GÖTEBORGS UNIVERSITET

Intestinal Mucosal Immunology of Salmonids

Response to Stress and Infection and Crosstalk with the Physical Barrier

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Dissertation abstract

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The effect of environmental factors and pathogens on the intestinal epithelium of fish has received increased attention in recent years. Studies focusing on effects of stress, nutrient uptake as well as vegetable ingredients in fish feed have all shown that the intestine is affected by environmental factors. The signs of inflammation during exposure to detrimental environmental conditions have brought to attention the local immune system in the gut. The gut is further one of the main routes for pathogen infection in fish. Therefore this thesis aims at investigating the mucosal immune factors and systems that are affected by environmental stressors and pathogen interactions.

In this thesis the effect of long term environmental stress on the mucosal intestinal epithelium was investigated. Results showed an ongoing inflammation in the intestine that was manifested as a compromised barrier integrity, infiltration of immune cells and an affected immune response. Atlantic salmon was co-habitant infected with infectious pancreatic necrosis virus as well as immune challenged with the viral mimicker, double stranded RNA Poly I:C, where after the mucosal immune response was studied. Both treatments clearly demonstrated an antiviral response including alterations of IFN type I and the Mx protein. When the fish were exposed to a stressor and immune stimulation in combination, the fish immune response was delayed. This stresses the importance of minimize stressful situations for the animals in, for example aquaculture. The demonstrated increase in intestinal epithelial permeability together with inductions of the mucosal immune system raises the question of whether stress or inflammation is the causative agent of the barrier dysfunction.

To address this, the effect of the immune system on the intestinal epithelia was assessed using an in vitro Ussing chamber approach in which the intestinal epithelia was exposed to recombinant cytokines. Exposure to IL-1β and IL-6 showed negative impact on the intestinal permeability, suggesting that the immune system of the fish is contributing to the inflammation seen during prolonged stress. Further, the tight junction proteins create an extracellular net-work between the epithelial cells and by that controls the intestinal paracellular permeability was shown to be affected by the two cytokines.
The interactions between stress, the immune system and the epithelial barrier function are therefore highly complex and important for our understanding of the physiology of health, welfare and disease.

Keywords: Inflammation, IPNV, Poly I:C, Recombinant cytokines, CD8, MHC-I, Claudins, Permeability, Environmental stress, Cortisol, DNA constructs, IL, IFN
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Abbreviations

MALT	Mucosal-associated lymphoid tissue
GALT	Gut-associated lymphoid tissue
M cell	Microfold cell in the Payers patches
NF-κB	DNA transcription factor Nuclear Factor κB
IL	Interleukin
IFN	Interferon
TNF	Tumor necrosis factor
TGF	Transforming growth factor
Mx	Myxovirus resistance (by origin)
CD	Clusters of differentiation antigen on T cells. Different types on different cell types
B cell	Antibody producing lymphocyte
T cell	Lymphocyte derived from the thymus (T)
T_h	T helper cells
T_c	Cytotoxic T cells
$CD8^+$	CD8 positive – associated with Tc
$CD4^+$	CD4 positive – associated with Th

List of papers which are referred to in the text by their Roman numbers

I
Disturbance of the intestinal mucosal immune system of farmed Atlantic salmon (Salmo salar), in response to long-term hypoxic conditions. Fish and Shellfish Immunology (2011). Niklasson L.; Sundh H.; Fridell F.; Taranger GL.; Sundell K. Fish and Shellfish Immunology 31:1050-4648

II
High stocking density and poor water quality disturbs the intestinal physical and immunological barriers of the Atlantic salmon. Sundh H.; Niklasson L.; Finne-Fridell F.; Ellis T.; Taranger G L., Pettersen E F.; Wergeland H I.; Sundell K. (Under revision for publication in Fish and Shellfish Immunology)

III
Modulation of innate immune responses in Atlantic salmon by chronic hypoxia-induced stress (2013). Bjørn Olav Kvamme; Koestan Gadan; Frode Finne-Fridell; Lars Niklasson; Henrik Sundh; Kristina Sundell; Geir Lasse Taranger; Oystein Evensen. Fish and Shellfish Immunology 34:1095-9947

IV
Cortisol effects on the intestinal mucosal immune responses during cohabitant challenge with IPNV in Atlantic salmon (Salmo Salar). Niklasson L.; Sundh H.; Olsen R-E.; Jutfelt F.; Skjødt K.; Nilsen T O.; Sundell K. (Submitted for publication in PLOS ONE)

V
Recombinant cytokines interleukin 1 beta and interleukin 6 increases intestinal epithelial permeability in Rainbow trout (Oncorhynchus mykiss). Niklasson L.; Sundell K.; Martin S.; Secombes C.; Sundh H. (Manuscript)

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INTRODUCTION

Immunology of fish

The basic mechanisms behind an immune response follow certain patterns that are preserved throughout evolution. These include macrophage activity, cytokine or complement factor signaling as well as specific targeting of pathogens [1]. Fish represent approximately 50% of the total number of vertebrates known today with about 32 500 species [2, 3]. Fish species differ in physiology depending on e.g. habitat and lifecycle, stressing the need to examine each group of fish to increase the understanding of their specific biology. Fish immunology, is an expanding research area within the field of fish physiology and is focused on a few commercially important species (e.g. salmonids, carp, sea bass and sea bream) as well as model species (e.g. zebrafish and medaka). The present thesis focuses on two of the commercially important salmonid species: The Atlantic salmon, *Salmo salar*, and the rainbow trout, *Oncorhynchus mykiss*. There is increasing awareness in the aquaculture industry that knowledge about fish biology contributes to strengthen ethical and economic sustainability. Particularly with the growing challenges associated with health and welfare of farmed fish. Diseases, pathogens and parasites as well as potentially stressful husbandry conditions and feed composition are key issues that need to be addressed. A key factor in addressing the health and welfare challenge and to allow timely and appropriate intervention is the need for an increased understanding of the fish immune system. The number of reviews about fish immunology in recent years illustrates the increased interest in this area and also the state of the art and advances that have been made [4-9].

Lymphoid tissues in fish

The distribution of the main lymphoid tissues in fish differs from that in mammals. The most evident example is the hematopoietic tissue that generates the white blood cells. In mammals the leukocytes originate from the bone marrow, but this tissue is lacking in fish and the head kidney functions as the primary hematopoietic organ. The head kidney is the most proximal part of the kidney that is localized ventral to and along the spine. Other important lymphoid tissues in mammals and fish are conserved and

include the thymus, where the T lymphocytes (T cells) mature and the spleen, the blood depot where pooling of lymphocytes such as memory cells occurs [10]. Further, the liver has an important function in blood surveillance and in clearing the body of possibly harmful substances. The appearance of leucocytes has been studied in both mammals and fish [11]. Macrophages appear in early developmental stages, already prior to birth or hatching, and are part of the innate (native) immune function. The presence of recombination activating genes (Rags) that are essential for somatic recombination an important event that underlies the specificity of T cell receptors and antibodies appears in the thymus within weeks after hatching. T cells are part of the acquired (specific) immune function and are together with B-cells responsible for e.g. antibody production. The specific effectors of the acquired immune system (e.g. antibodies) in fish differ from those in mammals and this may contribute to an underestimation of the importance of the acquired system in fish immune function. In fish one of the essential steps in the development of the lymphocytes, that is in common with what occurs in mammals, is recombination of the V(D)J gene segments. This generates a functional exon encoding the variable region of the antibody determining antibody specificity. It has recently been suggested that the antibody repertoire in fish might be greater than previously thought [12, 13]. Furthermore, genome duplications have led to emergence of new or subdivision of function between duplicate genes and hence a greater variety in gene and protein function [14].

Mucosa associated lymphoid tissues

The function of secondary, or peripheral, immune tissues are similar when comparing mammals and fish even though organization differs. The mucosa-associated lymphoid tissue (MALT) of mammals consists of organized immune centras, or lymph nodes, of which the gut associated lymphoid tissue (GALT) has been most intensively studied [15]. Mammals possess a GALT with Peyer's patches, involutions in the epithelial cell layer where co-localization and interaction between different immune cell types take place. The Peyer's patches possess microfold cells (M cells) that are specialized in transport of particles or organisms (antigens) and resident lymphocytes that interact to mount an adequate response to the challenge at hand. M cells do not contain lysozomes and can therefore transport

foreign antigens intact across the cells. This property has not conclusively been shown in any epithelial cell of fish. However, the existence of M-cell like cells has been proposed for salmonids [16]. Also non-pathogenic bacteria can utilize the M cells to cross the epithelial barrier and the areas that contain Peyer's patches lack mucus secreting goblet cells characteristic of the rest of the epithelial lining of the intestine [17]. B lymphocytes are concentrated in the central region of the Peyer's patches while T cells and dendritic cells surround the area. The resident cells of the GALT attract T cells to the area and initiate contact [15]. These interactions are important for the initiation of tolerance and/or immune response. Several MALT have been identified in fish; they possess GALT, skin-associated lymphoid tissue (SALT) and gill-associated lymphoid tissue (GiALT) and the GALT, the key tissue in the present thesis which will be presented in more detail below [5].

The innate immune response

Fish rely to a large extent on a highly diversified innate immune repertoire controlled by cytokines [9]. Cytokines are small proteins that mediate the immune response and constitute the signaling network of the immune system. They are released from all cell types and the innate system constitutes an efficient defense for a wide range of pathogens. The innate immune response is based on native recognition of harmful substances and instant activation of certain cell types and mediators. The recognition of non-self-motifs, *i.e.* pathogen-associated molecular patterns (PAMPs), is made by pattern recognition receptors (PRRs) such as toll like receptors (TLRs). TLRs are abundant in both fish and mammals and constitute an initial contact between the animal and potential threats in their surroundings [18-20]. Their activation by an antigen (bacteria, virus or other harmful substance) is followed by activation of the complement system acting to clear the threat by cell lysis and at the same time signal to the immune system. The immediate innate immune response to the presence of a "foreign" agent includes inflammatory cytokines, acute phase proteins and antimicrobial peptides [21, 22].

The cell types involved in the innate immune response of fish includes monocytes (e.g. macrophages) and granulocytes (e.g. neutrophils) as well as

eosinophil granulocytes that resemble the mammalian mast cells. However, most mast cell like cells in fish does not produce histamine. At the cell level, a typical immune response starts with secretion of cytokines by tissue cells as well as of prostaglandin and other compounds from mast cells. The secreted substances activate macrophages in the tissue and stimulate recruitment of neutrophils from the circulation [23]. Macrophages neutralize foreign antigen by phagocytosis and secrete cytokines and chemokines (chemotactic cytokines) that attract neutrophils to the area. The neutrophils take over and act on the threat by engulfing debris, clear the area of pathogen and undergo apoptosis. In a later stage a shift in chemokine action occurs and monocytes are recruited and differentiate into macrophages that then re-populate the tissue [24].

In the early innate immune response certain transcription factors controls the expression of cytokines and effector proteins. Well studied transcription factors includes nuclear factor κB (NF-κB) and, in the case of viral infection, interferon regulating factor (IRF) [6, 25, 26]. These transcription factors promote transcription and translation of cytokines that on binding to cytokine receptors in surrounding cells activate them and magnify and diversify the response to neutralize the threat at hand. In mammals, NF-κB is involved in many different responses, the immune response being one of the most frequent. Prior to activation, NF-κB is situated in the cytosol bound to its inhibitor (IκB). Upon activation, NF-κB is released and translocates to the nucleus where transcription of several genes is initiated, one of them being the gene for the inhibitor, IκB, leading to self-regulation of activation. The presence of this mechanism in fish has been reported [27]. The cytokines produced during the invasion of a microorganism propagates the response and if necessary facilitates the switch to a more specific response as will be described later. Therefore, cytokines are important not only for activation but also for their timing in the response and the effect each cytokine has on different cells in the cascade, factors that all contribute to the sum response to a specific threat.

The acquired immune response

After the initial innate activation and if the threat remains the response switches to a more specific mode of action through T cell activation,

initiated by cytokines [28]. The acquired immune system starts to develop early in life but depends on gene recombination creating specificity to fit the encountered pathogens. Evidence that a similar mechanism operates in both mammals and fish has started to emerge [12] (Figure 1). T cells react to antigens presented by antigen presenting cells (APCs), which engulf and break down foreign antigens invading the tissue. An APC is any cell capable of engulfing and presenting antigen e.g. monocytes (macrophages and dendritic cells) or B cells. In T cell dependent activation the antibody production by B cells is controlled by T helper (T_h) cells that express the CD4 complex ($CD4^+$ cells; CD=cluster of differentiation). The antibodies produced after B lymphocyte activation attaches to the pathogens surface thereby marking it for destruction. Cytotoxic T cells (T_c) expressing the CD8 complex ($CD8^+$ cells) can also react directly by secreting toxic compounds that provoke cell lysis when an infected cell presents a "non-self" antigen. The T cells interact with the presented antigen through the T cell receptor (TCR) complexes. As part of the complex, T cells express CD3, a signal transducing complex of the TCR crucial for T cell activation and proliferation [29]. CD3 have been cloned in fish [30].

