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Francisella noatunensis ssp. *noatunensis* in Atlantic cod

- *Intracellular localization, innate immune responses and antibacterial proteins*

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ABBREVIATIONS AND ACRONYMS

AMP	Antimicrobial peptide
ACL-cells	Atlantic cod larvae cells
APC	Antigen presenting cell
CFU	Colony forming unit
CMI	Cell-mediated immunity
CR	Complement receptor
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
FcγR	Fc (Fc = region of immunoglobulins) gamma receptor
FPI	*Francisella* pathogenicity island
GAS	IFN-gamma activation site
IFN	Interferon
IL	Interleukin
IRF	Interferon regulatory factor
JAK-STAT	Janus Activated Kinase and Signal Transducer and Activator of Transcription
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MHC	Major histocompatibility complex
MR	Mannose receptor
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa B
NK cells	Natural killer cells
NO	Nitric oxide
OMV	Outer membrane vesicle
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
ROI	Reactive oxygen intermediates
SE-N	Surface-exposed nucleolin
SR-A	Scavenger receptor class A
TEM	Transmission electron microscopy
T_H cell	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TS-WGD	Teleost-specific whole genome duplication

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SUMMARY

Francisellosis has in the cod farming industry caused big economic losses because of no available vaccine or treatments. Understanding host-pathogen mechanisms is especially important when traditional vaccine strategies using inactivated bacteria are not functional.

The focus of this thesis has been to study interactions between the host and the pathogen causing the disease. The bacterium *Francisella noatunensis* subspecies *noatunensis* (here after called *F. noatunensis*) is the causative agent and a special feature with this particular pathogen is the intracellular lifestyle and the invasion of immune cells which are designed to kill pathogens. Cod macrophages were used as tools to investigate bacterial invasion, localization of the bacterium in the host cell, survival and replication. Additionally, expression of immune and antibacterial genes was measured after infection with the bacteria. LPS from both *F. noatunensis* and *E. coli* was used as immune stimulants and activation of macrophages with gamma interferon (IFN-γ) was conducted. Investigation of invasion, survival and replication of *F. noatuensis* in a cod cell line of epithelial-like cells (ACL-cells) were also included.

Important findings are that *F. noatunensis* is able to survive and replicate in both cod macrophages and ACL-cells. Inside macrophages the bacteria seems to release vesicles, disrupt the phagosomal membrane and escape into the cytosol. Pro-inflammatory responses in cod macrophages appeared to be low while an anti-inflammatory response was higher. Also *F. noatunensis* LPS are a poor inducer of pro-inflammatory cytokine in contrast to *E.coli* LPS. The low induction of inflammatory responses after *F. noatunensis* infection or treatment with its LPS, suggests that this bacterium may have evolved virulence mechanisms to subvert host immune responses in cod.

Uptake of the pathogen in macrophages is dependent of actin filaments and temperature appears important in invasion of cod macrophages. It has become clear that fish possess a well-functioning interferon system to regulate host defence against viral infections and also intracellular bacteria. Recombinant (r) IFN-γ from cod seemed to activate cod macrophages to increased uptake of *F. noatunensis* followed by a reduction of intracellular bacteria.

In addition, natural antibacterial peptides, produced by the organisms itself, were investigated. Lysozyme, cathelicidin and hepcidin were chosen as they are all identified in cod. Multiple goose-type (g-type) lysozyme genes have been found in cod and can be products of gene duplications. Analysis showed that cod seems to lack chichen-type lysozyme in contrast to

other fish like Atlantic salmon. Investigation further revealed that all three types of the studied antibacterial peptides are involved in cod host defence mechanisms *in vivo*. Infection of cod macrophages (*in vitro*) confirms the importance of g-type lysozyme, while expression of cathelicidin and hepcidin genes was at the same level as control. Even though both hepcidin and cathelicidin have antimicrobial roles in mammalian phagocytic cells it looks like *F. noatunensis* does not trigger expression of these genes in cod macrophages. This could mean that other cells or systems than macrophages are causing cathelicidin and hepcidin amplification in cod. G-type lysozyme on the other hand seems important in defence mechanisms in cod macrophages and several issues indicate an intracellular role of this peptide.

SAMMENDRAG

Francisellose har skapt store økonomiske tap ved oppdrett av torsk grunnet mangelen på både vaksine og behandling. Siden bruk av tradisjonell vaksinestrategi med inaktiverte bakterier ikke fungerer, er det spesielt viktig å bedre kunnskapen om vert-patogen mekanismen.

Fokuset i denne avhandlingen har vært å studere interaksjoner mellom verten og bakterien som forårsaker sykdommen. Det er bakterien *Francisella noatunensis* underart *noatunensis* (heretter kalt *F. noatunensis*) som er årsaken til sykdommen og spesielt for denne bakterien er dens intracellulære livsstil og invasjon av immunceller som i utgangspunktet er designet til å drepe patogener. Torskemakrofager ble brukt til å studere bakterieinvasjon, bakteriens lokalisering i vertscellen, overlevelse og replikasjon, i tillegg til utrykk av immungener og antibakterielle gener etter infeksjon med bakterien. LPS fra både *F. noatunensis* og *E. coli* ble brukt som immunstimulanter og aktivering av makrofager med interferon gamma (IFN-γ) ble utført. Det ble også inkludert en cellelinje på torsk med epitellignende celler (ACL-celler) for å studere invasjon, overlevelse og replikasjon av *F. noatunensis* i disse cellene.

Viktige funn er at *F. noatunensis* er i stand til å overleve og formere seg i både torskemakrofager og ACL-celler. Inne i makrofagene ser det ut til at bakterien skiller ut vesikler, ødelegger den fagosomale membranen og rømmer ut til cytosol. Pro-inflammatoriske responser i torskemakrofager synes å være lave mens anti-inflammatoriske responser var høyere. LPS fra *F. noatunensis* fremkaller også lavt genuttrykk av pro-inflammatoriske cytokiner i motsetning til hva LPS fra *E. coli* gjør. Denne svake inflammatoriske responsen etter både infeksjon med *F. noatunensis* eller behandling men bakteriens LPS kan tyde på at bakterien har utviklet mekanismer for å unngå vertens immunresponser.

Opptak av bakterier i makrofager er avhengig av aktinfilamenter og temperatur ser ut til å ha en innvirkende kraft på invasjonen i torskemakrofagene. Det er kjent at fisk har et velfungerende interferonsystem for å regulere vertens forsvar mot virusinfeksjoner i tillegg til intracellulære bakterier. Rekombinant (r) IFN-γ fra torsk ser ut til å aktivere torskemakrofagene til økt opptak av *F. noatunensis* etterfulgt av en reduksjon av intracellulære bakterier.

I tillegg ble naturlige antibakterielle peptider som verten selv produserer studert. Lysosym, katelicidin og hepsidin ble valgt med bakgrunn i at de er funnet i torsk tidligere. I torsk er det funnet mange såkalte «goose-type» (g-type) lysosymgener som kan være produkter av genduplisering. Ifølge analysene ser det ut til at torsk, i motsetning til Atlantisk laks, mangler «chichen-type» lysosym. Videre viser undersøkelsene at alle tre typene av de studerte antibakterielle peptidene er involvert i torskens forsvarsmekanismer *in vivo*. Infeksjon av torskemakrofager (*in vitro*) bekrefter betydningen av g-type lysosym, mens utrykk av katelicidin- og hepsidingener hadde samme nivå som kontrollen. Selv om katelicidin og hepsidin har en antimikrobiell rolle i fagocytter hos pattedyr, ser det ut til at *F. noatunensis* ikke trigger utrykk av disse genene i torskemakrofager. Dette kan bety at det er andre systemer enn fagocyttene som produserer katelicidin og hepsidin under infeksjonen *in vivo* i torsk. G-type lysosym ser derimot ut til å være en viktig forsvarsmekanisme i torskemakrofager og flere ting tyder på at peptidet har en intracellulær rolle.

LIST OF PAPERS

Paper I

Intracellular localisation and innate responses following *Francisella noatunensis* infection of Atlantic cod (*Gadus morhua*) macrophages.

Kathrine R. Bakkemo, Helene Mikkelsen, Marianne Bordevik, Jacob Torgersen, Hanne C. Winther-Larsen, Christin Vanberg, Randi Olsen, Lill-Heidi Johansen and Marit Seppola (2011)

Fish & Shellfish Immunology 31: 993-1004

Paper II

***Francisella noatunensis* subsp. *noatunensis* invade, survive and replicate in Atlantic cod cells.**

Kathrine R. Bakkemo, Helene Mikkelsen, Audny Johansen, Børre Robertsen and Marit Seppola

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Paper III

Multiple specialised goose-type lysozymes potentially compensate for an exceptional lack of chicken-type lysozymes in Atlantic cod.

Marit Seppola, Kathrine R. Bakkemo, Helene Mikkelsen, Bjørnar Myrnes, Ronny Helland, David M. Irwin and Inge W. Nilsen (2016)

Scientific Reports 6, Article number 28318

1. INTRODUCTION

Atlantic cod in aquaculture

The aquaculture industry, with the production of Atlantic salmon (*Salmo salar*) in front, is a highly important business in Norway. Norway is and has been a world leading manufacturer of salmonids and did in 2015 export salmon and trout worth NOK 47.7 billion NOK. Atlantic cod (*Gadus morhua* L.) was introduced to intensive aquaculture in the late 1980s with expansion of cod farming in the early 2000s. In Norway there has been a long tradition in wild-catch of cod and farming of cod was commercialized to serve the market with fresh cod the whole year around. The cod farming industry was, however, confronted with some difficulties. A high production cost of farmed cod required a steady market and high market price. With falling prices for wild-caught cod, farmers were facing decreasing profit because of the trade competition. Additionally, the industry had some biological and technological challenges like high mortality in early stages, early sexual maturation, escapes and bacterial diseases like vibriosis, atypical furunculosis and from 2004; francisellosis. The consequence was that while the production of Atlantic salmon continued to grow from almost 1 million tons in 2010 to 1.3 million tons in 2014, the cod farming industry declined from 20.621 to 1213 tons in the same time period. After the financial crises in 2008, there were just a few cod farming companies left.

In aquaculture, diseases give significant losses for the farmers and improvement of disease control is one of the identified success factors in farming of cod [1]. The bacterial disease francisellosis have caused big economically losses in the south Western part of Norway especially when fish ready for slaughters are affected. In contrast, there have been only a few cases of francisellosis in Northern Norway and they may have been due to transportation of juvenile fish from the south. Today there is no available vaccine or treatment against francisellosis in cod.

As an introduction to the field, a brief description will be given about the diseases in the Norwegian cod farming industry, about the host defence mechanisms of human and fish and about the pathogenesis of a closely related and well-studied human bacterium.

Diseases in cod

Both viral and bacterial diseases have been reported in cod while bacterial diseases have been the main problem. The most common bacterial pathogens causing mortalities in cultured Atlantic cod are *Vibrio* (*Listonella*) *anguillarum* (vibriosis), *Aeromonas salmonicida* (atypical furunculosis) and *Francisella noatunensis* subspecies *noatunensis* (francisellosis) [2]. Also viruses like nodavirus causing viral nerve necrosis (VNN) and infectious pancreatic necrosis virus (IPNV) can infect Atlantic cod but only VNN has been reported (Table 1).

Table 1: Reported outbreaks of the most important diseases on cod in Norway

Disease	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015
Francisellosis	4	7	8	14	8	3	3	2	1	1	0
Vibriosis	18	19	19	20	16	6	*	5	0	0	*
Atypical furunculosis	3	13	9	16	16	5	*	1	0	*	*
VNN	0	3	6	3	1	0	0	0	0	0	*
IPN	0	0	0	0	0	0	0	0	0	0	*

* Data not available. Source: reports on the health status in Norwegian fish farms from Norwegian Veterinary Institute [3-8].

Francisellosis

The fish disease francisellosis was first discovered in farmed Atlantic cod on the west coast of Norway in 2004 and the causative agent was determined to be most closely related to the human bacterium *Francisella philomiragia* [9]. Initially the bacterium was described as *F. philomiragia* subspecies (ssp.) *noatunensis* [10] and *F. piscicida* [11], but 16S rDNA sequences for both type strains revealed that the two published names represented the same species of bacterium [12]. The official name has now been modified to *F. noatunensis* ssp. *noatunensis* [12-14] and the name *F. noatunensis* will be used in this thesis.

F. noatunensis is characterized as Gram negative, coccoid (size ~ 0.5-1.7 µm), strictly aerobic, facultative intracellular bacteria [13, 15]. The bacterium grow on blood agar enriched with cysteine and growth is enhanced in the temperature range of 6 – 22˚C, with an optimum at about ~20˚C [9]. No growth at 37 °C has been reported, suggesting that the bacterium is unlikely to pose a risk of zoonotic infection [12, 15].

Affected fish lose appetite, have reduced swimming performance, dark pigmentations and in some cases skin ulcers [9, 12, 15]. Internal signs range from slightly swollen spleen and kidney to white granulomas covering and infiltrating many of the internal organs. Granulomas are seen especially in the spleen (Fig. 1), kidney and heart, but also in the liver, intestine, white muscle, gills, eyes and brain [9, 15]. Sero-haemorragic ascites may also be present [15]. However, the fish can be affected without clinical signs [15, 16]. The bacterium has also been detected in wild caught fish in Sweden [17] and Norway [16] and a surveillance study has revealed that francisellosis was present in wild cod in the southern North Sea already in the 1980s [18].

