics 2011 Mar;6(1):39-43.
- [136] Guy B. The perfect mix: recent progress in adjuvant research. Nat Rev Microbiol 2007 Jul;5(7):505-17.
- [137] Singh M, O'Hagan DT. Recent advances in veterinary vaccine adjuvants. Int J Parasit 2003 May;33(5-6):469-78.
- [138] Stills HF. Adjuvants and antibody production: Dispelling the myths associated with Freund's complete and other adjuvants. Ilar J 2005;46(3):280-93.
- [139] Barr TA, Carrington J, Heath AW. Co-stimulatory agonists as immunological adjuvants. Vaccine 2006 Apr;24(17):3399-407.
- [140] Bates JT, Uematsu S, Akira S, Mizel SB. Direct stimulation of TLR5(+/+) CD11c(+) cells is necessary for the adjuvant activity of flagellin. J Immunol 2009 Jun;182(12):7539-47.
- [141] Huleatt JW, Jacobs AR, Tang J, Desai P, Kopp EB, Huang Y, et al. Vaccination with recombinant fusion proteins incorporating Toll-like receptor ligands induces rapid cellular and humoral immunity. Vaccine 2007;25(4):763-75.
- [142] Cuadros C, Lopez-Hernandez FJ, Dominguez AL, McClelland M, Lustgarten J. Flagellin fusion proteins as adjuvants or vaccines induce specific immune responses. Infect Immun 2004 May;72(5):2810-6.
- [143] Taylor DN, Treanor JJ, Strout C, Johnson C, Fitzgerald T, Kavita U, et al. Induction of a potent immune response in the elderly using the TLR-5 agonist, flagellin, with a recombinant hemagglutinin influenza-flagellin fusion vaccine (VAX125, STF2.HA1 SI). Vaccine 2011 Jul;29(31):4897-902.
- [144] Janeway CA. Approaching the asymptote- Evolution and revolution in immunology. Cold Spring Harbor Symp Quant Biol 1989;54:1-13.
- [145] Midtlyng PJ, Lillehaug A. Growth of Atlantic salmon *Salmo salar* after intraperitoneal administration of vaccines containing adjuvants. Dis Aquat Org 1998 Mar;32(2):91-7.
- [146] Mutoloki S, Brudeseth B, Reite OB, Evensen O. The contribution of *Aeromonas salmonicida* extracellular products to the induction of inflammation in Atlantic salmon (*Salmo salar* L.) following vaccination with oil-based vaccines. Fish Shellfish Immunol 2006 Jan;20(1):1-11.
- [147] Mutoloki S, Alexandersen S, Evensen O. Sequential study of antigen persistence and concomitant inflammatory reactions relative to side-effects and growth of Atlantic salmon (*Salmo salar* L.) following intraperitoneal injection with oil-adjuvanted vaccines. Fish Shellfish Immunol 2004 May;16(5):633-44.