On both the APCs and T cells, the specific CD complexes act to produce the right key-lock between the cells. The cytotoxic T cell (T_c) response is promoted when a cell derived, or viral, antigen is presented by major histocompatibility complex class I (MHC-I). MHC-I interacts with a TCR and CD8 leading to a cytotoxic response by the Tc cell. The humoral response were antibodies are produced by activated B cells are promoted when an exogenous antigen is presented by MHC-II and interacts with a TCR and CD4 leading to antibody production and/or T_c cell promotion. All these cellular markers have been identified in fish and studies suggest a similar function in fish as seen in mammals [12, 31-34]. Two major subsets of $CD4^+$ T_h cells have been characterized which are called T_h1 or T_h2 depending on whether they promote cytotoxicity and inflammation or antibody production by B cells [12, 34, 35]. Over the years new T_h cell subsets have been identified. Regulatory T cells (T_{reg}) modify the T cell response and has an important role in the development of oral tolerance [36]. In peripheral tissues such as the mucosa, $CD8^+$ cells also play an important role in tolerance by down-regulating antibody production against antigen [37]. T_h17 cells secrete interleukin-17 (IL-17) that promotes inflammation by

inducing expression of pro-inflammatory cytokines and attracting neutrophils [38]. However, by inhibiting the T_h1 pathway, IL-17 can also protect against chronic inflammatory disorders [39]. Furthermore, the γδT cells are suggested to have a role in the early immune response as these T cells lack the regular TCRs and are activated by non-conventional pathways [40]. In addition to these responses there are also natural killer (NK) cells which derive from the T cell lineage but that are included in the innate response, and act in a less specific manner. The different classes of T cells are activated through sequential exposure to cytokines, which also control memory cell proliferation.

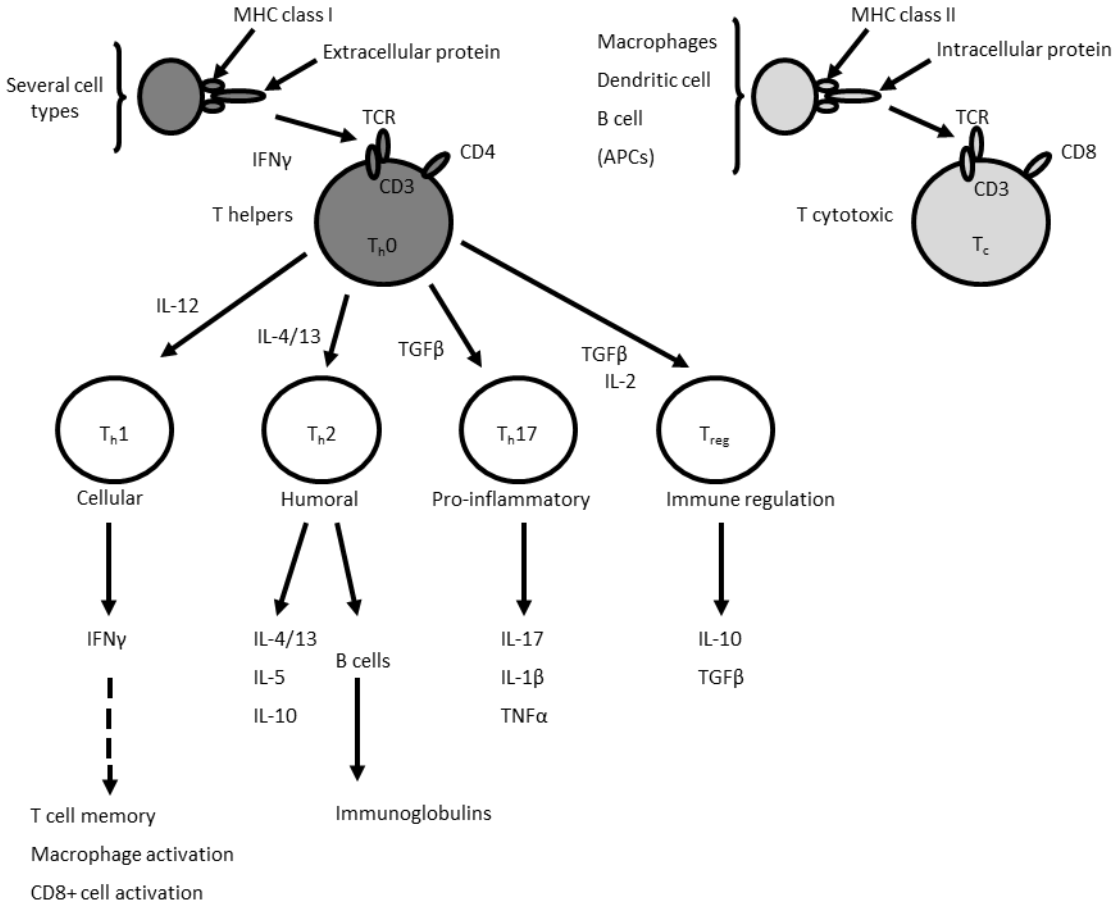

Figure 1.

The acquired immune response adopted from mammalian studies presenting the immune markers found in fish.

In fish, the broad spectrum of innate responses described above has been used to explain the relatively slow response of their acquired immune response compared to mammals. Ectothermia is another factor that may impair the possibilities to maintain a robust, long term specific response, a characteristic of the acquired immune response [42, 43]. Particularly since temperature fluctuations will also affect the pathogens and the degradation processes. The nature of the acquired immune response in fish represents a barrier to the development of vaccines for fish with a good response upon re-exposure [44]. Current research, however, suggests that the acquired immune response in fish may be underestimated [12, 44, 45].

Cytokines in fish

Cytokine regulation of the immune responses

The immune response in fish and mammals is based on communication between cells and the mediators are called cytokines. The innate immune response in both fish and mammals is initiated by cytokines such as IL-1β, tumor necrosis factor α (TNFα), IL-2, IL-6 and interferon γ (IFNγ) [45]. These are often referred to as pro-inflammatory cytokines due to their role in initiating and facilitating the inflammatory response (Figure 2). The immune response against virus is somewhat different and relies on other cytokines, the IFNs, for an appropriate response. Interferons induce anti-viral proteins and activation of Type I IFNs leads to the production of anti-viral protein Mx while Type II IFN (IFNγ) leads to production of anti-viral protein γ inducible protein (γIP also called IP10). However, IL-1β is also involved in the anti-viral response as shown by e.g. Haugland et al (2005) [46].

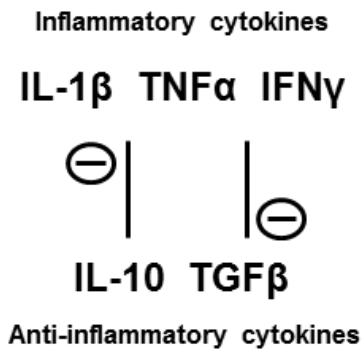

Figure 2.
The inflammatory
– anti-inflammatory balance.

In the innate response, IL-10 and transforming growth factor β (TGFβ) are induced and modulate the sum response in order to avoid an overreaction of the inflammatory response and for this reason are often referred to as anti-inflammatory cytokines (Figure 2) [47]. IL-8 is also activated in the innate immune response and aids the infiltration of immune cells into the tissue [48]. IL-8, or CXCL8, belongs to the chemokines that are generally classified based on their structure. CC chemokines have two adjacent cysteines near the N terminal of the protein, while the cysteines are separated by one amino acid in CXC chemokines. Both CXC and CC chemokines are found in fish although their action remains to be clarified [49, 50]. In the chemotactic process cytokines such as IL-1β, TNFα, IFNγ and IL-6 are involved in inducing expression of adhesion molecules for the cells that are about to enter the tissue e.g. neutrophils and thereby allowing them access to the area [51-53].

Also in fish, several cytokines are involved in modulating the acquired immune response (Figure 1). IFNγ, among others, initiates the activation of the acquired immune response. This cytokine together with IL-2 are expressed by T_h1 cells and promotes these, whereas T_h2 cells are promoted by and express IL-4, IL-5 and IL10 [54, 55]. The functionality of a T_h1/T_h2 relation as defined in mammals as well as the roles of other T_h cell subsets in fish has not yet been verified. However, the presence of cell markers and cytokine patterns suggests that these functions also exist in fish. In the present thesis key cytokines have been selected to examine the level of immune activation in the innate and acquired response. Key cytokines in fish are presented below with focus on their function in mammals and compared to what is known in fish.

IL-1β

IL-1β is produced and released by most cell types in the body and is a marker of inflammation, infection and overall immune activation. An increase in IL-1β leads to activation of the immune response, e.g. in mammalian Caco-2 cells activation of IL-1β leads to IκB degradation, NF-κB release and activation of the transcriptional process leading to expression of other cytokines as well as IL-1β itself [56]. In Atlantic salmon, activation of the IL-1β receptor induced NF-κB activity [57] and it further facilitates

transport of immune cells to the affected area by stimulating mobilization of cell adhesion molecules [52]. Recombinant IL-1β was used to stimulate rainbow trout monocytes/macrophages (the RTS-11 cell line) for large scale assessment using microarray [58]. Clusters of genes involved in immune response, defense response, transcription and signal transduction were shown to be activated [58]. Further, IL-1β was shown to promote genes involved in macrophage and neutrophil and, to a lesser extent, T and B cell function. IL-1β has also been shown to induce cortisol release and to be up-regulated during behavioral fever in rainbow trout suggesting a role in temperature selection in salmonids [59, 60].

TNFα

TNFα is another important cytokine in the early inflammatory response. Reports suggest a cooperative effect of TNFα and IL-1β in activation of the inflammatory response [61]. It is mainly produced by macrophages and is often used as a marker for inflammation in medicine. Recombinant IL-1β (rIL-1β) as well as lipopolysaccharide (LPS) from gram negative bacteria have been shown to induce TNFα expression in the head kidney macrophages of rainbow trout [62, 63]. In fish, TNFα expression induced immune activity in endothelial cells and is suggested to be involved in chemotaxis and adherence while little effect was seen on macrophages [64]. However, TNFα has been shown to up-regulate IκB in rainbow trout leucocytes, an indication of NF-κB activation [27]. The expression decreases over time, suggesting a control mechanism to avoid over reaction. TNFα has also been suggested to orchestrate down-stream effects in the anti-viral response inducing IFNs, Mx, IRFs, TNFs and ILs as well as JAK/STAT components [65].

At least three TNFα homologs exist in salmonids and are active in different tissues. The latest, TNFα3 was recently discovered and differential expression in different tissues has been suggested (Secombes personal communication). Up-regulation of TNFα1 seems to be the response to immune activation in lymphoid tissues but this form is less regulated in the intestine, while TNFα2 is up-regulated in intraepithelial cells (IECs) in response to bacterial challenge [19].

IL-6 and IL-8

IL-6 is released by macrophages, neutrophils and T cells but also by cells not belonging to the immune system [24]. Through inhibitory effects on IL-1β and TNFα, IL-6 can also function as an anti-inflammatory mediator [66]. However, effects of IL-6 on the intestinal epithelia also suggest that this cytokine is involved in increased epithelial permeability during stress in rats [67].

IL-8 attracts neutrophils and is for example found in endothelial cells where it is secreted in response to histamine as well as to IL-1β [48]. IL-8 is released by macrophages but also by other cell types such as epithelial cells upon IL-1β and TNFα stimulation (Figure 3). It has been shown that IL-1β and TNFα cooperatively increased expression of IL-8 from macrophages [68]. IL-8 can also be induced by IL-17 released from T_h17 cells [69]. In fish, induction of IL-8 has been reported in concert with IL-1β and TNFα after a bacterial challenge [19, 70]. Further IL-8 has been shown to induce IL-1β, TNFα as well as CC chemokines in rainbow trout and modulate their expression during viral infection [71, 72].

In mammals, IL-6 has been proposed to play an important role in the shift of phagocytes during inflammation [73] (Figure 3). This is accomplished

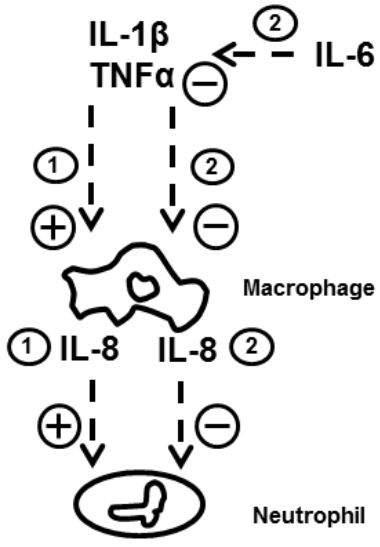

Figure 3.
The homing of neutrophils during an immune response in mammals

during neutrophil infiltration through induction of monocyte chemotactic protein 1 (MCP-1) as well as by increasing the oxidative burst and macrophage differentiation [73]. Further, IL-6 was shown to inhibit IL-1β and TNFα induced IL-8 secretion. Hence, secretion of IL-6 is part of the inflammatory response and has in the later stages of inflammation been

proposed to induce the shift from infiltration of neutrophils to infiltration of monocytes which subsequently differentiate into macrophages [24].