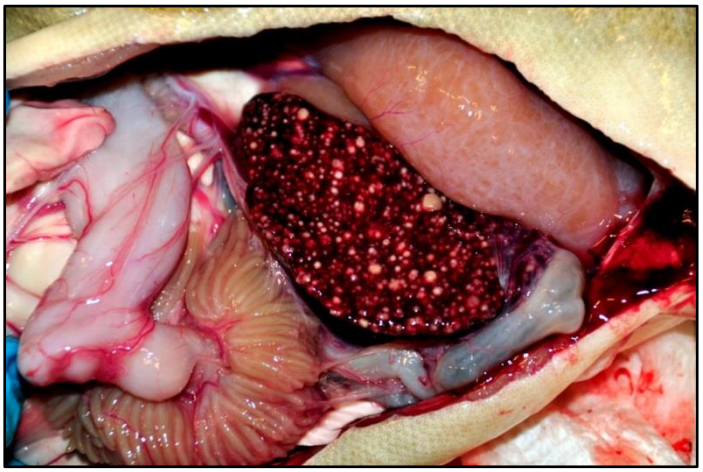

Figure 1: Swollen spleen with granulomas (white nodes) in cod after outbreak of francisellosis at Austevoll, Norway. Photo: Rama Bangera, Nofima AS

This severely systemic and chronic disease turned out to be a serious problem in commercial cod farming. In accordance with the decline in the production of farmed cod from 2008-2010, outbreaks of francisellosis have dropped. In 2012 the disease was only identified in two farms, one in Møre og Romsdal and one in Sogn og Fjordane, followed by one outbreak in Sogn og Fjordane in 2013 and one in Nord Trøndelag in 2014 (Table 1). In 2015 there were no outbreaks of the disease in Norway. However, we have reasons to believe that the disease will return as a problem if the production of farmed cod is expanding.

A number of other fish species including tilapia (*Oreochromis niloticus*), Atlantic salmon, hybrid striped bass (*Morone chrysops*/*M. saxatilis*), three-line grunt (*Parapristipoma trilinineatum*) and also the shellfish giant abalone (*Haliotis gigantea*) are reported to be affected by *Francisella* ssp. (reviewed in [12, 19]).

The immune system in vertebrates

It is commonly acknowledged that the immune system in vertebrates is divided into the **innate** immune system and the **adapted** (acquired) immune system. The innate immune system serves as the first line of host defence by allowing the rapid recognition of a broad spectrum of pathogens and stimulation of an antimicrobial response [20, 21]. The adaptive immune system, on the other hand, is involved in elimination of pathogens in the late phase of infection (after 5-7 days in human) and is composed of highly specialized lymphocytes like T-cells and B-cells that generate immunological memory [22].

Together these two systems have a close relationship, where the main function is firstly to distinguish microbial invaders from self and secondly induce the right type of innate and adaptive immune responses [23, 24]. The response of the immune system is an intricate and coordinated set of interactions among many different cells and proteins.

The immune system of fish is less studied compared to mammals, but a repertoire of innate and specific defence mechanisms have been described for several fish species [25]. Fish do not have bone marrow or lymph nodes. Myeloid cells are in fish derived from the head kidney and/or the spleen while the thymus, kidney and spleen are the major lymphoid organs [26].

Innate defence mechanisms

Skin and mucosal surfaces in both mammals and fish harbour the first line of defence against infections. In fish, the skin serves as a physical barrier while the mucosae contain antimicrobial substances like lysozyme, which kills microorganisms by disrupting the cell membrane [27]. When pathogens break through these barriers and enter the tissue or bloodstream, the innate immune system is designed to recognize the pathogen and induce effector mechanisms that kill the intruder. Spread of the pathogen through the bloodstream (or/and the lymph system in mammals) can lead to a systemic infection of the host.

Immune cells, traditionally called white bloods cells or leukocytes, identify and eliminate pathogens and coordinate the function of other part of the immune system by producing immunoregulatory cytokines. Innate immune cells include granulocytes, dendritic cells (DC), monocytes and macrophages, neutrophils and natural killer (NK) cells. All these cell types are found in teleost fish [26], but are much less characterized. Monocytes and macrophages will be discussed later as they are a major part of the research in this thesis.

Pathogen recognition

In the innate immune system microorganisms are recognized by a limited number of germline-encoded pattern recognition receptors (PRRs) expressed on effector cells like macrophages and dendritic cells, but also in various nonprofessional cells like epithelial cells and fibroblasts [28, 29]. These PRRs recognize different microbial structures (unique to microorganisms) like proteins, peptidoglycan, phospholipids, carbohydrates and nucleic acids known as pathogen-associated molecular patterns (PAMPs). Four different classes of PRR families have been identified and they all react with different specific PAMPs. These families include membrane-bound proteins such as (1) the Toll-like receptors (TLRs); (2) C-type lectin receptors (CLRs), and cytoplasmic proteins such as (3) the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs); and (4) nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (reviewed in [29, 30]).

TLRs are the largest family of the PRRs and play a central role as sensors of infection [31]. These receptors are located on the cell-surface membrane or in intracellular compartments of a variety of cell types like epithelial cells and antigen presenting cells (APCs) like dendritic cells (DCs) and macrophages [31]. In vertebrates there are currently around 20 TLRs family members [32] where humans possess 10 TLR members [31]. TLRs situated on the cell surface (TLR1, 2, 4, 5, 6 and 10) primarily detect bacterial components while TLRs in endocytotic vesicles and organelles (TLR3, 7, 8, 9, 11, 12 and 13) recognize different nucleotide species [30, 31].

TLR genes are found in several teleost species. Jault et al. (2004) discovered all orthologues (10 TLRs) of mammalian TLR genes in zebrafish (*Danio rerio*) [33]. Six teleost-specific TLRs (TLR14, 19, 20, 21, 22 and 23) have been identified in fish [34]. Still, several genes of this receptor family are absent in fish, like TLR4, TLR6 and TLR10. However, the understanding of the function of the receptors in fish is limited and some receptors seem to recognize more than one PAMP.

In Atlantic cod a unique composition and expansion of TLR genes was found in the genome [35]. Recent studies have used the Atlantic cod genome draft to characterize TLR genes [32, 36]. Sundaram et al. (2012) characterized and cloned 15 genes from the teleost-specific TLR21, 22 and 23 in cod [36] and Solbakken et al. (2016) demonstrated expansion of TLR7, 8, 9, 22 and 25 in addition to loss of TLR1/6, 2, 4 and 5 [32]. Whole genome and gene duplications are believed to have contributed to a greater genomic diversity in bony fish [34].

Whole genome duplication (WGD) leads to doubling of the chromosomal set and a teleost-specific (TS) WGD event is believed to have taken place 320 – 350 million years ago [37]. However, fish genome diversity was further increased after the TS-WGD by linage-specific events of genome duplications and/or duplications of single genes or set of genes [37, 38].

Lipopolysaccharide (LPS)

The bacterial endotoxin LPS is typically one of the first PAMPs to be detected by the host immune system [39]. LPS is a major component of the cell wall of Gram negative bacteria which stimulate the innate inflammatory response mainly via TLR4. It consists of a polysaccharides chain called the O-chain (O-antigen), an oligosaccharide core region and a hydrophobic lipid portion termed lipid A. The lipid A portion of LPS is responsible for the activation of the innate immunity in mammals and is one of the most potent PAMPs known [20]. LPS activates macrophage antimicrobial effector functions and the production of pro-inflammatory cytokines like tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β) and IL-6. In mammals, LPS is recognised by a cascade of receptor and accessory proteins. LPS binding protein (LBP) in the bloodstream transfer LPS released from the bacteria to CD14 on the surface of macrophages. CD14 then presents the LPS molecules to the TLR4-MD-2 complex [40].

The mechanisms of LPS recognition in fish are different from mammals [41]. It has long been recognised that lower vertebrates like fish are resistant to the toxic effect of LPS, while higher vertebrates are extremely sensitive to the endotoxin [42, 43]. A recent study confirms that a high LPS dose is needed to induce inflammatory responses in cod [44]. Genome sequences of Atlantic cod revealed absence of the TLR 4 gene [32, 35]. However, the overall increased number of TLRs in cod may represent functional shift of TLR copies and thus the mechanisms of the receptors recognitions may be different from that of mammals. This could be the case in zebrafish where TLR4 has been identified but it was not found to be a receptor for LPS [41]. The authors suggest that this can explain the tolerance of fish to LPS.

Despite the unusual LPS recognitions in fish, *Escherichia coli* LPS with different grades of purities induce immune responses in fish like cod and salmon monocytes/macrophages [44]. Additionally the same study reports similar responses *in vivo* in cod.

Inflammation

After the recognition of bacteria, an inflammatory response is induced to limit or prevent infection. The characteristic inflammatory response results in redness, swelling, heat and pain at the site of infection [29]. In mammals, these symptoms are caused by increased vascular dilation and blood flow (causing redness and heat), extravasation and deposition of plasma fluid and proteins (swelling), and leukocyte emigration and accumulation in the site of infection. In addition, the inflammatory response can lead to the formation of granulomas where the pathogens are encapsulated [26].

The inflammatory response is generated by a number of pro-inflammatory cytokines [29]. Cytokines are signal proteins and key regulators of both innate and adaptive immunity and can be produced by immune cells like macrophages and T-cells [45]. Chemokines are small protein molecules secreted by cells to induce chemotaxis in nearby cells [46]. In general, cytokines are secreted from one cell and bind to receptors on a target cell, or even on the cells producing the cytokine (self-stimulation).

Pathogen recognition of microbes by PRRs triggers a signalling pathway where genes involved in inflammation and microbicidal activity are upregulated. The transcription factors activator protein-1 (AP-1), nuclear factor κB (NF-κB) and interferon regulatory factor 3 (IRF-3) initiate and regulate the transcriptional cellular response to microbial infections [30]. These transcripts encode pro-inflammatory mediators, including cytokines and antimicrobial proteins. However, tight regulation of gene expression at the transcriptional levels is essential in the generation of a suitable immune response to ensure strong response early during infection as well as down regulation when needed at later stages [20].

In mammals there are several pro-inflammatory cytokines like TNF-α, IL-1β and IL-6 [24]. TNF-α is not found in cod but several of other cytokines are characterized such as IL-1β, IL-6, IL-8, IL-10 and IL-12p40 subunit. The subunit p40 refers to the size (kDa) of one of the covalently linked glycosylated chain of the heterodimer IL-12 [47]. A brief introduction to a selection of cytokines will be given below as they have been used as markers or primers in the following research.

IL-1β has a key role in initiating and maintaining the inflammatory response, by regulating expression of other cytokines and chemokines. In mammals, IL-1β is produced mainly by blood monocytes and tissue macrophages. The transcription of pro-IL-1β is induced by the transcription factor NF-κB. After recognition of bacteria, the activation of caspase-1 is

required for processing of IL-1 family [24]. Active caspase-1 cleaves the pro-inflammatory IL-1 family of cytokines into their bioactive forms, IL-1β and IL-18 [48]. The complex of proteins that activates caspase-1 is called the inflammasome. Inflammasomes consist of caspase-1, adaptor protein ASC (Apoptosis-associated speck-like protein containing a CARD) and an inflammasome sensor molecule (often NOD-like receptors; NRLs) [49]. IL-1β together with IL-6, activates hepatocytes (predominant cell type in the liver) to produce acute-phase proteins which activate complement and opsonize pathogens [24]. IL-6 is produced in several immune cells like monocytes, macrophages, B- and T-cells and endothelial cells. Furthermore IL-6 stimulates the proliferation of antibody-B lymphocytes and is important in T-cell responses [50].

IL-8 is a member of the CXC chemokine family and stands as an important pro-inflammatory cytokine. This tiny cytokine is produced by a wide variety of cells and induce the migration of leucocytes to the site of the infection [51].

Interferon gamma (IFN-γ) is a type II interferon and a key signal molecule for induction of antibacterial effects of both the innate and the adaptive immune system. Downstream IFN-γ signalling in macrophages results in activation of antimicrobial mechanisms. In early host defence IFN-γ is secreted from NK cells and APCs, stimulated by IL-12 and IL-18. Especially IL-18 is seen as a key factor in early host resistance against intracellular pathogens in mice [52]. Pathogen recognition by macrophages induce secretion of IL-12 and chemokines which attract NK cells to the site of infection [53]. Additionally IFN-γ is involved in cross-talk between nearby cells and possible self-activation since professional APCs, like macrophages and dendritic cells, secrete IFN-γ [53]. Later on in the adaptive immune response, T-cells are the major source of IFN-γ [54]. IL-12 also promotes the differentiation of $CD4^+$ T-cells to T_H1 cells that produce IFN-γ, important in cell-mediated immunity [52]. IFN-γ, IL-12 and IL-18 have thus a key role in linking innate and adaptive immune responses and are especially important in intracellular infections [53].

Since tissue and systemic lesions can occur during immunopathology, regulation of immune mechanisms is necessary. IL-10 has a key function in supressing the inflammatory response by down-regulating other cytokines mainly at the transcriptional level [55]. This anti-inflammatory cytokine is produced by macrophages, dendritic cells, B- and T-cells [56].

Altogether, the inflammatory immune response to microbial infections is a complex process in which the binding of PAMPs to PRRs promote a fine-tuned defence toward the pathogen without generating tissue damage, and at the same time stimulate adaptive immune responses.

Cytokines are good markers of inflammatory responses in the host. In teleost fish interleukins like IL-1β, 6, 8, 10, 12 and 18 are all characterised (reviewed in [45]). Analysis of the *Fugu rubripes* genome (Japanese pufferfish), the first vertebrate genome sequenced after human, identified many immune genes for the first time in fish [57]. Since then these inflammatory cytokines have been discovered in several teleosts. In Atlantic cod, IL-1β, IL-8, IL-10 and IL-12p40 subunit have been characterised [58, 59]. In addition, IFN-γ and interferon stimulating gene 15 (ISG-15) have both been cloned and characterised [60-62]. After *F. noatunensis* injection in Atlantic cod, measurement of IL-1β and IFN-γ showed up-regulation of gene expression in the spleen and head kidney [63]. IL-10 was also present and significantly up-regulated in the intestine after 60 days. Furthermore, cohabitant fish had increased gene expression of these cytokines.

Subcellular mediators of the innate immune system

Beside cellular components humoral constituents such as complement, antimicrobial peptides (AMPs), lactoferrin and acute phase reactants are examples of important mediators in the innate immune system. An introduction to complement and a selection of AMPs will be given below.