- [148] Mutoloki S, Reite OB, Brudeseth B, Tverdal A, Evensen O. A comparative immunopathological study of injection site reactions in salmonids following intraperitoneal injection with oil-adjuvanted vaccines. *Vaccine* 2006 Jan;24(5):578-88.
- [149] Mutoloki S, Cooper GA, Marjara IS, Koop BF, Evensen O. High gene expression of inflammatory markers and IL-17A correlates with severity of injection site reactions of Atlantic salmon vaccinated with oil-adjuvanted vaccines. *BMC Genomics* 2010 May;11:336.
- [150] Jiao XD, Cheng S, Hu YH, Sun L. Comparative study of the effects of aluminum adjuvants and Freund's incomplete adjuvant on the immune response to an *Edwardsiella tarda* major antigen. *Vaccine* 2010 Feb;28(7):1832-7.
- [151] Koppang EO, Bjerkas I, Haugarvoll E, Chan EKL, Szabo NJ, Ono N, et al. Vaccination-induced systemic autoimmunity in farmed Atlantic salmon. *J Immunol* 2008 Oct;181(7):4807-14.
- [152] Haugarvoll E, Bjerkas I, Szabo NJ, Satoh M, Koppang EO. Manifestations of systemic autoimmunity in vaccinated salmon. *Vaccine* 2010 Jul;28(31):4961-9.
- [153] Satoh M, Bjerkas I, Haugarvoll E, Chan EKL, Szabo NJ, Jirillo E, et al. Polyclonal hypergammaglobulinemia and autoantibody production induced by vaccination in farmed Atlantic salmon. *Fish Shellfish Immunol* 2011 Apr-May;30(4-5):1080-6.
- [154] Koop B, von Schalburg K, Leong J, Walker N, Lieph R, Cooper G, et al. A salmonid EST genomic study: genes, duplications, phylogeny and microarrays. *BMC Genomics* 2008;9(1):545.
- [155] Martin AG, Depoix F, Stohr M, Meissner U, Hagner-Holler S, Hammouti K, et al. *Limulus polyphemus* Hemocyanin: 10 Å cryo-EM structure, sequence analysis, molecular modelling and rigid-body fitting reveal the interfaces between the eight hexamers. *J Mol Biol* 2007;366(4):1332-50.
- [156] Killie JEA, Espelid S, Jorgensen TO. The humoral immune response in Atlantic salmon (*Salmo salar* R.) against the hapten carrier antigen NIP_LPH; the effect of determinant (NIP) density and the isotype profile of anti-NIP antibodies. *Fish Shellfish Immunol* 1991;1:33-46.
- [157] Killie JEA, Jorgensen TO. Immunoregulation in fish .2. Intermolecular-induced suppression of antibody responses studied by haptened antigens in Atlantic salmon (*Salmo salar* L). *Dev Comp Immunol* 1995 Sep-Oct;19(5):389-404.
- [158] Choi SJ, Kim HJ, Park KH, Moon TW. Molecular characteristics of ovalbumin-dextran conjugates formed through the Maillard reaction. *Food Chem* 2005;92(1):93-9.
- [159] Slutter B, Soema PC, Ding Z, Verheul R, Hennink W, Jiskoot W. Conjugation of ovalbumin to trimethyl chitosan improves immunogenicity of the antigen. *J Control Release* 2010 Apr;143(2):207-14.
- [160] Bal SM, Hortensius S, Ding Z, Jiskoot W, Bouwstra JA. Co-encapsulation of antigen and Toll-like receptor ligand in cationic liposomes affects the quality of the immune response in mice after intradermal vaccination. *Vaccine* 2011 Jan;29(5):1045-52.
- [161] Garlapati S, Eng NF, Wilson HL, Buchanan R, Mutwiri GK, Babiuk LA, et al. PCPP (poly di(carboxylatophenoxy)-phosphazene) microparticles co-encapsulating ovalbumin and CpG oligo-deoxynucleotides are potent enhancers of antigen specific Th1 immune responses in mice. *Vaccine* 2010 Dec;28(52):8306-14.
- [162] Schulke S, Waibler Z, Mende MS, Zoccatelli G, Vieths S, Toda M, et al. Fusion protein of TLR5-ligand and allergen potentiates activation and IL-10 secretion in murine myeloid DC. *Mol Immunol* 2010 Nov-Dec;48(1-3):341-50.
- [163] Vijay-Kumar M, Carvalho FA, Aitken JD, Fifadara NH, Gewirtz AT. TLR5 or NLRC4 is necessary and sufficient for promotion of humoral immunity by flagellin. *Eur J Immunol* 2010 Dec;40(12):3528-34.