A similar relationship can be seen also in fish [74, 75]. IL-8 is up-regulated in response to recombinant IL-1β in rainbow trout macrophages [75]. LPS, Poly I:C and rIL-1β strongly induce IL-6 expression in rainbow trout macrophages while the induction in the monocyte cell line RTS-11 [76] was weaker [76]. IL-6 increased macrophage proliferation and decreased IL-1β and TNFα expression while expression of IL-10, complement factors and anti-viral factors was up-regulated [74].

IFNs

IFNs are anti-viral cytokines that are secreted from virus infected cells and act as an alert for cells in their proximity by activating the JAK/STAT intracellular pathway [77, 78]. IFNs stimulate production of anti-viral proteins and are also capable of inducing a specific immune response. The activation pathways involve the transcription factors interferon regulatory factor (IRF) and NFκB [65].

The IFNs classified as type I are released from all cells in response to viral infection and includes several cytokines, e.g. IFNα and IFNβ [79]. IFN type I promotes apoptosis of infected cells [80] and stimulates production of e.g. anti-viral Mx protein as well as MHC-I, IRF and JAK/STAT components [6]. Studies have shown that although the IFN type I in fish are different from the mammalian counterpart, they share common induction patterns suggesting a common retained process for anti-viral activity including IFN and NF-κB activation [81].

In the innate immune response IFNγ is released by NK cells in response to activated phagocytes and infected APCs [6]. In rainbow trout, recombinant IFNγ induces expression and production γIP that attracts T cells [82]. To some extent IFNγ also stimulates the anti-viral protein Mx [83]. It further activates macrophages and initiates the acquired immune response by activating T cells. MHC-II, STAT1 (of the JAK/STAT) and MHC-I antigen presentation is all up-regulated by recombinant IFNγ [58, 82]. IFNγ are also released from T_h and T_c cells in response to MHC presented antigen [6].

Microarray studies have shown that IFNγ also activates genes involved in immune response, defense response, transcription, antigen presentation and catabolism in Rainbow trout RTS-11 cells [58].

IL-10 and TGFβ

The anti-inflammatory actions of IL-10 and TGFβ in mammals has been known for a long time [47]. These cytokines act on macrophages and inhibit the production of inflammatory cytokines. Studies also show that these cytokines can switch macrophage function from inflammatory towards anti-inflammatory actions [84, 85].

IL-10 modulates the inflammatory response and has a role in immune modulation [86]. It was first found to be secreted from CD4 positive cells of the T_h2 sub-population and inhibited IFNγ production by T_h1 cells via macrophage inhibition [87]. Mouse deficient in IL-10 readily develops enterocolitis mediated by T_h1 cells and is the causative agent in certain chronic inflammatory diseases when the T_h1/T_h2 balance is skewed [88]. Also NK cells are inhibited by IL-10. Studies on mice have shown that IL-10 is responsible for TGFβ secretion from T_{reg} cells that are involved in tolerance and modulation of T cell responses also known to induce chronic intestinal inflammation [89].

The anti-inflammatory TGFβ response is generally slower than the IL-10 response [47, 90]. Both cytokines function as immune modulators and the delay in the TGFβ response may be explained in part by the induction of TGFβ release by IL-10 [89]. TGFβ has been associated with T_h17 activation from naïve $CD4^+$ cells which could in part explain its role in promoting chronic inflammation [91]. Further, TGFβ has a critical role in the thymus were it promotes T cell development towards $CD8^+$ cells and NKT cells, a TCR expressing NK cell, related to the T_{regs}. TGFβ also induce differentiation of $CD4^+$ cells into T_{regs}, involved in self-tolerance, and also inhibits $CD4^+CD8^+$ cells to develop into T_h1, T_h2 and T_c cells thereby dampening the immune response [91].

Both cytokines are induced in rainbow trout upon immune stimulation by immersion in water containing plasmid DNA, lactoferrin and β-glucan [92]. The timing of up-regulation was found to follow the pattern seen in mammals.

IL-17

In mammals, IL-17 was first found to be expressed by CD4 positive T_h cells that were later shown to be a new T_h cell subset due to its unique properties in inflammation [93]. IL-17 is predominantly released by T_h17 cells but may also be released by γδT cells, NKT cells and neutrophils [94, 95]. IL-17 attracts neutrophils and functions as a bridge between the innate and adaptive immune processes through activation of pro-inflammatory pathways leading to tissue inflammation [69]. IL-17 was detected in Atlantic salmon thymus and intestine and induction of expression by LPS was found in spleen and head kidney [96]. Further, IL-17 mRNA expression increases in fish during soy-bean meal induced enteritis [97].

Summary

Complement of ILs in mammals and fish is similar. There is evidence from a few fish species and a relatively limited number of challenge studies that there may be conserved function between mammals and fish. However, taking into consideration the complexity of the immune system in fish, their habitat diversity and evolutionary diversity and species specificity of infection considerable work will be required to characterize cytokines family members and their action in fish.

The mucosal immune system of salmonid fish

Carnivore fish, such as the salmonids, possess a short, tube-like intestine divided into a proximal and distal part (Figure 4). The proximal part is thin walled and secretes a modest amount of mucous relative to the distal intestine and this part of the gut is responsible for the active absorption of nutrients, ions and water. The distal intestine is thicker and produces larger amounts of mucus and is the site of final ion/water exchange with the lumen contents. In the distal intestine exocytosis is evident and suggests macromolecular uptake and sampling of molecules occurs [5, 7].

The dynamics of nutrient transport and bacteria interactions in the intestine leads to a constant contact between the immune system and the luminal content. Immune cells as well as intra epithelial cells (IECs) readily

sample antigen from the lumen and present them to the immune system. The principles of oral tolerance in the gut is complex and results in a fine tuned equilibrium that differs from what is seen in other tissues.

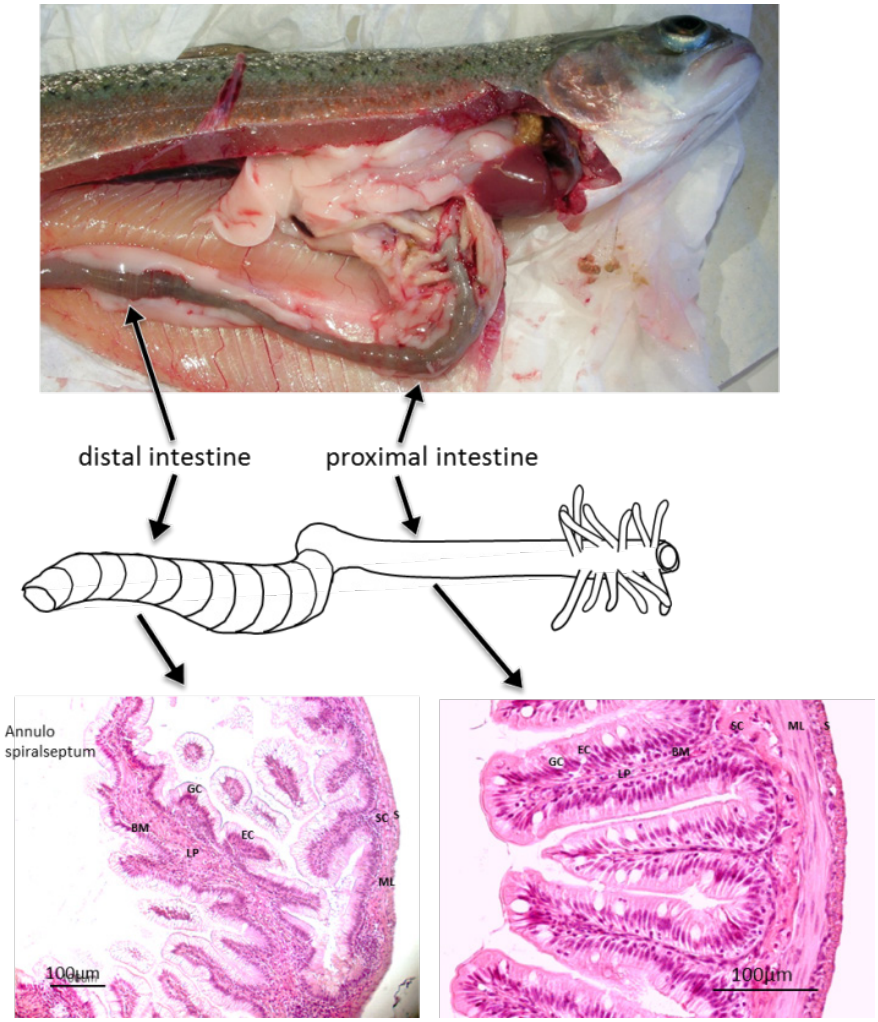

Figure 4.
The proximal and distal intestine of salmonids
A. Micrograph of the distal intestine showing a typical *annulospiral septa*
B. The proximal intestine
Epithelial cells (EC) lines the villi with goblet cells (GC) interspersed and underneath the epithelia the Lamina propria (LP) can be found. Further shown is the Basal membrane (BM), Stratum compactum (SC), Muscle layer (ML), Serosa (S) and Intestinal lumen (IL)

The GALT in fish

Apart from the innate and acquired systemic immune systems there is also immune systems present in the peripheral tissues that faces the

surrounding i.e. the mucosal immune systems. The most important is the gut-associated lymphoid tissue (GALT) due to its multifunctionality, antigen load and the endogenous microflora. The GALT of fish is more loosely arranged and the lymphoid follicles present in mammals have not been detected in fish [7]. This often leads to the false conclusion that the GALT mucosal immune system of fish is less effective. However, fish possess several immune cell markers in the gut equivalent to mammalian immune cells and antigen presenting cells suggesting they have a similar function [7, 16, 98]. As the IECs are constantly exposed to self and foreign antigens due to the exposure to the intestinal luminal content these cells are therefore in a more or less activated state at all times and possess dendritic cell like functions such as antigen sampling as well as antigen presentation [99]. Goblet cells are interspersed between the epithelial cells and secrete mucus containing antibacterial enzymes and antibodies. Similar to mammals, intraepithelial leucocytes e.g. Mast cell like cells, macrophages and lymphocytes, can be found between the epithelial cells as well as basally to the epithelia [5]. The mast cell like cells contain vacuoles with broad spectrum anti-pathogenic substances while macrophages are signal transducers and phagocytes. The layer underneath the gut epithelia is called the basal membrane which is followed by the lamina propria. The lamina propria is a layer of loosely arranged connective tissue where most of the mucosal immune cells reside (Figure 4). In mammals, B cells from this area secrete, into the mucus, immunoglobulin A, the major mammalian secretory antibody. The fish equivalent to IgA was long believed to be IgM although this conclusion has been revised in recent years with the discovery of IgH derived IgT/IgZ [100].

The intestinal epithelium

Together with the mucosal immune system, the intestinal epithelia should constitute an effective primary barrier against pathogens. The intestinal epithelium is composed functionally of an intrinsic and an extrinsic barrier [101]. In a healthy fish, these barriers constitute sufficient protection against pathogens in the surroundings. There is a constant but mainly controlled translocation of antigens across these physical barriers for presentation to the underlying immune barrier. However, these barriers are known to be affected during stress and infection which may lead to

impaired barrier function with increased permeability and cellular damage [102]. The intestinal epithelial immune system is also affected by stress e.g. suppression of IFNγ in intraepithelial leucocytes (IELs) has been reported after repeated stress in mice [103].

The intrinsic barrier - the epithelial cells and the tight junctions

A single layer of epithelial cells constitute the intestinal lining separating the circulation from the intestinal lumen. Between these epithelial cells there are several junctional protein complexes that hold the epithelium together and regulate the paracellular flux of small hydrophilic molecules, water and ions. The junctional protein complexes, called tight junctions (TJs), separate the mucosal side of the epithelial cells, the one facing the intestinal lumen, from the serosal or tissue side. Thus, two distinct compartments are created with an apical side and a basolateral side. These two sides are significantly different from each other resulting in differences in cell membrane composition and transport between the two sides. The TJs are attached to the actin filaments of the cells, these can contract and rapidly affect the intestinal permeability. This process is, among other factors, regulated by myosin light chain kinase and increases in response to pathogenic compounds [104, 105]. Further, the composition of the TJs can be altered resulting in changes in size and charge of the pores constituting the paracellular pathway [106]. The TJs are composed of a set of transmembrane proteins together with the ZO-1 proteins which are localized close to the cell membrane and cross link the cells actin filaments to the TJ complex [107, 108]. The protein occludin binds directly to ZO-1 [109] and occludins, tricellulin as well as a variety of claudins forms an extracellular network between the cells [106]. The extracellular network regulates the paracellular permeability the epithelium to hydrophilic molecules. The number and relative proportion of the involved pore forming and tightening proteins will dictate the pore size. The type of claudin isoform expressed will also influence the electrochemical characteristics of the TJs and dictate both ion and size selectivity [110] (Figure 5). In Atlantic salmon, 26 different claudin genes were detected using expressed sequence tag libraries [111]. The junction-associated membrane proteins (JAM) are another family of proteins that are proposed

to regulate transepithelial transport of for example lymphocytes in mammals [112].