Complement

The complement system is an important arm of the innate immunity against pathogens located in the humoral compartment. The main functions are lysis of the microbial cell membrane through the complement membrane attack complex (MAC), opsonization of microbes for enhances phagocytosis and generation of inflammatory responses [64].

The complement system can be activated by the classical, alternative or lectin pathways. The classical pathway is activated by binding of antibodies to bacteria while the lectin pathway is activated by binding of lectin. The alternative pathway is independent of antibodies and lectins and relies upon spontaneous activation of complement factor 3 (C3) which is abundant in the blood plasma. All three pathways differ in how they are initiated, but all results in the same effector function. Briefly, regardless the means of activation, C3 is cleaved into C3a and C3b. The newly formed C3b binds to the microbe and interact with plasma proteins (dependent of the activation pathway) that result in the formation of C3 convertase. This is a

proteolytic enzyme that cleaves more C3 leading to a massive deposition of C3b into the microbial surface. C3 convertase and C3b can form C5 convertase that is able to cleave C5 into C5a and C5b, which finally generates the MAC involving the terminal factors C5b, C6, C7, C8 and C9 leading to cell-lysis [65].

Opsonization of bacteria occurs when the protein C3b binds to the surface of the bacteria. C3b act as an opsonin and contribute to enhanced phagocytosis by interacting with complement receptors (CRs) expressed on phagocytes. C3 is the most abundant complement protein in the blood and CRs are important receptors involved in host cell recognition of several serum opsonized intracellular bacteria [66]. In addition, serum components like C3a and C5a have been shown to induce leukocyte migration [46]. Complement proteins also play an important role in modulating adaptive immune responses like B- and T-cell activation [65].

In teleost fish, the complement system can be activated by all three pathways [65]. In contrast to mammals, complement in teleosts is active at very low temperatures and C3 in fish are present in multiple isoforms that seems to provide a broader recognition of microorganisms [65].

Antimicrobial peptides

In addition to complement proteins, the host also produce other antimicrobial peptides (AMPs) that play an important role in the first line of host defence against invading pathogens. AMPs are often cationic, which facilitates adhesion to the negatively charged bacterial surfaces. There are many examples of AMPs, but lysozyme, hepcidin and cathelicidin are all previous studied in Atlantic cod and will be described below as they are used as tools to study immune responses after infection.

Lysozyme is an important defence molecule involved in innate defence. It is defined as a bactericidal enzyme that catalyse the hydrolysis of the β-1,4 linkages between N-acetylmuramic acid and N-acetylglucosamine in the cell walls (peptidoglycan layers) of Gram positive bacteria resulting in lysis. Gram negative bacteria can also be affected by lysozyme in combination with complement and other enzymes exposing the inner peptidoglycan layer [27].

There are several types of lysozymes, but only chicken-type (c-type) and goose-type (g-type) have been reported in vertebrates [27]. Both types differ in amino acid sequence, biochemical and enzymatic properties [67]. In cod only g-type lysozyme has so far been identified, while

salmon have both g- and c-type [68]. Larsen et al. (2009) found two variants of g-type lysozyme transcript in Atlantic cod and both genes were characterised and cloned [69]. Recently Irwin (2014) reported, after studying 118 different vertebrate species, that most of the species had two genomic sequences similar to g-type lysozyme [70]. However, the genome sequences of two bony fish (gar; *Lepisosteus oculatus* and tilapia; *Oreochromis niloticus*) revealed no g-type lysozyme genes, while single g-type genes were found in the genomes of many teleosts [70]. Atlantic cod had most g-type lysozyme genes with as many as 11 potential genes. Since these genes are distributed on small genomic contigs, it is difficult to identify how many proteins they actually encode. Like mammals, lysozyme in fish is mainly produced by leukocytes such as monocytes, macrophages and neutrophils. A recent finding indicates that g-type lysozymes in cod also are produced by macrophages since g-type lysozyme was strongly associated with macrophages inside granulomas [71]. The enzyme is present in the skin mucus, spleen, serum, gills, liver and muscle (reviewed in [27]). Cod is also known to have low increase of antibody titre after vaccination and still possess protection. Earlier Caipang et al. (2008) found that serum from Atlantic cod had antibacterial properties after vaccination with heat-killed *L.* (*Vibrio*) *anguillarum*, which implies that cod may have other components of the immune system than antibodies dealing with bacterial infections [72]. In 2009, Caipang et al. found enhanced expression of g-type lysozyme, in combination with others, in the spleen of Atlantic cod vaccinated with heat-inactivated *V. anguillarum* [73]. Consequently, antibacterial peptides and enzymes seem to have important roles in the immune system of cod.

Hepcidin was first discovered as an AMP and later known to be a key regulator in iron homeostasis [74]. It is a peptide hormone produced in the liver in response to inflammatory stimuli and iron overload [75]. It is well established that pathogens acquire iron for growth and pathogenicity and therefore iron have a regulatory role in the immune system [76]. The effector mechanism of hepcidin involves regulation of the cellular iron exporter molecule, ferroportin-1 [75]. Several fish species possess hepcidin-like genes [77-79]. Gene expression of hepcidin in winter flounder (*Pseudopleuronectes americanus*) and Atlantic salmon increased after challenge with *A. salmonicida* suggesting an antimicrobial role of the peptide [80]. Also high levels of hepcidin expression were detected in Atlantic cod tissues 2 days after injection of inactivated *V. anguillarum* [81].

Cathelicidins are a well-studied family of antimicrobial peptides [82]. The peptide is expressed in several cell types like neutrophils, macrophages, monocytes and epithelial cells

[83]. Cathelicidins possess a highly conserved region (cathelin domain) and a variable C-terminal antimicrobial domain which bind to negatively charged groups of the bacterial surface causing cell lysis [82]. In fish several cathelicidins have been identified and *V. anguillarum* and *A. salmonicida* have both been reported to increase expression of antibacterial genes like cathelicidin, g-type lysozyme and hepcidin in gill epithelial cells from cod [84].

The adaptive immune system

The initial inflammatory response is followed by an adaptive immune response with highly specific receptors. The adaptive immune system can be divided into two different arms; the humoral immune system that includes antibodies and B-lymphocytes (B-cells), and the cellular immune system (or cell-mediated immunity; CMI) that provide defence against intracellular microbes by T-lymphocytes (T-cells). Adaptive immune recognition is mediated by two types of antigen-specific receptors: B-cell receptors and T-cell receptors which recognize different chemically structures. B-cell receptors have a broad specificity for different macromolecules such as proteins, lipids, carbohydrates and nucleic acids, including small parts of these molecules. In contrast, T-cell receptors recognize only peptides displayed on APCs.

Since pathogens may be present and replicate both extracellularly (most bacteria, fungi and parasites) and intracellularly (viruses and some bacteria and parasites), different components of the immune system have evolved to combat different types of pathogens.

In the humoral immune system (related to the body fluids), B-cells are responsible for the production of antibodies (immunoglobulins). Antibodies provide protection by neutralising the pathogen and avoid attachment to host cells, increase phagocytosis (opsonization) and activate complement. After exposure to a pathogen, B-cells divide into memory cells or plasma cells. These cells are long-lived and when re-exposed to the same pathogen they rapidly proliferate to produce antibody-producing plasma cells.

CMI involves clonal expansion of specific T-cells marked with the co-receptor CD4 or CD8. All cells of the immune system have their origin in the bone marrow (head kidney in fish) and the precursor T-cell migrate to the thymus where they differentiate into $CD4^+$ T helper lymphocytes (T_H-cells) and $CD8^+$ cytotoxic T lymphocytes (CTL). Native T_H cells can differentiate into several effector/regulatory cells where the best described mammalian

subtypes are T_H1, T_H2, T_H17 and regulatory T cells (T_{Reg}) [24, 85]. The effector response is controlled by cytokines produced in response to PRR activation in the innate immune system. IFN-γ and IL-12 are the main mediators in CMI and promotes differentiation of $CD4^+$ and $CD8^+$ T cells into the T_H1 and CTL subtypes, respectively. T_H1 cells are involved in inflammation by recruiting and activating phagocytes while T_H2 cells stimulate B-cells to produce antibodies. T_H1-cells are needed for activation of $CD8^+$ T- cells to CTLs by secreting IL-2 [22].

The major histocompatibility complex (MHC) class I molecule, present on the cell surface of all nucleated cells, detect and express foreign substances called antigens, to CTL. MHC class II are present on the surface of professional APCs, like macrophages, DCs and B-cells. On the surface of APCs, antigens from digested bacteria are presented on MHC class II to T_H cells.

CMI is seen as most important in protection against intracellular pathogens [22]. The type of effector T-cell response is dependent on the intracellular localization of the bacteria. Antigens from pathogens in the cytosol, presented on MHC class I, activate CTLs, and phagosomal or vesicular pathogens, presented on MHC class II, activate T_H1 cells [22]. Immune responses that protect against intracellular pathogens are often referred to as "type 1 immunity" and include cytotoxic functions like NK cells, T_H1 and CTL activity in contrast to "type 2 immunity" that control extracellular parasite infections involving activation of mast cells, granulocytes, T_H2 cells and secretion of antibodies [85].

In fish the knowledge of infections with intracellular bacteria and the subsequent immune response is limited. However genes encoding immunoglobulins, MHCs and T-cell receptors have been identified in several teleosts (reviewed in [26]). Atlantic cod on the other hand seems to lack MHC class II, CD4 and invariant chain (Ii) which are essential for antigen presentation to T_H2 cells to initiate the humoral immune response.

Cod have a unique immune system

As already mentioned, the immune system in cod is different from human and from other species of teleosts. Early studies indicated that cod produced low levels of specific antibodies following immunization (reviewed in [2, 86]). In addition there was also stated that the concentrations of natural antibodies and immunoglobulin M (IgM) was much higher in cod serum. Pilström et al. (2005) hypothesized that deficiency of MHC class II could explain the lack of specific antibody response in cod [87]. Regardless of this, cod develop protective

immunity after vaccination with bacterial diseases, like vibriosis [88]. More recent studies have reported that Atlantic cod has specific antibody responses against *V. anguillarum*, *A. salmonicida*, *F. noatunensis* [89, 90] and inactivated *F. noatunenis* [63], but not against atypical *A. salmonicida* [91]. Larsen et al. (2016) have recently discovered specific antibodies in cod after infection with the intracellular pathogen *Brucella pinnipedialis* [92]. Known protective antigens inducing specific antibody responses in cod seems to be primarily against the LPS components of the bacteria [86].

In a recent study using high throughput sequencing technology of the cod genome, Atlantic cod seems to have lost the genes encoding MHC class II, CD4 and invariant chain (Ii) [35]. This supports the hypothesis from Pilström et al. (2005) that MHC class II is absent in cod [87]. The lack of genes encoding the MHC class II molecule suggests that cod also may lack the functional equivalents of T helper (T_H1, T_H2 and T_H17) and regulatory lymphocytes (T_{Reg}). All these components are conserved vital immune genes in jawed vertebrates and part of the adaptive immune system. The fact that Atlantic cod survive without these important immune genes suggests that mechanisms other than the classical adaptive immune response are responsible for protection. Hypotheses have been made that cod may compensate for their missing MHC II by having much more genes of MHC class I. However, it is difficult to distinguish such compensatory mechanisms especially since fish MHC class I and II genes are not encoded in the same genomic region [38].

In addition to the high expansion of MHC I genes, also an increased number of genes for TLR, fundamental in pathogen recognition, were found [35]. The majority of the TLR families found in the cod genome were receptors recognizing nucleic-acids, and represent the highest number of TLRs found in a teleost. As mentioned earlier, this unusual TLRs and MHC class I repertoire is most likely due to the TS-WGD and probably more recent genome duplication events. Taken together it is possible that cod rely more on cellular immune defence (MHC class I pathway) and non-specific innate mechanisms.

Phagocytosis and macrophage defences against intracellular pathogens

In addition to stimulate the inflammatory response, PAMPs also initiate phagocytosis. The innate immune system has effector cells named professional phagocytes like neutrophils, macrophages and monocytes (the macrophage precursor). Macrophages are present in all body compartments and are the first phagocytes to encounter the invading pathogens [93].

Phagocytosis

Phagocytosis (engulfment of large particles, usually over 0.5μm in diameter) is accomplished through a sequence of events in which the pathogens first binds to a receptor on the surface of the phagocyte and secondly the phagocyte engulfs the particle by cytoskeletal rearrangements (Fig.3). Pseudopods extended from the phagocyte surround the bacterium and finally enclose it in a phagosome [94].

Macrophages display a variety of membrane receptors, like TLRs, that allow them to recognize and engulf pathogens [28]. In addition they have cell-surface receptors for the Fc portion of the antibodies (Fc receptor-mediated phagocytosis), as well as the C3b component of complement (complement receptor-mediated phagocytosis). The mannose receptor (MR) on macrophages recognises mannose and fucose on the surface of pathogens (mannose receptor-mediated phagocytosis) and scavenger receptors such as SR-A recognises lipoprotein. Ligand binding to any of these receptors leads to the polymerization of actin at the site of pathogen attachment, causing the phagocyte plasma membrane to surround the pathogen in a large membrane-enclosed phagosome [95].

The mechanisms of phagocytosis are complex. The receptors involved may recognize more than one structure, have dual functions and cross-talk [95]. Phagocytic receptors may bind to pathogens directly or via opsonins.

Macrophages in teleosts

Phagocytes like macrophages play a central role in the defence against microbial infections in fish. The primary function of phagocytes is to destroy pathogens and digest their remains. The monocytes/macrophages internalise particles effectively and initiates the innate immune response by secretion of pro-inflammatory cytokines and chemokines, and stimulates the later adaptive responses by antigen-presentation and cytokine secretion. In mammals the circulating monocytes are derived from hematopoetic organs (bone marrow) and migrate into tissues where they differentiate to macrophages. Since teleosts do not have bone marrow or lymph nodes, monocytes are produced in the head kidney [26]. Tissue macrophages are found in lymphoid organs like the kidney, spleen and thymus, but also in other tissues. Both monocytes and macrophages are able to perform phagocytosis and microbial killing, but macrophages are more efficiently and live longer.