- [164] Ianeselli L, Zhang FJ, Skoda MWA, Jacobs RMJ, Martin RA, Callow S, et al. Protein-protein interactions in ovalbumin solutions studied by small-angle scattering: Effect of ionic strength and the chemical nature of cations. *J Phys Chem B* 2010 Mar;114(11):3776-83.
- [165] Rosenberg AS. Effects of protein aggregates: An immunologic perspective. *AAPS J* 2006;8 (3):Article 59.
- [166] Beutler B. Microbe sensing, positive feedback loops, and the pathogenesis of inflammatory diseases. *Immunol Rev* 2009;227(1):248-63.
- [167] Oda K, Kitano H. A comprehensive map of the toll-like receptor signaling network. *Mol Syst Biol* 2006;2:2006 0015.
- [168] Ryckaert J, Bossier P, D'Herde K, Diez-Fraile A, Sorgeloos P, Haesebrouck F, et al. Persistence of *Yersinia ruckeri* in trout macrophages. *Fish Shellfish Immunol* 2010 Oct;29(4):648-55.
- [169] Raida MK, Buchmann K. Innate immune response in rainbow trout (*Oncorhynchus mykiss*) against primary and secondary infections with *Yersinia ruckeri* O1. *Dev Comp Immunol* 2009;33(1):35-45.
- [170] Wennerberg K, Rossman KL, Der CJ. The Ras superfamily at a glance. *J Cell Sci* 2005 Mar;118(5):843-6.
- [171] Karin M. Signal transduction and gene control. *Curr Opin Cell Biol* 1991;3(3):467-73.
- [172] Bohmann D. Transcription factor phosphorylation - A link between signal transduction and the regulation of gene-expression. *Cancer Cell Mon Rev* 1990 Nov;2(11):337-44.
- [173] Makela SM, Strengell M, Pietila TE, Osterlund P, Julkunen I. Multiple signaling pathways contribute to synergistic TLR ligand-dependent cytokine gene expression in human monocyte-derived macrophages and dendritic cells. *J Leukoc Biol* 2009 Apr;85(4):664-72.
- [174] Stockhammer OW, Zakrzewska A, Hegedus Z, Spaink HP, Meijer AH. Transcriptome profiling and functional analyses of the zebrafish embryonic innate immune response to *Salmonella* infection. *J Immunol* 2009 May;182(9):5641-53.
- [175] Hansen TE, Jorgensen JB. Cloning and characterisation of p38 MAP kinase from Atlantic salmon - A kinase important for regulating salmon TNF-2 and IL-1 beta expression. *Mol Immunol* 2007 May;44(12):3137-46.
- [176] Hansen TE, Puntervoll P, Seternes OM, Jorgensen JB. Atlantic salmon possess three mitogen activated protein kinase kinase 6 paralogs responding differently to stress. *Febs J* 2008 Oct;275(19):4887-902.
- [177] Wiens GD, Glenney GW. Origin and evolution of TNF and TNF receptor superfamilies. *Devel Comp Immunol* 2011; In press, Corrected proof(doi:10.1016/j.dci.2011.03.031).
- [178] Hardie LJ, Chappell LH, Secombes CJ. Human tumour-necrosis-factor-alpha influences rainbow-trout *Oncorhynchus-mykiss* leukocyte responses *Vet Immunol Immunopathol* 1994 Jan;40(1):73-84.
- [179] Tafalla C, Figueras A, Novoa B. Viral hemorrhagic septicemia virus alters turbot *Scophthalmus maximus* macrophage nitric oxide production. *Dis Aquat Org* 2001 Nov;47(2):101-7.
- [180] Mulero V, Meseguer J. Functional characterisation of a macrophage-activating factor produced by leucocytes of gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol* 1998 Feb;8(2):143-56.
- [181] Garcia-Castillo J, Chaves-Pozo E, Olivares P, Pelegin P, Meseguer J, Mulero V. The tumor necrosis factor alpha of the bony fish seabream exhibits the in vivo proinflammatory and proliferative activities of its mammalian counterparts, yet it functions in a species-specific manner. *Cell Mol Life Sci* 2004 May;61(11):1331-40.