The extrinsic barrier – the mucus and excretory products

The mucus layer is the first barrier that pathogens encounter in the gut. Non-pathogenic bacteria live in close contact to the mucus and occupy most of the luminal area during normal conditions and this decreases the opportunity for pathogenic bacteria to settle [101]. The mucus is secreted from goblet cells although components within the mucus may be produced elsewhere. The major part of the mucus however, originates from the goblet cells and are high molecular weight glycoproteins called mucins [113]. The glycoproteins contain oligosaccharides that are known to be involved in pathogen adhesion. Mucins isolated from Atlantic salmon intestines have been shown to bind both A. salmonicida and IPNV (Padra personal communication). Furthermore, preliminary studies suggest that the majority of the intestinal mucins in the salmon intestine constitute a loose layer and thus that secretion of mucus may lead to physical removal of pathogens and toxins ([101], Padra personal communication). Interactions between different types of gram negative bacteria and the epithelia have also been shown to differentially alter the composition of glycoproteins in common carp [113]. Further, the mucus contains reactive oxygen species, hydrogen peroxidase, complement factors and broad spectrum antimicrobial peptides (AMPs). Complement factors and AMPs may opsonize bacteria and/or be involved in innate immune activation [114]. AMPs such as hepcidin and cathelicidins are induced upon bacterial infection in the intestine of Atlantic salmon [115-117]. In contrast to bacteria, viruses are small and may readily pass through the mucus layer [101]. However, anti-viral defenses include the ability of the mucins to bind viruses, complement factors, hydrolytic enzymes and IFNs that are secreted into the mucus [114]. Secretory immunoglobulins (antibodies) are also present in the secretions and attach to pathogens upon recognition, marking them for destruction [13]. Until recently, IgM was considered to be the secretory antibody of fish. However, IgM seems to be quickly broken down in the gut mucus [118] and recently a new secretory antibody IgT/IgZ, is suggested to be more important than IgM in the intestine [12, 100].

The impact of the immune system on barrier function

There are clear links between the mucosal immune system and the epithelial barrier. The stress induced increase in the intestinal permeability will most likely lead to an increased antigen influx that triggers mucosal immune responses [119]. Further, it has been shown that mucus production decreases during stress leading to an increased exposure to the luminal content [120, 121]. In mammals, mediators of the immune system are in turn known to affect the physical barriers. In mammals, IFNγ and TNFα increase paracellular permeability [122, 123]. The mechanism behind this is not fully elucidated but IFNγ inhibits occludin and ZO-1 expression as well as cellular Na^+,K^+-ATPase activity [124]. This may lead to increased intracellular Na^+ levels and a swelling of the cells which can result in an increased permeability [124]. Further, occludin and ZO-1 expression was decreased. The synergistic effects of IFNγ and TNFα are further suggested to depend on increased MLCK activity, leading to contraction of the actin filaments attached to ZO-1 in the TJs which thereby increases permeability [125, 126]. The cytokine IL-1β also increases TJ permeability in the human colon derived CACO-2 cells [56]. The mechanism suggested for this effect is an activation of NF-κB which decreases the expression of occludin and

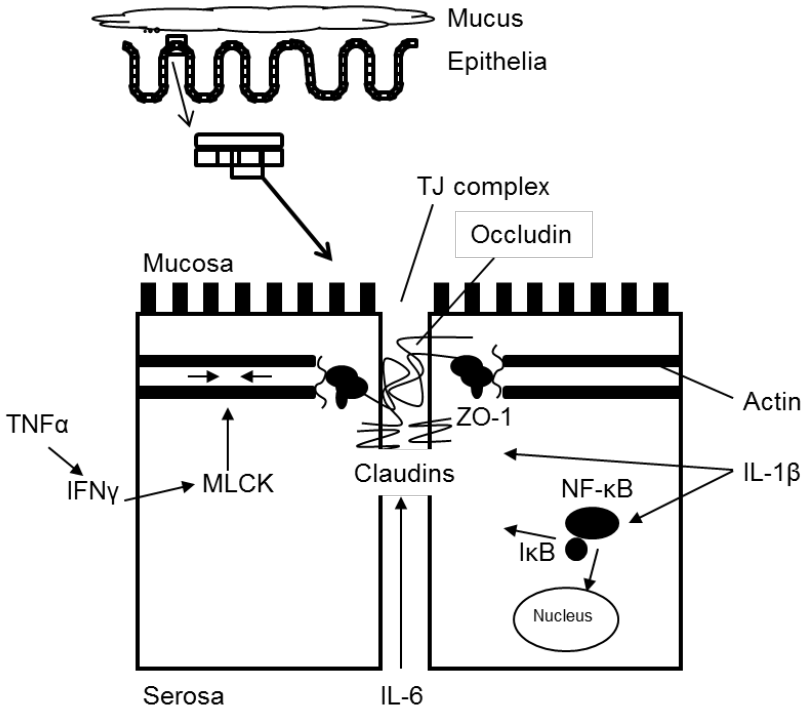

Fig 5. Selected possible ways of regulation of the tight junctions (TJ). In mammals cytokines are known to regulate intestinal permeability by effects on TJ proteins ZO-1, occludins and claudins.

increases expression of claudin-1, a barrier forming claudin known to be leaky [56, 127]. Increased permeability is also an effect of IL-6 treatment, which causes decreased ZO-1 expression in mice [67]. Further, IL-6 increased claudin-2 expression in CACO-2 cells [128]. These regulations are both known to increase the permeability (Figure 5).

The effect of the immune response on the intestinal barrier and the increased permeability by stress may alter the crosstalk between the epithelia and the immune cells. However, stress and increased cortisol levels may also directly affect the mucosal immune system. One of the direct effects of cortisol in mammals is mast cell degranulation [129] and stress related neuropeptides are shown to increase the number of mucosal mast cells [130]. These mast cells have been shown to have a marked effect on colonic epithelial permeability [131]. These cells release a wide range of ILs, macrophage stimulating factors as well as inflammatory molecules such as prostaglandins and leukotrienes [132]. In salmonids, mast cell like cells has been observed that might empty their vacuoles upon stress stimuli. Atlantic salmon possess mast cell like cells in stratum granulosum that infiltrate lamina prop and the epithelia in e.g. IPNV exposure. Hence, stress may directly affect the intestinal immune system also in fish.

To conclude, a stress response or an infection may lead to a diminished barrier that induces an immune response and the immune system in turn induces increased intestinal permeability. A “negative spiral” is created that is attenuated by both the immune and endocrine systems. These events result in chronic inflammation and are propagated as the two systems act against each other as they try to restore tissue homeostasis. The cross-talk is therefore an important part for understanding chronic inflammation in fish.

Aquaculture of salmonids

Salmonid lifecycle and the immune system

Many salmonid species are of commercial importance and many of them, e.g. the Atlantic salmon are anadromous and have a lifecycle that includes migration between fresh water and the marine environment [2]. The change from fresh water to a sea water environment demands a switch in water and ion transportation and this process is regulated by hormones.

During the salmonid life cycle, this developmental change is called the parr-smolt transformation. (figure 6).

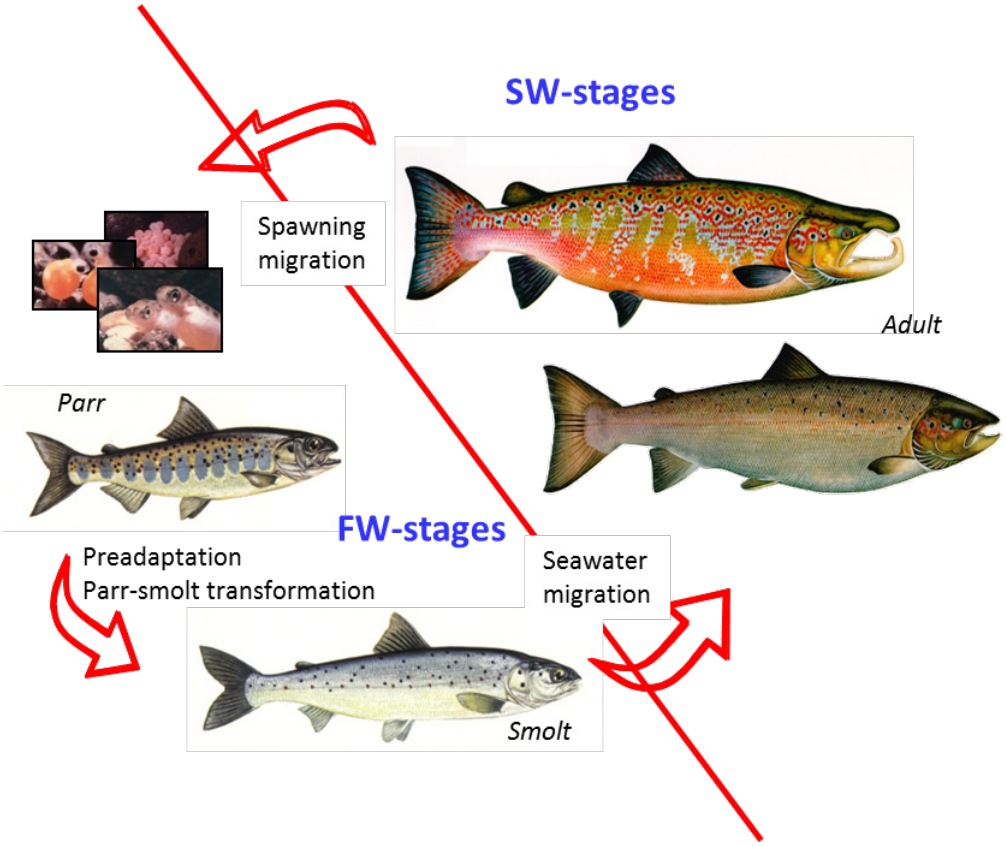

Figure 6.
The salmonid life cycle

An increased day length and temperature sets in motion a hormonal cascade including transient increases in plasma levels of thyroid hormones, growth hormone, IGF-I and cortisol. During this period the physiology of the animal is altered to prepare it for the change in environmental salinity. At the level of the gut the parr-smolt transformation involves changes in intestinal ion transporting activities and permeability [133, 134]. At the endocrine level, this thesis will focus on cortisol as this hormone, in addition to its role during development, also is one of the major stress hormones in fish. From the immunological point of view, an interesting time interval is the first period after sea water transfer. This is a sensitive period of the fish life cycle as new environmental challenges are present. Further, the sea water environment brings about exposure to other groups of viruses and bacteria which potentially can cause disease outbreaks. It is

therefore important to understand how the developmental changes in circulatory cortisol as well as the actual sea water transfer affect the immune as well as the endocrine system of fish.

Stress

The stress response

There is a constant interaction between the endocrine and the immune system in fish, as in other animals. Important endocrine events are known to influence immune function and vice versa [135]. For example the stress response is accompanied by effects on the inflammatory response and in mammals cortisol is widely used in medicine to counteract inflammation [136]. The immune response can further affect the stress response. Recombinant IL-1β and LPS have been shown to increase cortisol levels in rainbow trout plasma after 8h of exposure with a decrease back towards basal levels by 24h [109]. Irrespective of type of organism, the endocrine influence on the immune system is of interest and particularly possible effects of stress and the stress hormones as this may alter the organisms ability to respond to an infection or inflammation.

A stressful event induces a primary stress response with a transient increase in catecholamines followed by a transient increase in cortisol. In fish, a corticosteroid mediated stress response starts with an environmental or internal cue that affects the hypothalamic-pituitary-interrenal (HPI) axis [10]. This leads to release of corticotrophin releasing hormone (CRH) from the hypothalamus [10]. CRH acts on cells in the pituitary to release ACTH that stimulates cortisol release from inter renal cells [8]. Cortisol induces secondary stress responses such as immunological changes, increased blood pressure and decreased epithelial barriers [8]. The subsequent tertiary effects are for example diseases, reduced growth and impaired reproduction. Stress in aquacultured fish often leads to a threat to the homeostasis and previous studies have shown that one important part of this disturbance is through impairment of the intestinal epithelial integrity during both short term and long term stress [119, 137]. In fact, elevated permeability occurs in the Atlantic salmon intestine during stress and even when levels of the stress hormone, cortisol, have returned to basal levels [119].