Macrophages play an important role also in adaptive immune responses because of their antigen-presenting function and secretion of cytokines. As mention earlier, the release of

cytokines attracts immune cells from blood and haematopoetic tissues to the infection site. The presence of phagocytic cells in teleost, like macrophages and monocytes, are well documented over the years (reviewed in [96]).

Activation of macrophages

The process of transformation macrophages to highly potent killer cells is referred to as macrophage activation [97]. Over 50 years ago George Mackaness invented the term macrophage activation [98], but in 1983 Nathan et al. proved that the start signal is conducted by the secreted cytokine IFN-γ [99]. During a microbial infection, a cytokine-dependent set of reactions induce the synthesis of a number of highly toxic molecules. Microbial stimuli as well as IFN-γ may also activate macrophages [100]. Activation is then induced through the detection of microbial structures by different PRRs on the surface of the macrophage.

IFN-γ is important in regulation of antimicrobial responses. Binding of IFN-γ to its receptor (IFN-γR or IFNGR1 and 2) leads to activation of Janus Activated Kinase (JAK) and Signal Transducer and Activator of Transcription (STAT) pathway and expression of IFN-γ controlled genes (Fig. 2) [52]. IFN-γ binds to the receptors and activates JAK1 and JAK2, which in turn phosphorylate STAT1s leading to dimerization. STAT1 then directly translocates to the nucleus and bind IFN-gamma activated site (GAS) resulting in expression of transcription factors like IRF-1, IRF-2 and IRF-9 and other antimicrobial proteins [54, 101].

In mammals the activation of macrophages by IFN-γ is well studied [102-104]. Macrophages isolated from mice had an increased antimicrobial activity after priming with IFN-γ [105]. In fish IFN-γ seems to have similar function as mammalian IFN-γ [50, 100]. The IFN-γ receptor, IFN-γR1, has been found in the genome of zebrafish, pufferfish (*Fugu*) and three-spined stickleback (*Gasterosteus aculeatus*) and interaction of IFN-γ and the receptor has been confirmed [106]. Additionally, STAT1 and STAT2 protein, JAK1 and JAK2, Tyk2 and IRF9 have been found in zebrafish [107]. Also IFN-γ increases the expression of MHC class I and II on trout macrophages, induce gene expression of many ISGs and enhance the nitric oxide responses of phagocytes in fish [106]. Recombinant IFN-γ has shown to inhibit *Edwardsiella tarda* infection in olive flounder (*Paralichthys olivaceus*) and induce expression of immune related genes *in vitro* in kidney leukocytes [108].

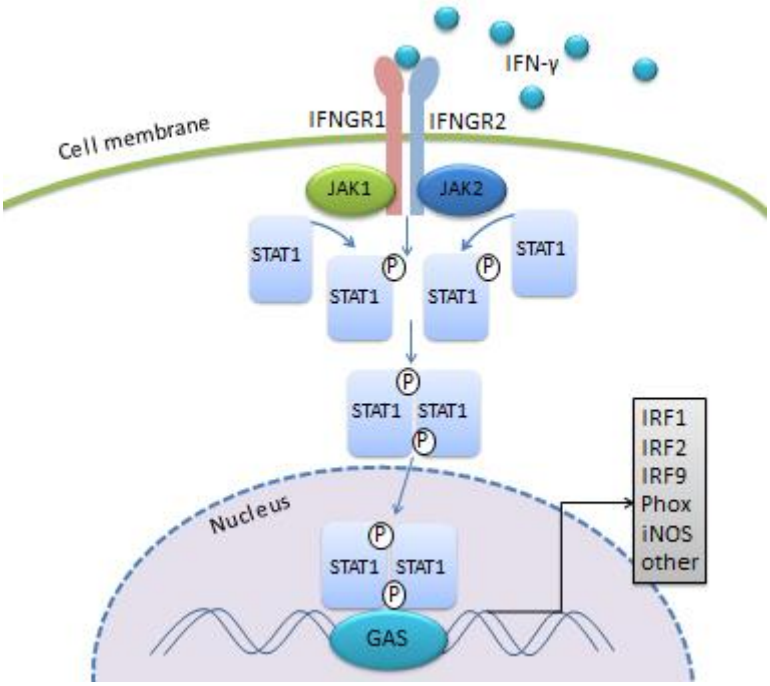

Figure 2: The JAK-STAT pathway. IFN-γ interacts with the receptors IFNGR1 and IFNGR2 and activates JAK1 and JAK2 which phosphorylate STAT1. STAT1 forms a homodimer that travel to the nucleus and bind to promoter IFN-gamma activation site (GAS) to initiate transcriptions of IFN-γ regulated genes (drawn by Kathrine R. Bakkemo).

Killing mechanisms by macrophages

Destruction of pathogens is possibly the most important effector function of macrophages. After bacterial uptake into phagocytic cells, the newly formed phagosome interacts with the host cells endocytic pathway and goes through a maturation process. The phagosome, containing an internalized microbe, becomes the site of effector mechanisms with the purpose of killing the intruder.

Phagocytes have evolved elaborate killing mechanisms, like respiratory burst, nitric oxide (NO) production and antimicrobial molecules released from lysosomes. Also nutrient deprivation and phagosome acidification are host defence mechanisms against pathogens [109]. After phagocytosis the bacterium enclosed in a phagosome is processed through the endosomal lysosomal degradation pathway by interaction with endocytic and lysosomal vesicles [110]. This normally includes three maturation steps; (1) early endosomes, (2) late endosomes and (3) lysosomes (see Fig. 3).

The process of phagosomal maturation and fusion is complex and not fully understood. The early endosome stage, which is regulated by the Rab5 GTPase, is followed by late endosome,

controlled by Rab7, and finally fusion with lysosomes where bacteria are degraded [111]. In the early endosome Rab5 GTPase is a small marker that helps downstream maturation by recruiting early endosomal gen 1 (EEA1), binding N-ethylmaleimide sensitive factor (NSF), Rabex5, Rabaptin5 and syntaxin13 to form a fusogenetic complex with endosomes [112]. In the late endosome Rab7A is a downstream effector protein that initiates a complex that is needed in fusion with phagosomes [112]. Additionally, in the late endosome a proton ATPase pump is causing acidification by importing H^+ into the phagosome [113]. In the last step the phagosome containing the pathogen fuses with the lysosomes, forming a new inclusion, a phagolysosome. Lysosomes inside macrophages contain a variety of degradative enzymes and antimicrobial proteins like proteases, phosphatases, nucleases, lipases and lysozymes that strongly inhibit or kill internalized bacteria [114].

Respiratory burst

Reactive oxygen intermediates (ROIs) are important components of the antimicrobial repertoire of macrophages and it is well established that the respiratory burst is a potent antimicrobial response. The respiratory burst results in the release of several oxygen containing compounds, such as superoxide anion (O_2^-), hydroxyl radical ($OH^\cdot$) and hydrogen peroxide (H_2O_2) [77]. This mechanism is based upon the activation of the enzyme NADPH oxidase (phox) which catalyses the reduction of oxygen (O_2) to O_2^- by using NADPH as electron-donor:

$$2O_2 + NADPH \rightarrow 2O_2^- + NADP^+ + H^+$$

All macrophages express this multicomponent enzyme on the cell membrane. Some of the superoxide anion is converted to H_2O_2 by the enzyme superoxide dismutase (SOD):

$$2O_2^- + 2H^+ \rightarrow 2H_2O_2 + O_2$$

In addition, H_2O_2 can react with O_2^-, causing the formation of the $OH^\cdot$:

$$O_2^- + H_2O_2 \rightarrow OH^\cdot + OH^- + O_2$$

ROIs like O_2^-, H_2O_2 and $OH^\cdot$ have the property to destroy a variety of biomolecules, resulting in metabolic defects [115]. This process is often referred to as oxidative stress.

It is well established that fish phagocytes also produce bactericidal ROIs during phagocytosis (reviewed in [77, 97, 116]). Stimulating fish phagocytes with recombinant fish cytokines like TNF-α, IFN-γ, and IL-1β is reported to give ROI responses [77]. Also cloning, sequencing

and phylogenetic analysis of NADPH-oxidase has been reported in several teleosts [116]. Increased respiratory burst were detected in Atlantic cod after treatment of macrophages with *A. salmonicida* LPS [117] and infection of cod with *F. noatunensis* seems to limit respiratory burst [118].

Nitric oxide production

In addition to ROI production, macrophages also produce microbicidal reactive nitric oxide (NO), which displays biochemical and physiological similarities to the response induced in mammalian phagocytes [26]. The enzyme iNOS (inducible nitric oxide synthase) is central in catalyzing the conversion of NO from L-arginine. NO and its derivatives nitrite, nitrate and nitrosamines are named reactive nitrogen species (RNS). The capability of fish phagocytes to produce NO as a microbial response has been well established (reviewed in [77, 116, 119]). In a recent review, it is stated that the iNOS gene transcript has been cloned in several fish species and the expression of iNOS in teleost macrophages is upregulated which results in production of NO after pathogenic and cytokine stimulations [77].

Intracellular survival of pathogens – sleeping with the enemy

The main goal for the bacterium is to replicate. Since the extracellular environment can be harmful in which the pathogen is exposed to host defence mechanisms like complement, antibodies and recognition by phagocytes, several pathogens have evolved strategies to infect eukaryotic cells. Since macrophages are amongst the first cells at the site of infection, the survival of pathogens rely on their ability to prevent the macrophage-mediated antibacterial mechanisms [77]. The ability to survive intracellularly inside phagocytes is essential in the pathogenesis of several bacteria. There are numerous of strategies to avoid the host cells antimicrobial program like tolerating low pH in phagosomes, degrading antimicrobial proteins, production of detoxifying enzymes to subvert respiratory burst, overcoming nutrient deprivation and escaping from the phagosome to the cytosol [77].

Intracellular bacterial pathogens like *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Salmonella typhi, Rickettsia rickettsia, Brucella* spp. and *F. tularensis* have the ability to avoid destruction within host immune cells, like macrophages (reviewed in [120-123]). While *Mycobacteria* and *Salmonella,* survive and replicate in membrane-bound vacuoles [23, 111] *Listeria, Rickettsia* and *Francisella* escapes the phagosomes and reside in the less hostile cytoplasm of macrophages [120, 124, 125].

Inside the macrophage phagosome, *M. tuberculosis* (causing tuberculosis), among other survival strategies, avoids fusion with lysosomes and survives and grows inside a vacuole. This means that the bacteria avoid the acidic environment in the phagolysosome [126]. The route of entry into host cells may decide the subsequent intracellular fate of the organism. CR is the receptor of complement-opsonised mycobacteria and CR-mediated phagocytosis seems to result in inhibition of respiratory burst and consequently appears to be the favorable route of entry [127].

The food-borne human intracellular bacterium *L. monocytogenes* uses cytoplasmic replication as a strategy to avoid detection and destructions in macrophages. Phagosomal escape is performed by lysis of the phagosomal membrane through the activity of the bacterial listeriolysin O (LLO) and phospholipases A and B [125]. They then escape from the endosomal compartments into the cytoplasm to replicate.

Some bacteria interfere with macrophage functions to facilitate their intracellular survival. Bacteria like *Brucella* spp. inhibit IFN-γ-mediated signalling, which are important in macrophage activating. Inside macrophages, *Brucella* resides in a vacuole. After interactions with the endocytic pathway, they finally reach the endoplasmic reticulum (ER) where they replicate [123].

Several fish pathogens have been reported to resist killing by macrophages, however less is known regarding intracellular survival and replication in these cells. In 2008, McCarthy at al. described survival and replication of *Piscirickettsia salmonis* (piscirickettsiosis or salmonid rickettsial septicaemia (SRS)) in rainbow trout macrophages by using transmission electron microscopy (TEM) [128]. It has lately been reported that *P. salmonis* replicate within membrane-bound vacuoles in infected cells [129].

Yersinia ruckeri, causing enteric redmouth disease (ERM) in salmonids, induce reactive oxygen responses and is able to survive in trout macrophages [130]. Although the invasion induced reactive oxygen responses, it was not sufficient to kill the intracellular bacteria during the first 24 hours after infection. It therefore seems like the bacteria is capably of avoiding ROI responses in the host cell.

The intracellular fish pathogen *E. tarda* is known to avoid the phagocyte reactive oxygen response by expression of catalase enzymes. The bactericidal molecule H_2O_2 may be converted to O_2 and H_2O by microbial expressed catalase [77]. The genome annotation of *E.*

tarda revealed two catalase encoding genes, Kat B and Kat G, and perturbation of both genes resulted in significant reduction in replication [131].

The causative agent of fish mycobacteriosis, *Mycobacterium marinum*, has several strategies to manipulate its host cells like arrest of phagosome maturation and acidification, escape into the macrophage cytosol and manipulating cytokine responses (reviewed in [77]).

Francisella tularensis

The zoonotic disease **tularemia** ("rabbit fever" or in Norwegian known as "harepest") is caused by the Gram negative, highly virulent, intracellular bacterium *Francisella tularensis*. *F. tularenis* is classified into four subspecies; *tularensis* (Type A), *holarcica* (Type B), *novicida*, and *mediasiatica*. While *F. tularensis ssp. novicida* only is virulent in mice, Type A and B are the major cause of disease in human, where Type A is the most virulent (reviewed in [109, 110, 132, 133]). Even very low dose, as few as 10 CFU, can infect humans. Consequently, this organism is seen as a potential weapon of biological terrorism [134]. The bacterium is capable of infecting many mammalian species like hares, rabbits and rodents. It is assumed that the vectors come from flies, ticks and mosquitos and the natural reservoir for several of the ssp. seems to be related to water [132]. Humans catch the disease by handling infected animals, by insect bites, ingestion of contaminated water or inhalation of contaminated aerosols. There is no licensed vaccine available, but most infections can be treated successfully with antibiotics including streptomycin, gentamicin, doxycycline and ciprofloxacin [135].