- [182] Yin Z, Lam TJ, Sin YM. Cytokine-mediated antimicrobial immune response of catfish, *Clarias gariepinus*, as a defence against *Aeromonas hydrophila*. *Fish Shellfish Immunol* 1997 Feb;7(2):93-104.
- [183] Bird S, Zou J, Wang TH, Munday B, Cunningham C, Secombes CJ. Evolution of interleukin-1 beta. *Cytokine Growth Factor Rev* 2002 Dec;13(6):483-502.
- [184] Nakae S, Asano M, Horai R, Iwakura Y. Interleukin-1 beta, but not interleukin-1 alpha, is required for T-cell-dependent antibody production. *Immunology* 2001 Dec;104(4):402-9.
- [185] Sangrador-Vegas A, Martin SAM, O'Dea PG, Smith TJ. Cloning and characterization of the Rainbow trout (*Oncorhynchus mykiss*) type II interleukin-1 receptor cDNA. *Eur J Biochem* 2000 Dec;267(24):7031-7.
- [186] Falzacappa MV, Muckenthaler MU. Hepcidin: Iron-hormone and anti-microbial peptide. *Gene* 2005 Dec;364:37-44.
- [187] Maier VH, Schmitt CNZ, Gudmundsdottir S, Gudmundsson GH. Bacterial DNA indicated as an important inducer of fish cathelicidins. *Mol Immunol* 2008 Apr;45(8):2352-8.
- [188] Lovoll M, Dalmo RA, Bogwald J. Extrahepatic synthesis of complement components in the rainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunol* 2007 Oct;23(4):721-31.
- [189] Papanastasiou AD, Zarkadis IK. Gene duplication of the seventh component of complement in rainbow trout. *Immunogenetics* 2005 Oct;57(9):703-8.
- [190] Kong HJ, Hong GE, Nam BH, Kim YO, Kim WJ, Lee SJ, et al. An immune responsive complement factor D/adipsin and kallikrein-like serine protease (PoDAK) from the olive flounder *Paralichthys olivaceus*. *Fish Shellfish Immunol* 2009 Sep;27(3):486-92.
- [191] Soanes KH, Figuereido K, Richards RC, Mattatall NR, Ewart KV. Sequence and expression of C-type lectin receptors in Atlantic salmon (*Salmo salar*). *Immunogenetics* 2004;56(8):572-84.
- [192] Bristol JA, Morrison TE, Kenney SC. CCAAT/enhancer binding proteins [alpha] and [beta] regulate the tumor necrosis factor receptor 1 gene promoter. *Mol Immunol* 2009;46(13):2706-13.
- [193] Skugor S, Glover KA, Nilsen F, Krasnov A. Local and systemic gene expression responses of Atlantic salmon (*Salmo salar* L.) to infection with the salmon louse (*Lepeophtheirus salmonis*). *BMC Genomics* 2008 Oct;9.
- [194] Workenhe ST, Rise ML, Kibenge MJT, Kibenge FSB. The fight between the teleost fish immune response and aquatic viruses. *Mol Immunol* 2010 Oct;47(16):2525-36.
- [195] Pulendran B. Learning immunology from the yellow fever vaccine: innate immunity to systems vaccinology. *Nat Rev Immunol* 2009 Oct;9(10):741-7.
- [196] Buonaguro L, Pulendran B. Immunogenomics and systems biology of vaccines. *Immunol Rev* 2011 Jan;239:197-208.
- [197] Petrovsky N, Aguilar JC. Vaccine adjuvants: Current state and future trends. *Immunol Cell Biol* 2004;82(5):488-96.
- [198] Perrie Y, Mohammed AR, Kirby DJ, McNeil SE, Bramwell VW. Vaccine adjuvant systems: Enhancing the efficacy of sub-unit protein antigens. *Int J Pharm* 2008;364(2):272-80.
- [199] Anderson DP. Adjuvants and immunostimulants for enhancing vaccine potency in fish. In: Gudding R, Lillehaug A, Midtlyng P, Brown F, editors. *Fish Vaccinology*. Basel: Karger, 1997: 257-65.
- [200] Midtlyng PJ. Vaccinated fish welfare: Protection versus side-effects. In: Gudding R, Lillehaug A, Midtlyng P, Brown F, editors. *Fish Vaccinology*. Basel: Karger, 1997: 371-9.
- [201] Song L, Zhang Y, Yun NE, Poussard AL, Smith JN, Smith JK, et al. Superior efficacy of a recombinant flagellin:H5N1 HA globular head vaccine is determined by the placement of the globular head within flagellin. *Vaccine* 2009;27(42):5875-84.
- [202] Huleatt JW, Nakaar V, Desai P, Huang Y, Hewitt D, Jacobs A, et al. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a

References

recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. *Vaccine* 2008;26(2):201-14.

Paper I

Paper II