Cortisol effects on immune function

Cortisol has an important role in modulating the immune response by suppressing the inflammatory processes [10]. It has been shown in mammals that the glucocorticoid receptor (GR) is involved in the effect of cortisol on the immune system [138, 139]. Receptor activation is known to inhibit IL-1β as well as the kinase that cleaves IκB [138]. Further, it has been proposed that the receptor translocates to the nucleus where it inhibits acetylation, a process essential for transcription, and suppress NF-κB activity which can lead to dampening of inflammatory processes [139].

During parr-smolt transformation there is a rise in cortisol levels and as the HPI axis is activated. [140]. There is a lower number of splenic antibody producing cells during the parr-smolt-transformation and fish vaccinated during this period have lower levels of antibody titer after 6 months compared to fish vaccinated prior to this event [141]. However, increases in immune mediators TNFα, COX-2, IFN type I, Mx, IFNγ and γIP have been observed suggesting that other endocrine factors such as GH might have a stimulatory effect on the innate immune responses during the parr-smolt transformation [142, 143].

Disease

In intensive aquaculture fish are dependent on adequate water exchange to ensure sufficient oxygen levels and metabolite clearance. The environmental conditions as well as handling may result in stressful situations for the animal. There is also an increased risk for transfer of pathogens in water with fish maintained under high density conditions. The skin, gills and the intestine are all possible routes of invasion and infection by pathogens.

The effects of vegetable ingredients in fish feed has been one initiating factor for the recent increased attention given to the intestinal mucosal immune system in salmonids. Vegetable proteins have been shown to cause increased epithelial permeability and enteritis in fish [144]. During soy bean meal enteritis the distal intestine is affected and shows signs of severe inflammation [97, 144]. Evidence for an increased inflammatory response and T cell presence during disease has been reported [97, 145]. Further the

injuries in the distal intestine in fish reared at 12°C as assessed using histology technique was more severe than fish reared at 8°C suggesting a temperature effect on the inflammation [146].

Viral infections with focus on IPNV

The yearly outbreaks of IPNV in Norwegian aquaculture have been stable around 150-200 cases a year since 2002 [147]. The economic cost of the virus for the aquaculture industry has led to an increased scientific interest in characteristics and infection mechanisms of the virus [148, 149].

IPNV belongs to the *Aquabirnavirus* of the *Birnaviridae* family. It consists of a 60 nm, non-enveloped, icosahedral virion containing double stranded RNA (bi-rna; [150]). The two strands contain three open reading frames of which VP2 and 3 is situated in the A strand and are cleaved to produce two capsid proteins, whereas VP1 is situated in the B strand and codes for the viral polymerase.

To reduce viral outbreaks vaccines against IPNV has been developed [151]. The effect of IPNV vaccination has increased in the past ten years due to increased knowledge about infection mechanism and virus properties. In a recent study all vaccinated fish survived viral challenge while in non-vaccinated cohabitants there was a mortality of approximately 30% which is normal during these types of challenges [152]. Vaccination of Atlantic salmon was shown to increase metabolism and cell signaling with a subsequent inflammatory response manifested by an increase in IL-1β [46].

The anti-viral immune response towards IPNV is similar to other viral infections and involves IFN type I activation and subsequent activation of the anti-viral protein Mx [77]. The Mx protein is a dynamin-like GTPase that is important in the early anti-viral defence [153]. The Mx1 protein is involved in transcriptional control of viral genes and resides inside the nucleus, whereas MxA and Mx2 reside in the cytoplasm [154]. The activated human MxA can interfere with normal viral nucleocapsid formation and seize the viral proteins into complexes, thereby restraining viral propagation [153, 155]. In recent years, knowledge on Mx functions has increased suggesting more roles for the protein in the cell machinery [156]. In fish, the role of Mx in different cellular processes is less clear. An antibody recognizing Mx1, 2 and 3 have been used in a study on Atlantic

salmon gill [157]. The protein was localized in the cytoplasm and in the apical areas of the epithelial cells. Atlantic salmon anti-viral protein Mx is significantly up-regulated by Poly I:C, an IFN type I inducing viral dsRNA mimic [158]. In Atlantic salmon, Mx as well as IFN levels have been monitored during the parr-smolt transformation and increased constitutive expression of IFNs as well as Mx is evident during this period [142].

Concomitant with an increase in the IFN driven anti-viral response, APCs present products from virus degradation through the antigen presenting major histocompatibility complex I (MHC-I; [159]). The complex is recognized by the T cell receptor (TCR) bound to the co-factor CD8 on T_c cells and activates the cellular response of the acquired immune system. Recently, cellular defense mechanisms have been proposed as an equally important part of the defense against the virus [34]. Differentiation of naïve T_h cells into T_h1 cells are characterized by the transcription factor T-bet that also stimulates IFNγ and chemokine expression [160]. The differentiation of naïve T_h cells into T_h2 cells is driven by the transcription factor GATA-binding protein 3 (GATA3), which also has a diversity of other non-immune functions [161]. In this study increased antibody levels correlated with an increase in GATA-3 and a decrease in T-bet while viral infection was correlated with an increase in T-bet. Up-regulation of T_h2 cell markers, the antibody producing T cell pathway, correlated with an increased protection against IPNV while activation of T_c cells and T_h1 cells correlated with increased viral infection.

Bacterial infection with focus on gram A. Salmonicida

Interaction with bacteria is a part of everyday life for cells in any epithelium facing the surrounding environment. Transfer of gram-negative bacteria, such as *A. Salmonicida*, occurs through transcytosis [101]. Lipopolysaccharides are molecules attached to the characteristic outer membrane of gram negative bacteria [162]. These are versatile structures that acts as endotoxins (toxins bound to the bacteria) eliciting strong immune responses upon recognition. During this thesis LPS has been proven to induce strong response of IL-1β mRNA up regulation in head kidney of Atlantic salmon and rainbow trout (unpublished results). The interaction with the epithelia starts when surface structures of the bacteria

such as LPS binds to TLRs in the epithelia. A cross-talk is initiated as well as vesicle release from the bacteria containing toxins, adhesion molecules and LPS [163]. This induces changes in the bacteria as well as the host cell that will result in repression of the bacteria or invasion [164]. In mouse macrophages, LPS is known to down regulate NF-κB which could be an evasive maneuver and allow bacteria to by-pass the immune system of the host [165]. In rainbow trout leucocytes LPS and TNFα have been shown to increase IκB transcription in fish in a similar manner [27]. The immune response to bacterial infection is initiated by activation of pro-inflammatory factors such as IL-1β, TNFα, IL-2, IL-6 and IFNγ. Chemokines, such as IL-8, are also activated in this initial step as well as anti-inflammatory cytokines such as IL-10 and transforming growth factor β (TGFβ) that modulate the sum response to avoid an overreaction. Similar responses are seen towards non-pathogenic bacteria and can result in a full scale immune response in a leaky epithelia during pro-longed stress and subsequent chronic inflammation [166].

The intestine as infection route

The intestine is a passage way for nutrients and osmolytes and possesses channels and transport routes for a variety of molecules [110]. At the same time the fish intestine is constantly exposed to the environment through digestion of antigen. The intestinal tract is an important port of entry for infectious pathogens as well as other antigens [133, 167]. Translocation of IPNV has been demonstrated after mucosal exposure both *in vivo* and *in vitro* [167]. Furthermore, exposure of rainbow trout to the furunculosis causing bacteria, *A. salmonicida*, resulted in translocation of the bacteria across the intestine [133]. Thus, both viral and bacterial pathogens use the gastro intestinal tract as a route of infection in salmonid fish. The translocation of pathogens across the intestinal barrier is facilitated by virulent factors like enzymes and toxins, secreted by the pathogens. IPNV exposure has been shown to increase the intestinal permeability of Atlantic salmon and severely damage the epithelial cells [167]. Similarly, enterocyte damage and detachment was found in the Atlantic salmon intestine after exposure to *A. salmonicida* [168] and the rainbow trout intestine had reduced permeability in response to the extracellular products and endotoxins of a virulent strain of *A. Salmonicida*. This suggests a host-

pathogen interaction resulting in pathogen facilitated transcellular or paracellular transport [169].

Despite the recent advances in the knowledge of viral-immune-endocrine interaction and indications of viral modulation of the intestinal immune functions, direct effect of IPNV and cortisol on the intestinal epithelial immune response has not been thoroughly examined.

AIM OF THE THESIS

The mucosal intestinal immune system of mammals has been intensively studied in the light of stress related chronic intestinal diseases. The impact of this knowledge on medicine has led to resolving or at least dampening the effects of stress related and infectious intestinal inflammation. These processes are less well understood in fish although there is an increasing body of literature suggesting similar stress related effects on the fish intestinal physical barrier. These findings have provided insight into stressful situations, how the fish can cope with stress and possible ways to circumvent stress. However, knowledge about the impact of stress, infection and a disturbed physical barrier on the mucosal epithelial immune and inflammatory responses in fish is scarce.

The main objective of this thesis was therefore to **investigate the immune response in the intestinal mucosa of salmonids**.

The intestinal epithelium is truly multifunctional with roles in absorption but also in prevention of pathogen entry, in which the mucosal immune system is vital. In this context a specific aim of the present study was to **investigate the basal level expression of key cytokines in the intestinal mucosa.**

Stress affects the systemic immune system through effects of stress hormones on the expression of immune mediators. However, knowledge about the effect of stress hormones on the mucosal immune response is limited. External stressors impact on the physical barrier of the intestinal epithelium and make it leakier, and this probably affects the underlying immune barrier. A specific aim of the present thesis was therefore to **characterize the pattern of cytokine expression in the intestinal mucosa in response to stress** both **directly by the stress hormone cortisol** and **as a consequence of increased leakiness of the physical barrier.**

The intestinal epithelium is one of the main infection routes for both viral and bacterial infection and an efficient immune barrier within this primary barrier can substantially decrease the susceptibility of fish to infection. A specific aim of the present study was therefore to **investigate how the intestinal immune system responds to infection** and also **how this**

response is affected by external stressors in combination with an infection.

Changes in the physical barrier will no doubt affect the underlying immune barrier but there will also be an impact of cytokines secreted from the mucosa on the physical barrier. A final aim was thus to **investigate the possible crosstalk between the immune response and the epithelia**.

MAIN FINDINGS AND DISCUSSION

Methodological considerations

Gene expression studies

Gene expression analysis can give insight into how the cells in a tissue respond to a certain stimuli or environment and they, when translated into proteins will contribute to restore homeostasis. Genes are up-regulated or down-regulated in response to different stimuli leading to an increased or decreased possibility of protein expression respectively. However, in the process there are multiple checkpoints that will influence the actual physiological response to the stimulus and it is important to bear this in mind when interpreting gene expression data.

An important aspect of the present thesis was the validation of the qPCR technique used to quantify transcript abundance. A number of factors will affect the outcome of qPCR experiments and these starts with the sampling of tissue for RNA extraction. The sampling time is of outmost importance to secure the quality of the sensitive RNA (Figure 7). There are two primary ways of storing samples for RNA extraction, by flash freezing or by stabilizing the RNA using RNAlater buffer. By comparing RNA quality from the same samples using both methods Flash freezing has been suggested as the best way to preserve tissue RNA (Andersson and Nilsen personal communication). Yet other tests of the two methods have shown variations in quality depending on stabilizing method and the tissue investigated [170] and for practical reasons RNAlater is often better suited for field work. Both techniques have been used in the present thesis and were shown to result in equally high quality RNA. RNA quality in head kidney samples were shown to be better in flash frozen samples compared to

samples stabilized using RNAlater. On the other hand, liver samples retained better quality when stored in RNAlater. RNA extraction is performed either by using the spin column kits or by phase separation using Trizol. The column method usually results in highly pure RNA while the purity may be lower but the yield of RNA is frequently higher using Trizol. Quality and quantity controls are therefore important and in the present thesis flash freezing and subsequent Trizol based RNA extraction was chosen when possible.