Because of the concern about bioterrorism, virulence mechanisms and host immunity to *F. tularensis* are well studied and mostly by using murine models and infection by an attenuated live vaccine strain (LVS). The LVS is derived from *F. t. holarctica* (Type B). The most virulent ssp. (Type A) is less used because of the requirement of facilities with biological safety level 3 (BSL3), the second highest level.

Overview of the life cycle of *F. tularensis*

Although macrophages are considered to be an important target of *F. tularensis*, the bacteria can infect non-phagocytic cells, like epithelial cells [66, 136-138]. In mammals, the alveolar epithelial cells are the first barrier during respiratory infections and Hall et al. (2008) found bacteria in these cells 1 day post infection by using flow cytometry [139]. Recently, Faron et al. (2015) observed internalized bacteria in alveolar epithelial cells 16 h after infections, by

using TEM [140] .While there has been many studies on the interactions of *F. tularensis* with various host cells, the passing through the epithelia to enter the bloodstream and the role of macrophages in this process is still poorly understood *in vivo* [140].

Briefly, *F. tularensis* gains entry to macrophages via phagocytosis where they prevent maturation of the phagosome and escape into the cytosol to grow (Fig. 3). Initially the bacterium binds to receptors on the surface of the macrophage. *F. tularensis* then enters macrophages through opsonin dependent and independent mechanisms [113]. Serum-opsonized bacteria are taken up by macrophages through the complement receptor 3 (CR3) and antibody-opsonized bacteria interact with the Fc gamma receptor (FcγR). Phagocytosis of non-opsonized bacteria depends on the mannose receptor (MR) and surface-exposed nucleolin (SE-N) [141-143]. The route of entry has a profound impact on the intracellular fate of the bacteria and the outcome of the infection [144]. Uptake of *F. tularensis* is significantly enhanced by both serum (complement) - and antibody opsonisation [94, 141, 142, 145, 146]. FcγR-mediated uptake induce oxidative burst and pro-inflammatory responses, while complement-receptor-mediated phagocytosis is associated with neither an oxidative burst nor pro-inflammatory response [145]. However, opsonized bacteria seem to replicate modestly in the cytosol compared to non-opsonized bacteria. Regardless of a decreased replication in the cytosol by opsonised bacteria, the use of complement opsonin to gain entry into cells is likely to be an important virulence mechanism of *F. tularensis* [39].

In phagocytes the bacteria are taken up via pseudopod loops [94]. The process is dependent on filamentous actin and fusion of the pseudopod loop with the plasma membrane results in enclosure of the bacteria within a spacious vacuole near the surface of the macrophage. Ultra-structural analyses have revealed that the vacuole rapidly shrinks, moves inwards the centre of the cell and is remodelled into a tight phagosome [66].

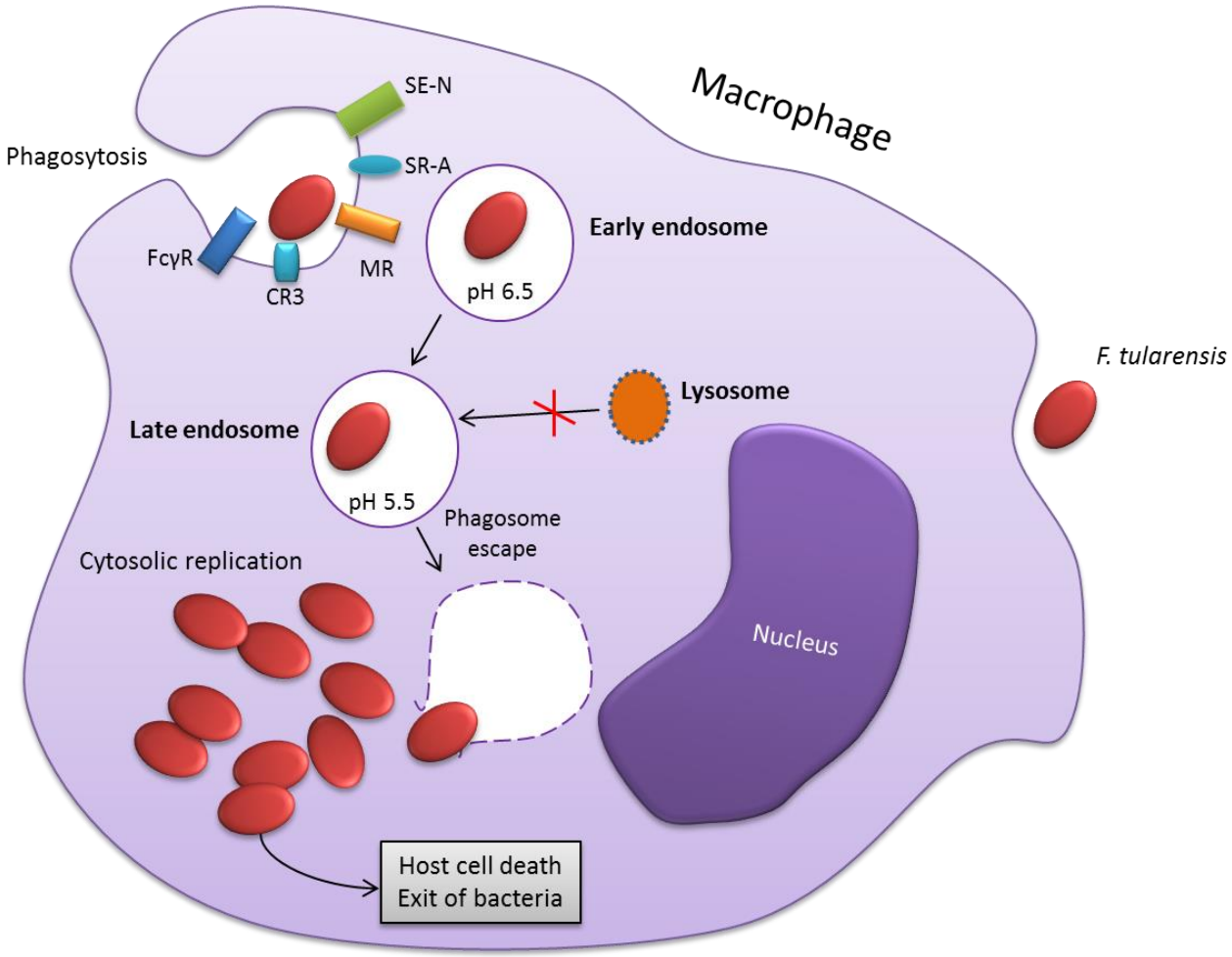

Figure 3: A simplified model of the intracellular life cycle of *F. tularensis* inside a macrophage. Single or multiple macrophage receptors can recognize the bacterium before engulfment by a pseudopod loop mechanisms. The bacteria then reside in a vesicle called phagosome that interacts with the host cell endocytic pathway, early and late endosomes, causing acidification of the phagosome. *F. tularensis* does not fuse with lysosomes but disrupt the phagosomal membrane and escape into the cytosol to replicate (drawn by Kathrine R. Bakkemo).

Usually during pathogenesis of intracellular pathogens the bacteria-containing phagosome undergoes a series of maturation steps during the endocytic pathway. This starts with the early endosome stage, followed by late endosome and finally fusion with lysosomes where bacteria are degraded [114]. In the late endosome a proton ATPase pump is causing acidification. The *Francisella*-containing phagosome interacts with early endosome but has limited interactions with late endosomes and does not fuse with lysosomes (reviewed in [113, 124, 132, 144]).

Disruption of the phagosomal membrane might be triggered by acidification of the phagosomal compartment. Even though the mechanisms involved are not known, it has been showed that many genes are essential during escape into the cytosol [147]. These genes are

encoded from a genomic region called the *Francisella* pathogenicity island (FPI), but the molecular mechanism(s) are still not clear [148]. Within 30 to 60 minutes after entry the bacteria escape to the cytosol which prevent host immune defences like ROIs and antimicrobial peptides [147]. Intracellular proliferation eventually leads to release of bacteria to the extracellular space through induction of apoptosis.

Survival mechanisms of *F. tularensis*

Francisella spp. is known to subvert host immune responses. In the extracellular compartments *F. tularensis* seems to block complement activation by converting C3b (attached to the bacteria) into C3bi and C3d and promote uptake by phagocytes instead of MAC formation [109]. This escape from the extracellular antimicrobial environment is believed to relieve the bacterium from complement, antibodies and AMPs, although this pathway of entry limits intracellular replication [109, 145].

As described above phagosomal escape is an essential mechanism in the survival strategy of *F. tularensis*, since mutants with reduced ability of escaping have defective intracellular growth [144]. The escape from phagosome gives the bacteria access to nutrients needed for intracellular replication.

The bacteria also interfere with the inflammatory responses early during the infection. *F. tularensis* within the phagosome induce the secretion of TNF-α in macrophages mediated by the PI3K/Akt pathway, which also leads to activation of NF-κB. Activation of NF-κB results in secretion of cytokines that limit phagosomal escape, however after escape into the cytosol the bacteria suppress NF-κB activation and the secretion of TNF-α, IL-6, IL-8 and IL-12 (reviewed in [113]). In addition, *F. tularensis* interfere with the IFN-γ signalling to evade the immune system. Early during infection IFN-γ signalling is down-regulated by up-regulation of SOCS3 which is a negative regulator of IFN-γ signalling [110].

Throughout infection of human monocytes with *F. tularensis* several TLRs are down-regulated (TLR1, 4-8) while TLR2 is upregulated, resulting in reduced immune response. Additionally, the bacteria have LPS with weak endotoxic activity and do not stimulate TLR4 [110].

Intracellular pathogens have at least two strategies to control the oxidative burst: (1) prevent the production of ROIs, or (2) detoxifying ROIs as they are formed [122]. *F. tularensis* uses both strategies to inhibit ROIs. The productions of ROI are disrupted by modifying the

NADPH oxidase activation and detoxify ROI using proteins like catalase and SOD [109, 124]. This inhibition of oxidative burst increases the survival of the bacteria in phagocytes.

A large-scale view by microarray analyses on transcripts from human blood monocytes infected with *F. tularensis* (Type A), revealed reduced expression of several host response genes, such as those associated with IFN-γ signalling, TLR signalling, autophagy and phagocytosis [149]. Altogether, *F. tularensis* possesses a number of mechanisms that alter the immune response in its favour, adapting it to the intracellular environment.

Francisella in aquatic organisms

The genus *Francisella* can be divided into two major linages; *F. tularensis* and *F. philomiragia*. The *F. philomiragia* linage is closely related to currently known fish pathogenic species [12]. Bacterial strains isolated from warm water fish species such as tilapia are mainly *F. noatunensis* ssp. *orientalis* (synonymic to *F. asiatica*), while isolates from cold-water species such as Atlantic cod and Atlantic salmon belongs to *F. noatunensis* ssp. *noatunensis*.

Water temperature seems to play a significant role in development of francisellosis. Clinical signs of the disease have not been observed in Atlantic cod unless the sea water temperature exceeds 15 °C, although the bacterium is found both in farmed and wild populations at low temperatures [9]. In hybrid striped bass the pathogenesis of *Francisella noatunensis* ssp. *orientalis* appears restricted to 20-28 °C [150] and in tilapia an experimental infection revealed a higher mortality at 15 °C than at 30 °C [151]. Salinity does not seem to be vital since both *F. noatunensis* and *F. noatunensis* ssp. *orientalis* has been isolated from fish in both fresh water (Atlantic salmon [152], hybrid striped bass [150] and tilapia [153]) and salt water (Atlantic cod [15] and three-lined grunt [154]).

There are no commercial vaccines available to limit the spread of francisellosis in aquaculture. However, live attenuated *F. asiatica* induce protection of fransiellosis in tilapia [155]. The attenuated strain of *F. asiatica* in this study is an iglC-mutant. The IglC-gene, located on the FPI in the genome, is required for phagosomal escape of *F. tularensis* and subsequent growth in mammalian macrophages [156]. Vaccination trial with the *F. asiatica* iglC mutant resulted in protection against wild-type of *F. asiatica*, and the vaccine is now patented by two of the authors [155].

Infections in fish by bacteria belonging to the genus *Francisella* is associated with extensive granuloma formation in their hosts [12, 19]. The bacterium causing francisellosis in tilapia, *F.*

asiatica, is able to enter, survive and replicate in head kidney-derived macrophages, and finally kill the cell by apoptosis [153]. The same study also reported that tilapia autologous normal serum (not heat-inactivated) increased internalisation of the bacteria into the macrophages, while mannan (blocking the mannose receptor) decreased internalisation, indicating an involvement of both CR and MR. In Atlantic cod, *F. noatunensis* has been shown to infect adherent Atlantic cod leukocytes *in vitro* [157]. Recently, by flow cytometry, Vestvik et al. (2013) found an increase in fluorescence in leukocytes isolated from head kidney infected with *F. noatunensis*, indicating replication of the bacteria in cod immune cells [118]. The bacterium is reported to inhibit the respiratory burst of Atlantic cod leukocytes [118] by using an indirect assay established by Kalgraff et al. (2011) [158]. The survival within immune cells and inhibiting of killing mechanisms inside leukocytes indicate that phagocytes are important target cells. However, both B-cells and neutrophils of Atlantic cod have phagocytic capacity [159] and *F. noatunensis* are able to infect B-cells *in vitro* [160]. Taken together, the interactions of bacteria within the genus *Francisella* with fish phagocytes seem to correspond with the well-studied human pathogen *F. tularensis*.

2. AIMS OF STUDY

Main objective:

The purpose of this project was to study the intracellular lifestyle of *F. noatunensis* in Atlantic cod cells, with particular focus on cod macrophages. Primary macrophages derived from the head kidney of Atlantic cod and a cod cell line (ACL-cells) were used as tools to better define the host-pathogen interactions. A better understanding of how the bacteria interfere with the host cells is essential for development of prophylactics and treatments.