To quantify the amount of mRNA expressed in tissues under different conditions, the amount of the gene of interest, *i.e.* the target gene needs to be related to a reference mRNA. This refers to a transcript that has a constant expression in the same tissue independent of the treatment. The choice of reference gene is important and it should be selected prior to every experiment. In the course of the thesis several studies were aimed at selecting appropriate reference genes that have a stable abundance

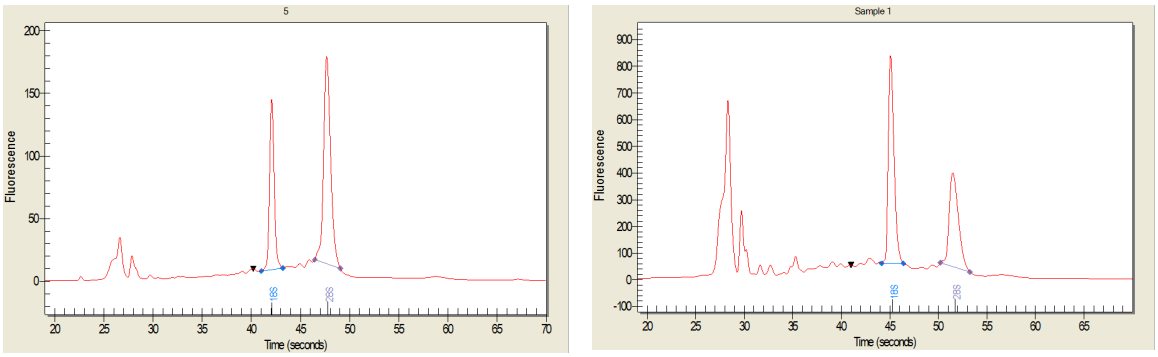

Figure 7.
Peaks corresponding to 18S and 28S rRNA. RNA quality check as measured by the Experion platform (Bio-Rad, Sunbyberg, Sweden).
Left: Normal sampling time, RQI quality index 9,9 (scale 1-10). Right: Degradation after 2-5 minutes. RQI quality index 7

between experiments and tissues. The most commonly used reference genes are genes that are important for the cell cycle, which are expected to be stable irrespective of experimental treatment. Examination of the most common genes used as references in Atlantic salmon studies suggested two forms of elongation factor α (ELFα) to be most stable between tissues [171]. ELF1α is a GTP dependent, abundant and highly conserved translational factor that promotes tRNA binding to ribosomes [172]. It is also known to

be constant in all phases of the cell cycle. Olsvik et al [171] further concluded that β-actin, a cytoskeletal protein, and ribosomal RNA (e.g. 18S) showed intermediate variation in Atlantic salmon samples whereas GAPDH, a glycolytic enzyme, was the most variable [171]. In the same study the reference genes were also examined in salmon undergoing smoltification and yielded similar conclusions. Lovoll et al [173] investigated the same genes, with the exception of GAPDH, during a 6 weeks long viral challenge and the outcome indicated that ELFα was the most stable reference gene followed by 18S RNA, β-actin and RPS20. For intestinal samples Olsvik et al [171] concluded that β-actin was the most stable gene with ELFα next in line and 18S was most variable. However, during viral challenge Lovoll et al showed that 18S was the most stable in the intestine and that ELF1α can sometimes be regulated. Nonetheless, other studies have shown 18 S to be the most stable and also the most suitable reference gene in tissues with high cell turnover such as the intestine [174-177]. Both Olsvik and Lovoll showed that ELF1α was the most stable in the head kidney with 18S and β-actin showing increased variation. Ingerslev et al [178] performed a similar test on cells and tissue in control and LPS treated fish and ELF1α was the most stable and 18 S the least stable gene. Regulation of ELF1α after LPS treatment was low. In the present thesis ELF1α was chosen based on validation experiments that showed it to be overall the most stable reference gene in these studies (**I-IV**). However, in situations where the reference gene was shown to be regulated 18S was used as a complementary reference gene, based on the literature presented (**IV**).

Protein expression studies

Monitoring the protein expression is important to verify if changes in gene transcription are linked to changes in protein expression. Different tissues will respond to specific stimuli in different ways and the treatment, time point of sampling or environmental factors may affect the protein expressed by regulating post-translational and post secretory modifications. Hence, the response seen in gene expression may not necessarily lead to changes in protein expression. Therefore it is important to investigate both gene expression and protein expression during stimulation of a certain response. In the present thesis protein expression was investigated using immunohistochemistry (IHC). One advantage of IHC

is that it is possible to determine the cellular localization of the expressed proteins and this can provide clues about the mechanisms of action in cells of importance to the immune response. In the present thesis, antibodies developed for immunohistochemistry in different animals have been used. In all cases when species specific antibodies were available these have been used, often in collaboration and through kind gifts by the designer and manufacturer of the specific antibody. The Atlantic salmon specific antibody against MHC-I, produced and validated by Karsten Skjødt, at University of Southern Denmark, is published for the first time in paper **IV**. For some proteins, like the tight junction proteins, mammalian antibodies have been used as these were shown to cross react with the salmon proteins [107].

In order to investigate the innate immune response in the Atlantic salmon intestine a monoclonal, Atlantic salmon specific antibody directed against neutrophils (E3D9) was used [179]. These cells are attracted to an affected area by chemokines such as IL-8 as previously stated. The neutrophil antibody is used in paper **I** and **II**.

To examine the protein expression levels of anti-viral proteins an antibody directed against the Atlantic salmon Mx protein was used (**IV**). This species specific anti-body recognizes the three forms of Mx, Mx1, 2 and 3, found in Atlantic salmon. The antibody has been used in previous studies and in the present study had a similar tissue and cellular localization [157].

Ussing chamber methodology

To study the impact of the immune system on the paracellular permeability of the intestinal epithelia, an experimental model of a functional epithelium is of utmost importance. An advanced Ussing chamber system has been designed and developed in our lab in order to study barrier functions in an intact and metabolically active intestinal epithelium. These chambers are specially developed to ensure accurate measurements of electrical parameters in a low-potential and low-resistance tissue as the fish intestine (**V**) [134, 185].

Using the Ussing chamber technique, there are two major ways of measuring the paracellular permeability over the intestine: The transepithelial resistance and the transfer rate of the inert, hydrophilic marker molecule, in these studies ^{14}C mannitol was used. In brief, the

intestinal epithelia is mounted between two half chambers that are filled with temperature and pH adjusted Ringer solution. Using platina electrodes situated as far away from the tissue as possible, alternating and random DC voltages are applied to the epithelium. This will create corresponding random currents alternating between positive and negative values with a specific min and max range, resulting in zero net current load on the epithelium. The transepithelial potential (TEP) created by each of these currents across the tissue can be detected using KCl electrodes and the measurement system UCC-401 (UCC-labs Ltd. [110]). The system compensates for the background resistance and using linear regression of the current-voltage curves created as well as Ohms law (U=R*I), the short circuit current (SCC) and the transepithelial resistance (TER) can be calculated. The transfer rate of the inert, hydrophilic mannitol is a way to measure paracellular permeability since this is the passage way for this molecule. The radioactive label ensures the ability to measure minute amounts transferred across the epithelium. This molecule can be added to the mucosal side of the chamber and by taking serial samples from the serosal side of the chamber the accumulation rate of mannitol can be further assessed [110]. The diffusion rate of mannitol is determined by the concentration gradient of the molecule and the permeability of the paracellular pathway. The apparent permeability (P_{app}) is calculated using the formula:

$$Papp = dQ/dt * 1/AC_0$$

where dQ is the steady-state appearance rate of the compound on the serosal side, C_0 is the initial concentration of the compound on the mucosal side and A is the exposed tissue area between the two half chambers [134].

There is a reversed relationship between the transpeithelial resistance and paracellular permeability *i.e.* a high TER corresponds to a low paracellular permeability. The apparent permeability of uncharged molecules, like mannitol, on the other hand directly reflects the paracellular permeability. Depending on the different cellular and molecular mechanisms behind the two ways to measure paracellular permeability the two measures are complementary. The additional information given by the two parameters can aid in the understanding of the TJ regulatory mechanism. Some evidence of an increased TER by heavily mucus secreting epithelia have also been gathered through experience with the Ussing chamber method,

indicating that a flow of small charged molecules across the paracellular pathway may be dampened by increased mucus secretion from the goblet cells in the epithelia.

The use of recombinant proteins and DNA constructs

To study the effect of the immune system in the intestinal physical barrier recombinant cytokines as well as vectors bearing DNA constructs of key cytokines were used. These results are partly presented in paper **V**.

The effects of recombinant cytokines on live and metabolically active whole intestinal epithelia of salmonids have not been conducted previously and represent a new and interesting way of examining immune-epithelial crosstalk (**V**). The recombinant proteins were used for in vitro exposure of the intestine in the Ussing chambers. To simulate an increase in cytokines levels in the epithelia recombinant IL-1β (rIL-1β), rIL-6, rIFNγ, rTNFα and rIFN type I was added to the serosal side of the preparation.

The use of recombinant cytokines in cell culture is well established in fish cell culture experiments [82, 186]. Further, the use of specific peptides derived from cytokines have contributed to understanding of viral host-pathogen interactions [187], functional differences in different domains of cytokines [188] as well as differential response in cells of different origin. These studies demonstrate the potential of using this approach to stimulate cells or epithelia *in vitro*.

To bring about a constitutive expression of cytokines at a whole animal level is a state of the art technique. The use of constructs, *i.e.* plasmids bearing the coding sequence for a specific peptide, is a promising way to induce specific parts of the immune response and evaluate their effects on e.g. cytokine expression. In the present thesis, a construct for IL-1β was used in a pilot study. The main aim of this experiment was to assess the effect of constitutive expression of this cytokine on intestinal epithelial permeability as well as to look down-stream at the effect on other cytokines and elements of the immune response. The construct was applied by intra muscular (i.m.) injection, previously shown to be the best way to achieve an effect whereas intraperitoneal (i.p.) injection has proven less effective [151].

Results and discussion

Basal immune function in the gut

Innate immune cells

The intestinal epithelium is continuously exposed to a diversity of antigens from ingested food and water as well as from the non-pathogenic, commensal microflora. Although the extrinsic barrier with mucus, AMPs and antibodies protect the epithelia from direct contact with the endogenous microflora as well as foreign compounds and organisms, interaction is inevitable and the barrier sometimes breach. This will lead to exposure of the second physical barrier, the epithelia. The load of microorganisms in the intestine is high. Fish have around 10^5-10^8 bacteria ml^{-1}, in mammals 10^{12} [101]. Thus, the intestine is a tissue that continuously samples antigen and interact with the microflora [189]. This results in a symbiotic relationship between the intestine and its endogenous microbes and will in some senses result in a constant state of low level inflammation. This increased basal immune tonus is further affected by the level of permeability in the intestine. The fish intestine is defined as a leaky barrier which results in a relatively high exposure of antigen to the immune cells [110]. Thus, even without the presence of pathogens the mucosal immune system needs to be on constant alert to intruding threats and at the same time avoid overreaction [190, 191]. Presence of neutrophils within the intestinal wall in un-stressed fish (**I**, **II**) in the control fish supports this idea (Figure 8). The immunohistochemical analysis revealed regions of the intestine containing neutrophils scavenging the underlying layers under the epithelia. Further, neutrophils stretching out between the epithelial cells were also present. There was a decreased bacterial translocation of *A. Salmonicida* in response to high stocking density and low water (**II**). The infiltration of neutrophils together with the up-regulation of cytokines suggests a sufficient protection towards the pathogen. This supports the idea of constant active inflammatory machinery. One could speculate that due to the seemingly lack of Peyer's patches in fish, the mucosal immune system is different in the sense that immune cells need to be interspersed throughout the intestine to be able to cover the whole area. This may also

influence the speed at which the antigen presentation and acquired immune response occur in fish [9].

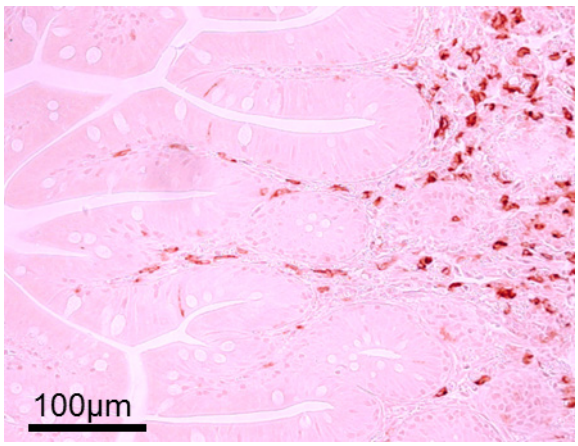

Figure 8.
Neutrophils (dark red) in the lamina propria as detected in control fish

Inflammatory cytokines

The basal mucosal immune response in the intestine was examined as the gene expression of immune markers and presence of immune cells. This thesis clearly shows that an array of cytokines is expressed in the intestine even without immune stimulation a result that further adds to the idea of a constant activation. Constitutively, a differential expression in different intestinal regions was detected **(I, II)**. IL-1β, IFNγ and TNFα all showed a higher basal level of expression in the distal intestine compared to the proximal intestine. These are all inflammatory markers belonging to the innate immune response. In a recent work, the distal intestine of Atlantic salmon had high expression levels of the IL-17 receptor and the γδT cell receptor, TCRγ, than the proximal intestine [97]. The T_h17 cells promote inflammation and the δγT cells are also suggested to be involved in the T cell driven innate responses [38, 40]. In contrast to the proximal intestine were a more leaky epithelia assures a constant exposure to antigens and were the immune reactivity seems more pronounced the distal intestine may have a lower exposure to antigens and also a slower reactivity due to the high basal levels of inflammatory cytokines. The literature and the results presented here suggests that the distal intestine is predisposed to respond to antigens by T cell mediated, pro-inflammatory pathways. It could be that the distal intestine is not readily exposed to antigens partly

due to the large mucus production, thereby having a sufficient protection by an innate-like response. IL-17 further suppress T_h1 activity known to promote chronic inflammation which could be a way to avoid overreaction in the distal intestine [39].