Sub-objectives:

- Establishments of an **invasion assay** in both primary macrophages and ACL-cells as tools for functional studies.
- Determine the intracellular **localization** of *F. noatunensis* inside cod macrophages.
- Examine intracellular **survival** and **replication** of *F. noatunensis* in both cell types.
- Study the involvement of **actin filaments** in uptake of *F. noatunensis* in cod macrophages.
- Explore the involvement of **IFN-γ** in cod macrophage activation.
- Study the involvement of *F. noatunensis* and its LPS on **inflammatory responses** in cod macrophages.
- Antibacterial proteins in cod:
    - Study the gene distribution of **lysozyme**, **cathelicidin** and **hepcidin** in cod.
    - Examine whether ***in vitro*** and ***in vivo*** **infection** of *F. noatunensis* or LPS from the extracellular bacterium *E. coli* stimulate the expression of antibacterial genes in cod.
    - Find out if priming of cod macrophages with **recombinant IFN-γ**, before infection with *F. noatunensis*, influence antibacterial gene expressions.

3. ABSTRACT OF PAPERS

Paper I

Intracellular localisation and innate responses following *Francisella noatunensis* infection of Atlantic cod (*Gadus morhua*) macrophages.

The facultative intracellular bacterium *Francisella noatunensis* causes francisellosis in Atlantic cod (*Gadus morhua*), but little is known about the survival strategies and how the bacteria evade the host immune response. In this study we show intracellular localisation of *F. noatunensis* in cod head kidney derived macrophages using indirect immunofluorescence techniques and green fluorescent labelled bacteria. Transmission electron microscopy revealed that *F. noatunensis* was enclosed by a phagosomal membrane during the first 1-3 hours of infection. Bacteria were at a later inflectional stage found in large electron-lucent zones, apparently surrounded by a partially intact or disintegrated membrane. Immune electron microscopy demonstrated the release of bacterial derived membrane vesicles from intracellular *F. noatunensis*, an event suspected to promote degradation of the phagosomal membrane and allowing escape of the bacteria to cytoplasm.

Studies of macrophages infected with *F. noatunensis* demonstrated a weak activation of the inflammatory response to take place as measured by increased expression of the Interleukin (IL)-1β and IL-8 genes. In comparison, a stronger induction of expression was found for the anti-inflammatory IL-10 indicating that the bacterium exhibits a role in down-regulating the inflammatory response. Expression of the p40 subunit of IL12/IL17 was highly induced during infection thus suggesting that *F. noatunensis* promotes T cell polarisation. The host macrophage responses studied here showed low ability to distinguish between live and inactivated bacteria, although other types of responses could be of importance for such discriminations. The immunoreactivity of *F. noatunensis* lipopolysaccharide (LPS) was very modest, in contrast to the strong capacity of *Escherichia coli* LPS to induce inflammatory responses. These results suggest that *F. noatunensis* virulence mechanisms cover many strategies for intracellular survival in cod macrophages.

Paper II

Francisella noatunensis subsp. *noatunensis* invade, survive and replicate in Atlantic cod cells.

Systemic infection caused by the facultative intracellular bacterium *Francisella noatunensis* subsp. *noatunensis* remains a disease threat to Atlantic cod (*Gadus morhua* L). Future prophylactics could benefit from better knowledge on how the bacterium invades, survives and establishes infection in its host cells. Here, and facilitated by the use of a gentamicin protection assay, this was studied in primary monocyte/ macrophage cultures and an epithelial-like cell line (ACL cells) derived from cod. The studies showed that *F. noatunensis* subsp. *noatunensis* is able to invade primary monocyte/ macrophages and that the actin-polymerisation inhibitor cytochalasin D blocked internalisation, demonstrating that the invasion is mediated through phagocytosis. Interferon (IFN)-γ treatment of cod macrophages prior to infection enhanced bacterial invasion, potentially by stimulating macrophage activation in an early step in host defence against *F. noatunensis* subsp. *noatunensis* infections. We measured a rapid drop of the initial high levels of internalised bacteria in macrophages indicating the presence and action of a cellular immune defence mechanism before intracellular bacterial replication took place. Low levels of bacterial internalisation and replication were detected in the epithelial-like ACL cells. The capacity of *F. noatunensis* subsp. *noatunensis* to enter, survive and even replicate within an epithelial cell line may play an important role in its ability to infect live fish and transverse epithelial barriers to reach their main target cells - the macrophage.

Paper III

Multiple specialised goose-type lysozymes potentially compensate for an exceptional lack of chicken-type lysozymes in Atlantic cod.

Previous analyses of the Atlantic cod genome showed unique combinations of lacking and expanded number of genes for the immune system. The present study examined lysozyme activity, lysozyme gene distribution and expression in cod. Enzymatic assays employing specific bacterial lysozyme inhibitors provided evidence for presence of g-type, but unexpectedly not for c-type lysozyme activity. Database homology searches failed to identify any c-type lysozyme gene in the cod genome or in expressed sequence tags from cod. In contrast, we identified four g-type lysozyme genes (LygF1a-d) constitutively expressed, although differentially, in all cod organs examined. The active site glutamate residue is replaced by alanine in LygF1a, thus making it enzymatic inactive, while LygF1d was found in two active site variants carrying alanine or glutamate, respectively. *In vitro* and *in vivo* infection by the intracellular bacterium *Francisella noatunensis* gave a significantly reduced LygF1a and b expression but increased expression of the LygF1c and d genes as did also the interferon gamma (IFN-γ) cytokine. These results demonstrate a lack of c-type lysozyme that is unprecedented among vertebrates. Our results further indicate that serial gene duplications have produced multiple differentially regulated cod g-type lysozymes with specialised functions potentially compensating for the lack of c-type lysozymes.

4. DISCUSSION

In this project the main objectives was to study the intracellular lifestyle of *F. noatunensis* in Atlantic cod and some of the host immune responses. As methods and results are described in detail in the respective papers, the following text will be a summary of the main findings.

Establishment of a protocol for gentamicin protection assay

In an effort to study host-pathogen interactions in cod primary macrophages and Atlantic cod larvae cells (ACL-cells), an invasion and an intracellular replication assay were established. Both assays are based on the use of gentamicin to kill extracellular bacteria. This method of studying bacterial invasion in eukaryotic cells has been in use for many years [161]. However, the technique has to be adapted for each organism. In this case isolation of head kidney macrophages, bacterial growth medium and growth curve, sensitivity of *F. noatunensis* to gentamicin and detergents, infection time, type of lysate and others was optimised. The invasion assay was used in all three papers and in combination with three different subsequent methods; (1) gene expression by quantitative real-time PCR (qPCR) to study host immune responses, (2) microscopy to determine intracellular localization of bacteria in macrophages and (3) quantification of internalized bacteria by calculating colony forming unit (CFU) on agar plates to study host-pathogen interactions.

A disadvantage of using invasion assays with antibiotic treatments to quantify intracellular bacteria, is the potential for underestimating the number of intracellular bacteria [162]. Bacterial counting of CFU detects only viable bacteria and misses if there has been some intracellular killing of bacteria by the macrophage, or if the antibiotics have been leaking into the cells. Recently there have been published a study of antibiotic uptake by cultured Atlantic cod leucocytes [163]. In this report they used high-performance liquid chromatography (HPLC) to detection of drugs inside the cells. This study was designed to give knowledge about antibacterial treatment of fish infected with intracellular pathogens. The antibiotics oxolinic acid and flumequine, were therefore selected as they in general have fast in-and outflux in cells. Although both drugs were rapidly taken up by the cells, flumequine did not prevent intracellular replication of *F. noatunensis*. The aminoclycoside gentamicin has for a long time been known to be effective in a cell-free system, but less effective to kill intracellular bacteria and consequently the gentamicin has been used widely in invasion assays [164], also in fish cells [130, 165-169]. As it is a prerequisite to use an antibiotic that

kill extracellular bacteria in invasion assays, we tested the sensitivity of *F. noatunensis* to gentamicin and found it satisfying. Detection of gentamicin in primary macrophages and ACL-cells after treatment should preferably have been done in our experiments. However, since worst case scenario is underestimating of intracellular bacteria, we consider the results trustworthy regarding intracellular localisation, survival, replication and host immune responses.

Intracellular localization in cod macrophages

In **paper I** intracellular localization of bacteria in primary macrophages was investigated by the use of TEM and light (fluorescence) microscopy. The results revealed that the bacterium was enclosed by a tight phagosomal membrane during the initial stage of infection. TEM showed intact bacteria present in both a tightly enclosed vacuole and in a larger electron-lucent space, not surrounded by an intact phagosomal membrane 3 hours post infection (pi). Immune electron microscopy done 1, 3 and 24 hours pi also showed bacteria in both intact membranes and without a clear visible membrane. Immunofluorescence studies using anti-*F. noatunensis* (anti-FN) serum showed bacteria either clustered together or even distributed in large vacuoles 24 hours pi. Additionally, cod macrophages infected with green fluorescence protein (GFP)-expressing *F. noatunensis* confirmed intracellular localization. All together it seems like *F. noatunensis* inside cod macrophages escape to cytoplasm rapidly after entering the macrophages.

At the time this project was initiated, little was known about the intracellular localization of *F. noatunensis* in macrophages. However, some month before the submission of paper I, Furevik et al. (2011) reported intracellular location of *F. noatunensis* in macrophages isolated from the head kidney, spleen and blood in Atlantic cod [160]. Confocal microscopy indicated escape of bacteria into the cytoplasm based on clustered intracellular bacteria in the initial phase of infection followed by spread of the bacteria in late phase, confirming our results.

In mammals, the intracellular pathogen *F. tularensis* is known to escape from phagosomes into the cytoplasm in macrophages [146, 170]. Golovliov et al. (2003) studied the intracellular localization of *F. tularensis* in mouse and human macrophages by using TEM [170]. Microscopy pictures clearly show disruption of the phagosomal membrane inside monocytes/macrophages. Similar to our study in fish cells, bacteria were surrounded by an

electron-lucent space. TEM studies of phagosomal escape have also been reported in macrophages from tilapia infected with *F. asiatica* [153].

A notable finding of our study was the bacterial vesicles seen in infected macrophages. The use of immune electron microscopy demonstrated that anti-FN marked intracellular bacteria were surrounded by anti-FN marked vesicles, demonstrating bacterial origin. These vesicles were only present when the phagosomal membrane appeared partially degraded suggesting that the vesicles promote membrane degradation to allow escape of the bacteria to cytoplasm. Release of membrane vesicles, often referred to as outer membrane vesicles (OMVs), from microbial cells is conserved among organisms including Gram negative, Gram positive, archaea, fungi and parasites [171]. OMVs from Gram negative bacteria may contain signal molecules, LPS, lipoproteins, phospholipids, toxins and antigens. Recently, Brudal et al. (2015) demonstrated that *F. noatunensis* secrete OMVs both in broth-culture and in zebrafish embryos during infection [172]. In addition, OMVs containing virulence factors, like IglC, PdpD and PdpA, appeared to protect to some degree against francisellosis in a zebrafish model. The authors proposed the possibility of using OMVs as a vaccine against francisellosis. Small vesicles are also observed early in the infection of rainbow trout (*Oncorhynchus mykiss*) macrophages with the fish pathogen *P. salmonis*, and the authors suggested that the vesicles might be virulence factors that support intracellular survival of the pathogen [128]. The human pathogen *F. tularensis* have also been shown to produce vesicles in macrophages [170]. More investigation though is needed to conclude that bacterial vesicles contribute to intracellular survival of these bacteria.

Survival and replication of *F. noatunensis* in macrophages and ACL-cells

In **paper II**, by using the invasion assay followed by bacterial counting, we detected the viability of the bacteria after an intracellular phase. We found that live bacteria can be recovered from infected macrophages and ACL-cells.

The surface of the skin, gills and the gastrointestinal tract serve as portals of entry for pathogenic bacteria in fish. The ability to invade epithelial cells is a key factor of virulence for several human pathogenic bacteria and this capacity has also been demonstrated for fish pathogens like *Photobacterium damselae* ssp. *piscicida* [173, 174], *Aeromonas hydrophila* [175], and *V. anguillarum* [176]. In **paper II** we used a cell line from Atlantic cod larvae (ACL) with epithelial-like morphology. Earlier there has been demonstrated that this cell-line

is functional to use in host-pathogen interaction as Jensen et al. (2013) reported that the cells are susceptible to viral infections and express immune genes like IL-1β, IL-6, IL-8, IL-12p40, ISG15, Vig-1, IRF1, LGP2 and mda5 [177]. Also when subjected to bacterial crude *E. coli* LPS the ACL-cells expressed inflammatory genes like IL-1β, IL-6 and IL-8 and antibacterial genes like cathelicidin and hepcidin [44]. In order to investigate the ability of *F. noatunensis* to invade other cell types than macrophages we did an *in vitro* infection of ACL-cells. The results revealed that *F. noatunensis* has the ability to invade, survive and replicate in fish epithelial cells in the form of ACL-cells. Although the replication was significant 48 hours after infection, the invasion frequency was low, only ∼0.01 % of the adherent ACL-cells was infected. Our observation that *F. noatunenis* enters and replicate in ACL-cells suggest that interaction with non-phagocytic cells might play a central role in its virulence strategy. Regardless of the low frequency of invasion, the epithelial cells most likely provide a passage through the epithelia and into internal organs and target cells. The use of antibiotic in invasion assays is a prerequisite when calculating CFU of intracellular bacteria. We did test bacterial sensitivity to gentamicin to ensure sufficient killing of extracellular bacteria, but as mentioned before leakage of gentamicin into ACL-cells and primary macrophages was not determined. This implies that the actual invasion frequency might be higher than the calculated value.