Effect of temperature

External abiotic factors, like temperature are also affecting the basal expression level of key cytokines (**I**). The expression of IL-1β and IFNγ were lower in 16°C compared to 8°C in both proximal and distal intestine. This could suggest that the mucosal immune function is more dependent on innate immune functions at the lower temperature analogous to the theory that an efficient acquired immune system needs homeothermy [192]. The explanation for the lower innate function at 16°C could then be that there is a greater acquired immune response activity at this temperature and thus a less need for the innate response. This has been shown previously in a study where macrophage activity was high at 8°C and low at 12°C while antibody production was low at 8°C but high at 12°C [193]. During soy bean meal enteritis fish reared at 12°C had more severe damages than fish reared at 8°C as examined by histology scoring [146]. Taken together, this could indicate that at a sufficiently high temperature the fish starts to rely more on the acquired immune response compared to the innate immune response whereas the innate immune response is more important at lower temperatures. This could suggest that the innate and acquired immune activities change their basal level expression in response to changes in temperature cues from the surrounding. It could further suggest that the influence of temperature on the immune system has led to a predisposition towards the innate immune response in cold and temperate adapted fish species [193].

Anti-viral markers

Regarding constitutive expression of the anti-viral defense systems, Mx was shown to be present in the epithelial cells of non-challenged fish (**IV**). The localization of the protein close to the nucleus suggests a role in suppression of viral replication during infection. However, in the present thesis as well as in a previous study on Atlantic salmon there is additional

presence of the protein close to the apical membrane of the epithelial cells (**IV**, [157]). This implies that Mx also can act as a cell membrane localized barrier to infection or that it has a role in exocytosis as has been proposed

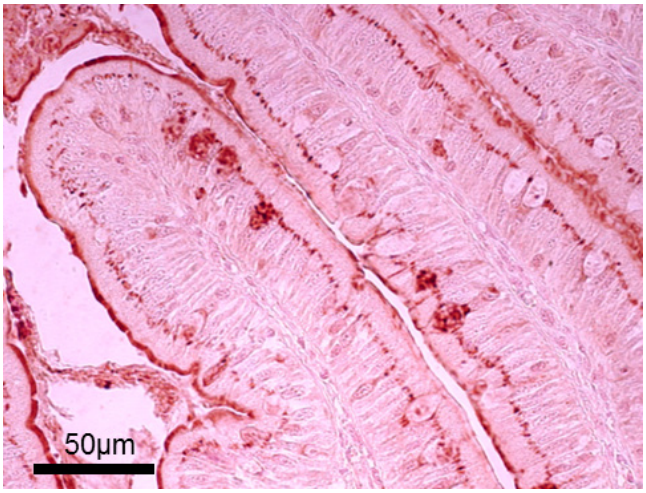

Figure 9.
Mx protein (dark red) localized supra nuclear in the cytoplasm of enterocytes as well as close to the apical membrane. Mx is also localized in the goblet cells

in mammals [154, 156]. Another interesting possibility for this apical localization of Mx immune staining is the function of Mx as a secreted product, which could exert anti-viral functions in a preventive fashion already in the intestinal lumen. The presence of Mx immune reactivity also in the goblet cells of the salmon intestine supports this new and intriguing role for Mx (**IV**) (Figure 9). Secretion of Mx has been suggested previously in mammals and is part of the newly discovered multifunctionality of the protein [156].

Acquired immune cells

Presence of $CD8^+$ cells was also found during control condition as suggested by positive CD8α immune staining in paper **IV**. The $CD8^+$ cells were found in the lamina propria close to the basal side of the epithelial cells (Figure 10). Expression of MHC-I was simultaneously apparent on the basolateral side of the epithelial cells (**IV;** Figure 10). This suggests a crosstalk between the two cell types in the epithelia where the IECs presents antigen to the resident $CD8^+$ cells. Expression of CD3, present on all regular T cells, have previously been found in close contact to the basal parts of the enterocytes

during soy bean meal induced enteritis [145]. Taken together, this suggests a functional T cell response in the intestine of Atlantic salmon. High mRNA

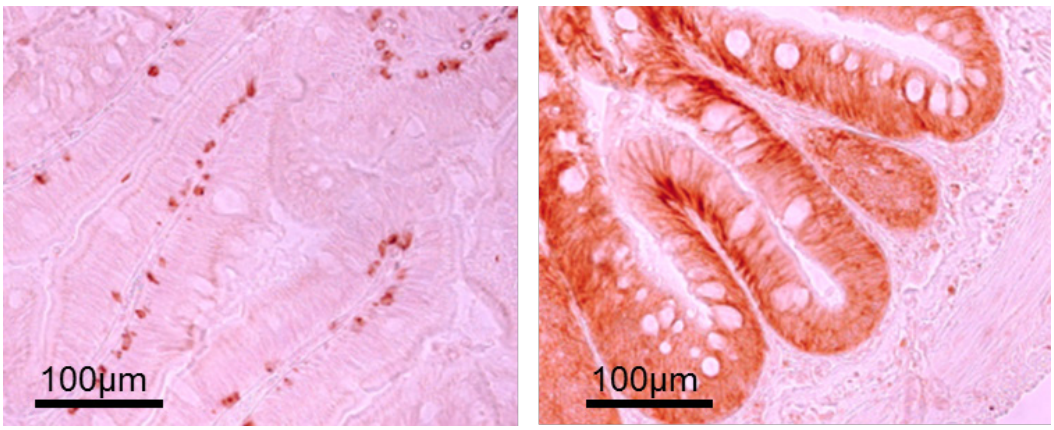

Figure 10.
Left: CD8 positive cells in the lamina propria close to the basal membrane of the entrocytes.
Right: MHC-I localized in the basal and lateral membrane of the epithelial cells

expression of both CD4 and CD8 has also been found during soy bean meal induced enteritis suggesting a correlation between CD3 protein and CD8 and CD4 mRNA expression [97, 145].

Summary

Overall, the presented expression of immune markers and cells clearly suggests a functional mucosal immune barrier in the intestine of salmonids. This thesis further suggests that the dynamics of the intestinal mucosal immune responses of salmonids are important for the fish to withstand challenges experienced from the environment. Reports on a functional intestinal mucosal immune system in fish have been presented previously but conclusive evidence of the basal level of immune marker expression was lacking. The present thesis shows that the intestinal mucosal immune response differs from the systemic and that the mediators necessary for an innate immune response in this first line of defense is present. The constant expression of cytokines and immune markers and abundance of neutrophils and $CD8^+$ cells further suggest a constant state of immune activation in the intestine. This contrasts the situation in other important lymphoid tissues and should be considered when analyzing results from immune stimulation experiments.

The impact of environmental stress on mucosal immunity

Effects of stress

The intestinal mucosal immune system is clearly affected by environmental stress as shown in paper **I**, **II** and **III**. The different stressful environments investigated in the present thesis are chosen to represent possible scenarios that can occur in aquaculture due to certain husbandry conditions [194]. Long-term stress created by hypoxic conditions, high fish densities and poor water quality were manifested as initial increases in plasma cortisol levels and maintained decreases in intestinal barrier function (**I**, **II**, **III**, [119]). Cortisol levels are often monitored and used as a marker for stress. In the acute phase cortisol increases and during repeated stress high levels of cortisol can be found up to a month [119, 137]. At the end of the experimental period, when intestinal samplings for immune analyses were performed, the plasma cortisol levels had declined to resting levels also in the groups with the most severe stress treatment (**I**, **II**, **III**, [119]). In terms of secondary stress responses the transepithelial resistance (TER) decreased in fish held in high stocking densities leading to bad water quality (**II**). Low TER was apparent when the cortisol levels already had dropped to basal levels (**II**). Therefore the secondary effects of stress are more robust and constitute a more reliable marker for pro-longed stress than for example cortisol (**II**, [119]). A subsequent immune challenge of fish that had been exposed to prolonged hypoxia treatment resulted in a second increase in plasma cortisol levels. This was an acute transient stress response evoked by the immune stimulation as phosphate buffered saline (PBS) injected fish remained at resting levels (**III**).

Effects on innate immune function

One long-term effect on the immune response of adverse environmental conditions that may appear in aquaculture (high water temperatures, high fish densities and poor water quality) is apparent in the down regulation of transcription factor activation (**I**, **II**). There is also a concomitantly lowered expression of early innate immune markers as well as an increased neutrophil infiltration during chronic stress. The down-regulation of IL-1β and IFNγ and up-regulation of IL-10 in the proximal intestine in response to longer periods of stressful environment suggests a protective mechanism to

minimize the damages otherwise caused by the immune system (**I**, **II,** [75, 195]). In mammals, the normal response to stress is the reduction of inflammatory and T_h1/T_c inductive cytokines TNFα and IFNγ concomitant with a promotion of T_h2 and anti-inflammatory IL-10 and TGFβ to avoid an overreaction [196]. Intestinal inflammatory diseases have been shown to be induced by either by the T_h1 or the T_h2 inflammatory pathway [197]. The causative agents in the T_h2 response are suggested to be cytokines such as IL4 and IL-6 that both are involved in creating an increased intestinal permeability [67, 198]. Hence, if fish possess a T_h1/T_h2 antagonism similar to mammals the modulatory actions in this antagonism to avoid an overreaction might in long-term stress lead to pathological stages.

Similar to mammals IL-10 may drive an immune response dominated by alterations of cytokine expression in macrophages and other APCs to avoid an over-reaction [199]. In the long-term however, IL-10 activation may lead to TGFβ activation [89]. This activation pathway in turn leads to differentiation of $CD4^+$ cells towards T_h17 and/or T_{reg} as well as proliferation of non-specific $CD8^+$ cell differentiation [91]. The detrimental downstream effect of this scenario, such as an impaired possibility for antigen sampling and normal macrophage function as well as an increased neutrophil infiltration, could account for the increased risk for stress related intestinal inflammations and overall bad health. This may lead to an inflammation driven by the T_h2 cell subset and T_{reg} and result in an IBD like situation like the one seen in mammals. As stated, the evidence for a T_h1/T_h2 balance in fish remains to be determined. However, the basis for such a system is at place [34].

Effects of cortisol

At the point of sampling in paper **I** and **II**, the visible signs of a disturbed homeostasis is present and paracellular permeability of the intestine and thus the barrier integrity is clearly damaged (**I**, **II**, [119]). This is also shown by a decreased villi height in the proximal intestine in paper **I**. The effect of stress on the physical barrier is often more apparent in the proximal intestine compared to the distal intestine [119, 137, 200]. Further, the modulation of the immune response found in the proximal intestine is accompanied by an overall suppression of immune markers in the distal

intestine (**I**, **II**). The origin of the stress effects seen in the proximal intestine is not known, but they could be a direct effect of the stress hormone cortisol. In the secondary response cortisol increases the intestinal permeability which may lead to an overall negative effect in long-term stress [110]. Direct effects of cortisol are also suggested by studies on the mammalian glucocorticoid receptor (GR) activation [103, 138]. High cortisol levels during an acute stress response may lead to suppression of NF-κB, IL-1β and IFNγ by GR activation [103, 138]. Further, in the epithelia it has been shown that IECs only partially degrade IκB, necessary for NF-κB activation, which may buffer the response to luminal antigen [201]. This could further add to the detrimental effects seen in a leaky barrier with increased antigen exposure, stimulation of the epithelial immune system, further increase in permeability and a subsequent inflammation. Moreover, IL-10 was found to be synergistically up-regulated by cortisol and immune stimulation in rainbow trout macrophage RTS-11 cell line [75]. This would suggest a favoring of T_h2 cell proliferation promoted by this cytokine. Also in the rat, activation of the glucocorticoid receptor promote T_h2 cell proliferation over T_h1 resulting in a decreased antibody production [136]. Thus, at least one reason for the propagation of intestinal inflammation indicated during seen during long term stress could be a direct effect of cortisol on the T_h1/ T_h2 system. Furthermore, even though the cortisol levels with time decline, the stressor and the disturbed intestinal integrity remain. Hence, there is probably a window of increased risk for negative effects of interaction between the mucosal immune system and the commensal bacteria as the overall mucosal immune function is suppressed. This might be an early effect that initiates the possibility of the later intestinal chronic inflammation seen in **I** and **II**. In fish, seemingly lacking the highly organized facilitated immune cell interaction through lymphoid follicles this might be of increased importance during stress [7].