A considerably higher invasion frequency was seen in macrophages, with an estimate of 1 bacterium/macrophage (∼100 % of the adherent macrophages). This higher level of invasion in macrophages, compared to in ACL-cells, may reflect that macrophages are the preferred host cell type and additionally possess phagocytic capabilities. After a 2-fold drop in intracellular bacteria from 2 – 24 hours pi there was a significant replication from 24 – 48 hours pi. Quantification of intracellular bacteria over time using qPCR with primers specific for the *F. noatunensis* outer membrane protein FopA and Fc50 targeting the 16S rRNA gene, confirmed an initial drop in expression followed by a slight increase during the succeeding sampling points (10-72 hours pi). The reduction of intracellular bacteria before the detected intracellular replication suggests that *F. noatunensis* at some level are killed by the macrophages, but still manage to replicate intracellularly.

The results of Vestvik et al. (2013) showing intracellular replication of *F. noatunensis* in Atlantic cod leucocytes using flow cytometry, support our results [118]. Kaldestad et al. (2014) also confirmed intracellular replication of *F. noatunensis* in cod leucocytes [163]. Recently, *F. noatunensis* has been shown to replicate during the late phase of infection in the amoeba *Dictyostelium discoideum*, which are cells similar to macrophages with similar

optimal growth temperature as the bacteria [178]. Taken together, the fact that *F. noatunensis* is able to invade both non-phagocytic and phagocytic cells implies that this bacterium is capable of interacting with specific receptors on the surface and trigger endocytosis. The intracellular replication in both cell types can explain the problems of finding successful treatments and vaccines against francisellosis in cod.

As expected, since outbreaks of the disease in cod have not been observed in sea water temperature below 15 °C, assay temperature influence the uptake of *F. noatunensis* in cod macrophages. Investigation of invasion at three different temperatures revealed a significant higher uptake at 16 °C that at 12 °C. However, whether the increase comes from reduced host responses or better invasive abilities of the bacteria at higher temperature is not clear.

Phagocytosis is actin dependent

In **paper I** we studied the phagocytic capacity of primary Atlantic cod macrophages by using inactivated *E. coli* cells conjugated to fluorescent beads. Fluorescence microscopy revealed that the capacity of phagocytosis was intact in cultured macrophages.

Recognitions of invading bacteria on the surface of macrophages create changes in the host cytoskeleton. Actin filaments are major components of the cell skeleton and phagocytosis requires actin polymerisation at the site of ingestion [179]. In **paper II** the treatment of cod macrophages with the actin-disrupting agent cytochalasin D almost completely inhibited the entry of *F. noatunensis* into macrophages in a dose-dependent manner. This confirms cellular invasion and the importance of host actin microfilaments in cytoskeletal function during phagocytosis. Invasiveness of other intracellular bacteria such as *Legionella pneumophila* [180] and *Campylobacter jejuni* [181] was also reduced by the same inhibitor. Uptake of *F. tularensis* is reported to be dependent of actin filaments and phagocytosis is sensitive to cytochalasin [66]. Cytochalasin D reduced cell-death in a dose-dependent manner of macrophages isolated from mice infected with *F. tularensis* [182]. In the latter study bacteria were already inside the macrophage when treated and inhibition of cellular motility hampered escape and proliferation in the cytosol, preventing death of macrophages. This demonstrated that the bacteria also are dependent of actin inside the macrophage.

Recombinant IFN-γ limits intracellular survival of *F. noatunensis*

IFN-γ is an important cytokine for induction of antibacterial host responses of both the innate and the adaptive immune system. The cytokine is primarily produced by NK cells and activated T_H1 phenotype $CD4^+$ and $CD8^+$ cells [183]. Downstream IFN-γ signalling in macrophages results in activation of antimicrobial mechanisms. Intracellular killing of the human pathogen *F. tularensis* by macrophages is believed to depend on IFN-γ-induced activation of macrophages and is thus important in host control of the bacterium [102, 104, 105]. In fish, IFN-γ is also a key activator of macrophages [100]. Functional analysis of rIFN-γ protein has shown protective effect against *E. tarda* in olive flounder and against *Nocardia seriolae* infection in ginbuna crucian carp, *Carassius auratus langsdorfii* [108, 184]. In **paper II** the cod macrophages were primed with rIFN-γ prior to infection to observe whether IFN-γ has an effect on invasion and replication of *F. noatunensis.* Treatment of macrophages with rIFN-γ induced gene expression of IRF1, which is a product in IFN-γ signalling. Priming of macrophages with rIFN-γ resulted in more than a ten-fold increase of *F. noatunensis* invasion followed by a twenty-fold reduction of intracellular bacteria ten hours later. Gene expression studies targeting the 16S rRNA gene (Fc50) confirmed the results. This significantly higher uptake of bacteria and effective destruction of intracellular bacteria implies that IFN-γ has an important role in activation of cod macrophages. Still the bacteria were not completely eradicated by the macrophages which indicated that IFN-γ-treatment alone is not sufficient to kill all bacteria in cultured cod macrophages. However, the possibility of using rIFN-γ as an adjuvant in vaccines against *F. noatunensis* may be relevant.

Innate immune responses in macrophages

Cytokines are released during inflammation in the host and are commonly used as markers of infection. In **paper I** the gene expression of inflammatory responsive genes was measured after infection of primary macrophages with both live and inactivated *F. noatunensis* and after treatment of macrophages with LPS from both *F. noatunensis* and *E. coli*. Five genes, IL-1β, IL-6, IL-8, IL-10 and IL-12p40 subunit, were monitored at different time points after infection.

Gene expression of cytokines in macrophages following *F. noatunensis* infection

The inflammatory response is highly regulated and initiated by pathogens or tissue damage. Pro-inflammatory cytokines such as IL-1β, IL-6 and IL-8 enhance antimicrobial functions in immune cells while anti-inflammatory cytokines such as IL-10 supress these responses [183]. IL-1β and IL-8 were weakly but significant upregulated from 6 – 12 hours pi (IL-1β to 24 hours pi) while IL-6 showed only modest and not significantly induction. After 48 hours gene expressions were almost diminished. The anti-inflammatory gene IL-10 showed delayed expression with a peak at 24 hours pi. IL-12p40 subunit is a factor associated with T cell responses [47] and was in the present study significantly elevated at all studied time points with a peak level at 24 hours pi. The high expression of the p40 subunit could imply that *F. noatunensis* activates T cell polarisation in cod since IL-12 is known to promote differentiation of CD^+ T-cells to T_H1 cells [52]. However, the possibility of cod lacking T_H cells suggests that this cytokine might have other roles in cod. IL-12 is also known to activate CTLs [185, 186] that might be more relevant in cod because of the functional MHC class I.

There were small differences in immune responses after exposure to live versus inactivated bacteria. Exposure of live *F. noatunensis* to cod macrophages seemed to induce a more marked, but not significantly different, expression level of the immune genes, especially for IL-1β, compared to inactivated *F. noatunensis*. Similar results were seen in human macrophages after exposure to live or killed *Francisella* sp. [48]. Live bacteria were necessary for IL1-β secretion, indicating activation of caspase-1.

Taken together, the low induction of inflammatory response and high increase of the anti-inflammatory gene IL-10 suggest that *F. noatunensis* have the ability to supress host immune responses. Such virulence mechanism is also seen in the well-studied *F. tularensis* (reviewed in [109, 113]).

Gene expression of cytokines in macrophages after LPS treatment

LPS are major PAMPs of Gram negative bacteria and commonly used to initiate inflammatory responses. The host cell usually recognizes LPS on the surface as a bacterial component. This initiates activation of signalling molecules and subsequent production of pro-inflammatory cytokines. In **paper I** the gene expression of IL-1β, IL-6, IL-8, IL-10 and IL-12p40 were monitored after exposure of cod macrophages to *F. noatunensis* LPS and *E. coli* LPS. The analysis revealed that *F. noatunensis* LPS is a poor inducer of expression of all

the genes. In contrast, high expressions were found after exposure to *E. coli* LPS. Similarly Seppola el al. (2015) found that crude and ultrapure *E. coli* LPS stimulated gene expressions of inflammatory genes like IL-1β, IL-6, IL-8 and IL-10 in cod macrophages. The low inflammatory response generated by *F. noatunensis* LPS suggest that this bacterium has evolved virulence mechanisms to suppress innate immune responses against this endotoxin. In mammals, similar observations have been made. Even though mammals possess TLR4, *F. tularensis* ssp. *novicida* LPS (share the same Lipid A structure with *F. tularensis* ssp. *tularensis*) have a weak endotoxic activity and activates neither TLR4 nor TLR2 [187]. Avoidance of TLR4 activation is possible a virulence factor of *F. tularensis* since administration of synthetic TLR4 agonists activated the TLR4 pathway in mice and caused inflammatory responses [188]. Based on these results it is assumed that the poor stimulatory effect gives *Francisella* sp. the ability to escape early detection by the host. In contrast, 10 years earlier LPS from *F. tularensis* was suggested to contribute to virulence since mice with destructive mutation of TLR4 gene (C3H/HeJ mice) were more susceptible to infection with the *F. tularensis* live vaccine strain [189]. The LPS receptor in fish is still unknown [35] and the expansion of other TLRs genes in the cod genome may compensate for the lack of TLR4 [32, 36].

Antibacterial proteins in cod

AMPs and enzymes are important in host defence against microorganisms and have been recognized as promising candidates for potential alternatives to antibiotics [190]. In **paper III** the antibacterial gene expression of g-type lysozyme, cathelicidin and hepcidin were investigated in cod tissues. Additionally, expression of these AMP genes were studied both during *in vivo* and *in vitro* infection experiments with *F. noatunensis* and exposure to *E. coli* LPS (only lysozyme genes). Priming of macrophages with rIFN-γ before infection with *F. noatunensis* was also done to study whether IFN-γ has an impact on antimicrobial gene expressions during infection.

Cod lack chicken-type lysozyme

Lysozymes have important roles in the innate immune system of all animals. It has been reported that cod have multiple genes of g-type lysozyme, actually as many as 11 potential genes (LygF1a-k), in contrast to the human genome that has only two [70]. Multiple g-type lysozymes have been found in several species and can be products of gene duplications.

Phylogeny of fish g-type lysozyme sequences revealed that the two genes LygF1b (reported in 2009 as *codg1&2* [69]) and LygF1d are closely related to each other, and most likely are products of independent gene duplication [70].

Gene duplications are important events in the evolution and doubling of genes or even whole genomes give large amounts of raw material that can generate genetic complexity. It is believed that the vertebrate genome has undergone two WGDs and that the teleost-linage has gone through a third WGD after the split from tetrapods [37]. Duplication of genes results in two identical paralogs but the genes can undergo different fates like losing function (pseudogenization) or acquire novel functions (neo-functionalization). Teleost are the most diverse vertebrate group and extra complexity in the genomic assembly may serve as an explanation of their success and diversity [34].

Only g-type lysozyme has been discovered in cod which leads to the question whether c-type is absent. When using a g-type inhibitor, PliG, and a c-type inhibitor, Ivy, when measuring enzyme activity in extracts from cod tissue, only use of PliG supressed lysozyme activity. This means that all lysozyme activity originated from g-type lysozyme (level of 40-50 Units/mg total protein). Furthermore, the presence of a c-type lysozyme gene was not found in the cod genome.

Most vertebrate genomes have both c-type and g-type lysozyme genes including Atlantic salmon [68, 70, 191]. The immune system of cod seems to differ from other teleosts with having more g-type lysozyme genes and lacking c-type lysozyme [70]. The fact that cod lack important components of the immune system such as MHC II, CD4 and several TLRs, without having difficulties surviving, suggest that cod compensate by having expansion and wider spectre of yet unknown components. It seems like g-type lysozyme gene expansion might be one of these compensatory mechanisms that can make cod more specialised. As mentioned earlier expansion of genes are also seen in the TLR family and MHC class I repertoire in cod [32, 35, 36].

Distribution of antibacterial peptides in cod organs and tissues

In **paper III**, BLAST search in the cod genome using the identified g-type lysozyme genes from cod and salmon, identified three complete and one near-complete genes of the previously named LygF1a-d. However, attempt to distinguish gene expression of these four genes turned out to be difficult because of sequence similarities between LygF1a and b, and

LygF1c and d. Consequently two primers were used that directed the co-amplification of LygF1a+b and LygF1c+d.

Expression of both pair of lysozyme genes, together with cathelicidin and hepcidin genes, were tested in different organs and tissues of non-infected cod. Additional testing of enzyme activity of lysozymes was performed as mentioned above. Both pair of g-type lysozyme genes (LygF1a+b and LygF1c+d) were expressed in all organs and tissues examined, although in different extent. Lysozyme activity was only detected in the spleen and head kidney and LygF1a+b expression was dominant.

Gene expression analysis of the hematopoietic organs head kidney and spleen, showed highest gene expression of LygF1a+b. LygF1c+d expression were dominated in the spleen, gills and blood. Cathelicidin had highest expression levels in the head kidney, spleen and blood while expression of hepcidin was highest in the liver and blood. Of all genes the highest fold increase was of hepcidin in the liver, which is not surprising since hepcidin is produced in the liver.

In accordance with our results, Myrnes et al. (2013) also detected lysozyme activity in the head kidney and spleen of Atlantic salmon in addition to gills and blood [68]. They also found gene expression of g-type lysozyme in the head kidney, spleen, gills and liver. Also cathelicidin and hepcidin have both previously been detected in cod [81, 192]. The highest expression of cathelicidin was detected in the head kidney and spleen of non-infected fish, similar to our results [192]. Hepcidin showed highest expression in the liver, like in our study, but also peritoneum and head kidney of control fish had high expression levels of hepcidin [81].

Differences in gene expression and enzymatic activity between the two pair of lysozyme genes suggest that the genes have different functionality. LygF1b is reported to have two alternative transcription start site which result in two products, one containing a signal peptide for secretion and one that lack this feature [69]. Most of the lysozyme that lack a signal peptide is suggested to have intracellular function [70]. No signal peptide sequence were identified in the three other lysozyme genes, LygF1a, c and d. These three genes also seem to be enzymatically inactive or low-active lysozymes since they have mutated catalytic sites in their sequences compared to LygF1b. This is in accordance with the observed higher lysozyme activity of LygF1a+b in both head kidney and spleen, compared to LygF1c+d.

LPS from *E. coli* does not induce expression of g-type lysozymes

Recently it has been shown that expression of Atlantic cod hepcidin and cathelicidin is highly induced by *E. coli* LPS both *in vitro* and *in vivo* [44]. In **paper III** live fish were injected with *E. coli* LPS followed by sampling of the head kidney at different time points for detection of g-type lysozymes. Also cod macrophages were incubated with *E. coli* LPS and harvested over time. The results revealed that *E. coli* LPS did not contribute to increased gene expression of the two pair of g-type lysozymes neither *in vivo* nor *in vitro*.

Atlantic salmon has both g-type and c-type lysozyme in contrast to Atlantic cod. Similar to our study g-type lysozyme was not detected in salmon macrophages after priming with *E. coli* LPS, while c-type lysozyme had increased expression [68]. The observation that LPS from the extracellular bacterium *E. coli* does not induce gene expression of g-type lysozymes, suggest that these genes require LPS signals through intracellular routes, in contrast to cathelicidin and hepcidin.

Preferably, LPS from the intracellular *F. noatunensis* should have been included in the experiments. However, as discussed above, *F. noatunensis* LPS is a poor inducer of inflammatory host responses in cod macrophages and was therefore excluded from the experiments.

***F. noatunesis* induce gene expression of antibacterial peptides**

Identification of natural *Francisella*-bactericidal factors in cod and especially in macrophages where the bacteria reside, open up for usage of such components in therapeutics. G-type lysozymes in fish have antibacterial properties and bacterial infections does often initiate expressions of this enzyme [70]. Also increased gene expression of the antibacterial peptides cathelicidin and hepcidin are reported during bacterial infections [80, 81, 84].

In vivo

Live Atlantic cod were in **paper III** infected (intraperitoneal) with *F. noatunensis* to study the effect on expression of antibacterial genes like LygF1a+b, LygF1c+d, cathelicidin and hepcidin. Surprisingly, gene expression of LygF1a+b was significantly down-regulated, in contrast to the expression of LygF1c+d, cathelicidin and hepcidin that increased significantly after infection. This outcomes are in accordance with high levels of hepcidin expression in Atlantic cod tissues 2 days after injection of inactivated *V. anguillarum* [81]. Also increased expression of antibacterial genes like cathelicidin, g-type lysozyme and hepcidin in gill

epithelial cells from cod infected with *V. anguillarum* and *A. salmonicida* show similarities to our results [84].

Taken together, it seems like all three types of antibacterial peptides, g-type lysozyme, cathelicidin and hepcidin, are involved in host defence mechanisms after *in vivo* infection of cod with the *F. noatunensis*. However, only one of the lysozyme gene pair, LygF1c+d, responded with up-regulation.

In vitro

By using the invasion assay, macrophages were infected with *F. noatunensis* with or without treatment of recombinant IFN-γ before infection, or with rIFN-γ alone. As mentioned earlier, rIFN-γ is known to activate macrophages and lysozyme is one of the effector mechanisms. Treatment of macrophages with rIFN-γ alone contributed to expression of LygF1c+d, but none of the other antibacterial genes. Also during *F. noatunensis*-infection of macrophages LygF1c+d was the only gene that was up-regulated. The gene expression of LygF1c+d was further increased with initial treatment of the macrophages with rIFN-γ, suggesting that the cytokine stimulate antibacterial activity in cod macrophages.

The infection of isolated cod macrophages confirms the importance of g-type lysozyme while cathelicidin and hepcidin genes were at the same level as control. Even though both hepcidin and cathelicidin have antimicrobial roles in mammalian phagocytic cells [75, 83], it looks like *F. noatunensis* does not trigger expression of these genes in cod macrophages. This could mean that other cells or systems than macrophages are causing gene expression of cathelicidin and hepcidin during infection *in vivo* in cod. G-type lysozyme on the other hand seems important in defence mechanisms in cod macrophages. However, it looks like the two pair of lysozyme genes have different roles in the defence against *F. noatunensis*. While LygF1b probably can be secreted from cells, there are several issues that indicate an intracellular role of LygF1c+d:

- ⇨ It is known that lysozymes can be located in the cytosol of monocytes and macrophages [193]. Since *F. noatunensis* most likely replicate in the cytosol of cod macrophages it is possible that increased gene expression of LygF1c+d serve a role in controlling intracellular growth of *F. noatunensis*.
- ⇨ The fact that LPS from the extracellular *E. coli* does not stimulate expression of cod g-type lysozyme genes, might point in the direction of intracellular roles of these peptides.

- ⇨ IFN-γ is known to activate macrophages and in paper III priming of macrophages with rIFN-γ alone contributed to gene expression of LygF1c+d, indicating that LygF1c+d are one of the effector mechanisms inside cod macrophages.
- ⇨ LygF1c+d probably lack a signal protein needed for secretion, which also suggest an intracellular function.

Three years ago, the use of immunohistological examination showed that g-type lysozyme in Atlantic cod was strongly associated with the intracellular bacterium *Yersinia ruckeri* in the core of granulomas after infection [71], also suggesting a role of the enzyme in defence against intracellular bacteria. Nevertheless, it is not known if cod lysozymes have lytic property on *F. noatunensis,* which is something that would need further investigation.

5. CONCLUSIONS

- *F. noatunensis* survive and replicate in both cod macrophages and ACL-cells and:
    - appears to disrupt the phagosomal membrane and escape into the cytosol soon after infection of cod macrophages
    - uptake of *F. noatunensis* in cod macrophages is actin filament dependent
- *F. noatunensis* seems to subvert and supress host immune responses by:
    - generating low induction of pro-inflammatory responses
    - higher expression of an anti-inflammatory response (IL-10)
    - poor induction of pro-inflammatory responses in cod macrophages after treatment with *F. noatunensis* LPS, in contrast to *E. coli* LPS
- rIFN-γ treatment of cod macrophages prior to infection:
    - increased bacterial invasion
    - stimulated the expression of one of the g-type lysozyme gene pair (LygF1c+d)
- Expression of AMPs in **non-infected** cod:
    - C-type lysozyme is not an essential enzyme in cod.
    - G-type lysozymes were expressed in all organs and tissues examined, although in different extent, while lysozyme activity was only detected in the spleen and head kidney.
    - Cathelicidin had highest level in the head kidney, spleen and blood while highest level of hepcidin was in the liver and blood.
- Expression of AMPs in cod **after infection** with *F. noatunensis*:
    - During ***in vivo*** infection of cod with *F. noatunensis*, the antibacterial peptides cathelicidin, hepcidin and one of the g-type lysozymes (LygF1c+d) appears to be important in the host immune response.
    - During ***in vitro*** infection of cod macrophages with *F. noatunensis*, only one pair of the g-type lysozyme genes (LygF1c+d), and not cathelicidin and hepcidin, was upregulated.
    - LygF1c+d genes in cod seem to have an intracellular role in host defence.

6. FUTURE PERSPECTIVE

In intensive high-density fish farming, effective disease control is needed. Vaccination in aquaculture has improved the production and reduced the usage of antibiotics dramatically, from 50 000 kg in 1987 to less than 2 000 kg in 1997, while the production increased from 50 000 tonnes to 350 000 tonnes in the same period [194, 195]. Essential for effective vaccine development is to induce specific immunity that protects the fish against the pathogen in the future.

A precondition for development of disease control is to understand the cellular and molecular mechanisms underlying the infection, like the involvement of receptors and signalling pathways. In the current work invasion assays were established as a tool for functional studies of host-pathogen interactions between Atlantic cod cells and *F. noatunensis*. Unfortunately researcher lack specific markers for cellular and molecular components of the fish immune system. This makes it difficult to investigate the downstream signalling from receptor activation and needs future attentions.

Antimicrobial proteins naturally produced by the organisms are potential alternatives to antibiotics. The results show that lysozyme, cathelicidin and hepcidin seems to play important roles in the innate immune defence against francisellosis. However, the antibacterial activity of purified, synthetic or recombinant forms of these proteins on *F. noatunensis* needs further research, in addition to whether lysozyme can prevent intracellular growth of the bacterium.

A live attenuated vaccine has shown protection against wild-type *F. asiatica* in tilapia. Live attenuated vaccines has the advantage of using the natural routes of attachment and initiate signalling cascades and induce protective immunity without bacterial proliferation. The whole-genome sequence of *F. noatunensis* ssp. *noatunensis* may reveal opportunities to develop an attenuated bacterium to use in vaccines in the future.

7. APPENDIX

In paper III, the resolution of the published Figure 1was significantly reduced compared to its original due to format changes during Scientific Reports article processing.

A higher resolution version of Figure 1 is therefore presented in this Appendix (next page).

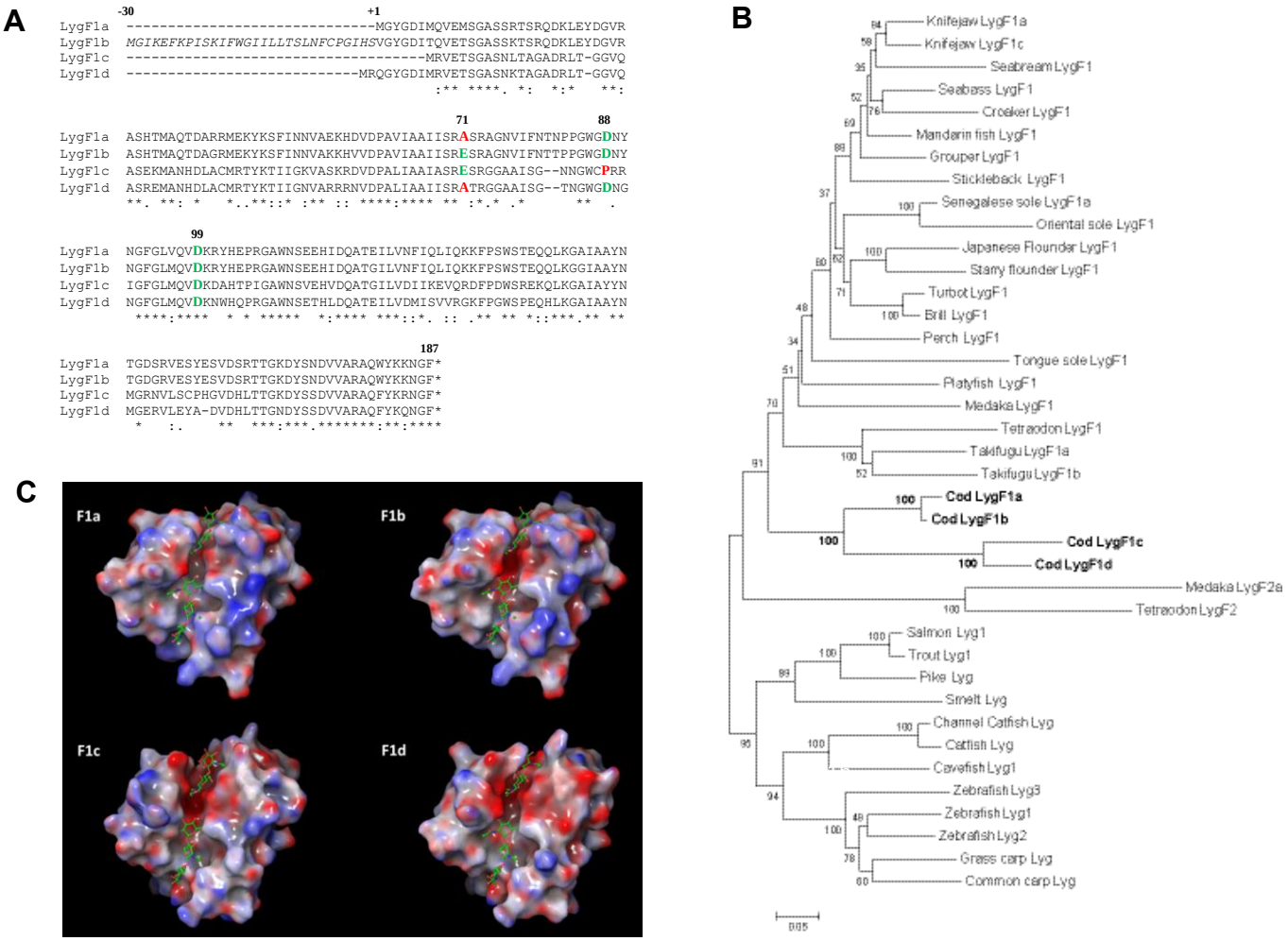

Figure 1. Atlantic cod g-type lysozymes and electrostatic potential. (**A**) Alignment of cod g-type lysozyme protein sequences. Amino acid sequence alignment was generated with Clustal Omega, with the symbols below the alignment complete conservation (*), sites with strongly conserved properties (:), and sites with weakly conserved properties (.). The The sequences are numbered from the N-terminus of the mature LygF1b proteins, with the signal peptide in italic and numbered backwards. The catalytic residues equivalent to E73 (E71 in the cod LygF1a/LygF1b sequences) and D86 and D97 (D88 and D99 in the cod LygF1c/LygF1d sequences) in the g-type sequence are indicated, with residues compatible with enzymatic function in green and those that should prevent enzymatic activity shown in red. **(B)** Phylogenetic relationships of g-type lysozyme from cod and other fishes. A bootstrapped (1000 replications) neighbour-joining tree based on maximum composite likelihood distances was generated using MEGA6.2[54]. Numbers at the nodes represent the number of bootstrap replicates (out of 1000) that supported each node. Branch lengths are proportional to amount of inferred change, with scale bar (changes per base) shown at the bottom. Trees were rooted based on previous phylogenetic analyses of g-type lysozyme sequences[14]. (**C**) 3-D models of LygF1a, LygF1c, and LygF1d were constructed based on the previously identified structure of LygF1b[26] and the electrostatic surface potential is indicated in red (negative) and blue (positive). NAG molecules are presented as sticks docking into the LygF1 models in correspondence with the observations in native LygF1b.

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