Effects on innate immune cells

Additional support for the presence of an inflammation in the proximal intestine after long term stress is found in the immunohistochemistry results demonstrating a significant increase in neutrophil infiltration in the proximal intestine as a response to low water oxygen levels (Figure 11). The neutrophils are present in high amounts to clear the inflamed area

from debris after apoptotic events. The homing of neutrophils supports that the leaky barrier in the proximal intestine have resulted in a long term inflammatory response (**I**, [119]). The seeming contradiction of increased neutrophil infiltration in combination with a lowered inflammatory immune function can be explained by the cells participating in the ongoing inflammation secreting chemokines and complement factors that attracts the neutrophils. As stated earlier, e.g. IL-17 is known to increase neutrophil presence in mammals and this cytokine has been shown to be up-regulated during soy-bean meal enteritis in Atlantic salmon [97, 202]. Further, in mammals IL-10 have been shown to decrease TNFα induced neutrophil infiltration into mucosal areas in the acute phase, but to increase inflammation and neutrophil presence during long-term inflammation [203]. The higher levels of IL-10 seen in paper **I** is thus well in line with an increased neutrophil infiltration.

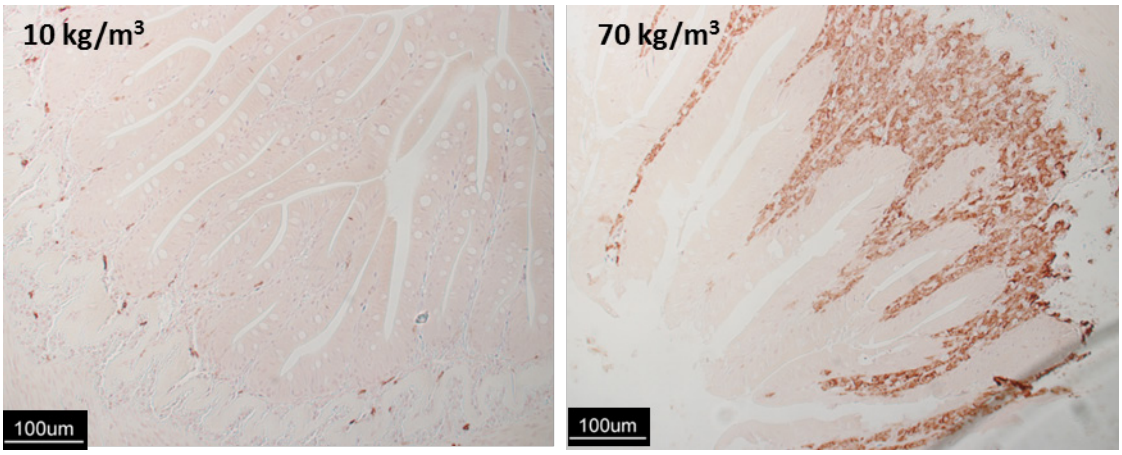

Figure 11.
Neutrophil infiltration (dark spots) in the proximal intestine in fish exposed to different stocking densities leading to bad water quality in the high stocking density group resulting in severe inflammation.

Summary

The immune system is vital for perseverance during infection. Inflammatory and anti-viral mechanisms contribute to an instant innate response that later is inhibited by anti-inflammatory markers [18]. These markers facilitate the switch to an acquired response. In chronic diseases, this mechanism can become deleterious, stressing the need to clear the pathogen or stressor in time. The T cells activated and differentiated during

the chronic situation leads to different types of chronic inflammation. The results in paper **I** and **II** suggests a T_h2-like response to long term environmental stressors in terms of bad water quality.

Mucosal immune responses to infection

A viral outbreak in juvenile and post-smolt salmonids leads to high mortalities and life-long latent virus bearing macrophages in survivors [204]. The resistance towards the virus seems to be a polygenic trait and may therefore vary within a population [205]. In general, whether or not a viral infection leads to disease depends on the cross talk between cells where a susceptive individual fails to avoid the accumulation and replication of the virus while a non-susceptible individual is able to turn of the viral replication and/or clear the body of viral components.

Cytokine responses to Poly I:C and IPNV

The intestinal immune response to a mimicked viral infection, accomplished through i.p. injection of poly I:C, constitutes of an up-regulation of IFNγ and Mx mRNA expression in fish kept at normal environmental conditions (**III**). The response to Poly I:C is rapid in both intestinal segments suggesting a responsiveness to viral infection in the whole length of the intestine. The dynamin-like protein Mx interferes with the viral replication and perform envelopment of viral products, as described previously, and has therefore been used as a marker for infection in numerous studies on vertebrates [153]. 6 days of cohabitant challenge, through fish i.p. injected with IPNV, demonstrated an active anti-viral response, manifested by an increase in IFN type I mRNA expression (**IV**). However, the Mx expression was not significantly up-regulated even though the variation was high suggesting an initiated Mx response in IPNV infected fish. This was supported by the fact that in some fish positive for virus after 6 days of cohabitant challenge showed a greatly elevated expression of Mx (**IV**). Further, the effects of viral change were predominantly seen in the proximal intestine whereas the distal intestine showed no significant up regulation. This indicates that the exposure to the antigen at this early stage is not evenly distributed throughout the intestine. This also indicate a difference in response to authentic virus as compared to Poly I:C

stimulation (**III**, **IV**). IPNV cohabitant challenge resulted in a moderate yet clearly present response to IPNV whereas the response is robust and present at all sampling points in response to Poly I:C (**III**, **IV**). During IPNV infection the IFN pathway is normally up regulated when the infection fails to circumvent the immune system. However, if the virus get the advantage IFN type I activity is inhibited at the level of Mx [187]. Hence, after six days of IPNV cohabitant challenge an IPNV favoring situation is found in the infected group (**IV**). However, an increased proliferation of the virus might also increase other immune factors, e.g. IL-1β that may induce an overall immune reaction as suggested by an increased in IL-1β expression in the head kidney (**IV**). The result seen for IL-1β is supported by assessment of the same gene in paper **III**. Here, an increased IL-1β expression was found in the head kidney 2 days post injection of Poly I:C in fish exposed to low water oxygen levels. IL-1β has recently been proposed to have anti-viral effects in salmonids [188].

For IL-10, no significant difference was found in head kidney after 6 days of cohabitant challenge. This might be due to an early sampling time as IL-10 is known to be up regulated in the head kidney cells after IPNV infection and is important in the latent phase of IPNV pathology [206]. The variation in mRNA expression was high and as for Mx, high variations might masque a potential effect in IL-10 in the IPNV infected group (**IV**). The head kidney macrophages are responding to IPNV by producing IFN type I and IL-1β while the IFN type I pathway may be inhibited at the level of Mx in other cell populations [204, 206]. In fish carrying the latent form of the disease the virus seems to replicate in small numbers and transfers from macrophage to macrophage [207, 208]. There is a decreased chemokine response in IPNV infected cells that may facilitate avoidance of a full scale immune activation [209]. The results presented in the present thesis thus shows that IL-1β is up-regulated in the head kidney suggesting an active innate immune response after 6 days of IPNV cohabitant challenge (**IV**). This is in line with the increased anti-viral response to Poly I:C as manifested by up-regulation of IFNγ at day 1 and of Mx day 1 and 2 (**III**). Further, the IFN type I pathway is up-regulated after 6 days of cohabitant IPNV challenge (**IV**) However this pathway is at least partly inhibited at the level of Mx. This effect could be a result of the time of sampling or an effect of the virus inhibiting Mx.

Effect on the acquired response

In the cohabitant challenge with IPNV (**IV**), an initiated T cell response was evident after 6 days post start of the challenge, as seen by the increased CD3γδ mRNA expression in the proximal intestinal mucosa of the cohabitee fish (**IV**). Certain immune markers such as CD3 subunits and the MHCs are known to be conserved through evolution [210, 211]. A recent report showed positive selection during evolution for CD3γδ, the surface protein of the TCR-CD3 complex. This suggests an important role for this component together with other immune genes including the major histocompatibility complexes (MHCs) that controls antigen presentation in adaptive immunity. In mammals, IL-10 has an important role during persistence of infected individuals [199]. The effect on T cell function is a decreased antibody production, T cell differentiation of CD4 positive cells towards T_{reg} and inhibition of $CD8^+$ cell proliferation [199]. $CD8^+$ cells in the mucosa are further known to be involved in oral tolerance as they suppress antibody production towards certain antigen [37]. These processes are also known to include $CD4^+$ Tregs that are proposed to control cellular immunity [212]. A similar mechanism has been suggested for sea lice exposed skin in the Atlantic salmon [213]. Results showed a time dependent decrease in mRNA expression of CD8 in the damaged area of the skin while CD4 mRNA expression was high. Recently, the transcription factor (Foxp3) known to be involved in Treg development was sequenced in Atlantic salmon and shown to be induced in central as well as peripheral tissues such as the gut [214]. In the proximal intestine, Atlantic salmon exposed to cohabitant challenge with IPNV showed a decreased CD8α expression at the protein level (**IV**). As for the head kidney, IL-10 showed high variations in the proximal intestine. Nevertheless, this shows that T cell populations are modified during viral infection also in fish intestine. Hence, rearrangement of the T cell population in the intestine occurs during IPNV infection as suggested by increased CD3 mRNA expression and decreased expression of CD8α expression.

Intestinal region differences

During infection, there are clear differences between the mucosal immune responses in the proximal intestine compared with the distal intestine. The immune response in the proximal intestine seems to be more pronounced and clear differences between treated and control groups can be found (**I**, **II**, **III**, **IV**). In the distal intestine there is a higher basal mRNA expression of almost all immune markers examined. As for changes in immune marker expression in the presence of a stressor or pathogen, there seems to be less effect in this region (**I**, **II**, **III**, **IV**). The distal intestine generally secretes more mucus than the proximal part and might therefore be more exempt from contact with antigens [101, 110]. There are however evidence for antigen sampling in this area and it is therefore as important to further investigate the immune response also in this region [5, 7]. In mammals, there is a clear pattern of differentiation in immune marker expression between the different intestinal segments [215]. In salmonids, Mulder et al [70] found IL-1β to be up regulated in the proximal intestine of Rainbow trout after 6-8 days of *Aeromonas salmonicida* (*A. Salmonicida*) exposure whereas no increase could be found in the distal part. IL-1β was also up-regulated in IECs as a response to *A. salmonicida* [19]. In Atlantic salmon activation of the IL-1β receptor by its binding protein induced activation of NF-κB [57]. Chemokine IL-8, attracting neutrophils were clearly up-regulated in the proximal intestine of Rainbow trout after *A. salmonicida* exposure whereas no effect was found in the distal part [70]. Further, TNFα was up-regulated in the proximal intestine day 8 post infection with *A. Salmonicida* after an initial decrease and up-regulation was also found in IECs [19, 70]. The same pattern, an increase over time, was found for IFNγ after exposure to *A. salmonicida* in the proximal intestine of Rainbow trout whereas there was a differential expression in the distal intestine [70]. In the same study TGFβ expression decreased in the distal intestine in response to infection.

The large regional differences in immune response between the proximal and distal intestinal as shown in the present thesis, stresses the importance of treating these two regions separately when examining the immune responses (I-V). Hence, even if the proximal intestine of Atlantic salmon is affected during IPNV infection there might be other processes keeping the balance in the distal intestine intact.

There is a higher expression of $CD8^+$ cells in the distal intestine compared to the proximal intestine of IPNV infected fish (IV). The expression is further not affected by treatment to the same extent in the distal intestine as it is in the proximal intestine. This suggests a preserved ability to initiate a T_c driven immune response in the distal intestine and that this response is kept at a controlled level. The clear reduction in CD8α seen in the proximal intestine indicates that $CD8^+$ cells are replaced or abolished during infection in this region (**IV**). This effect in the proximal intestine might suggest a skewed T_h balance towards a T_h2 cell driven response that leads to antibody production and T_h1 and T_c cell suppression [38]. During soy bean meal enteritis the inflammation is manifested in the beginning of the distal intestine [97, 144]. This indicates that differences in the distal intestine compared to the proximal intestine are causing the pathology. As discussed in an earlier chapter the distal intestine seems to rely to a large extent on innate immune functions and inflammatory T cell subsets. Also in mammals, differences in T cell subsets are found during infection and inflammation [215]. PolyI:C and viral dsRNA have been found to bind to toll like receptors on IECs and induce IL-15 secretion from these cells in the small intestine of mice. The IL-15 in turn and activate intraepithelial leucocytes (IEL; [215]). These IELs were shown to be $CD8\alpha\alpha^+$ cells, an unconventional T cell subset as opposed to previously known $CD8\alpha\beta^+$ cells. $CD8\alpha\alpha^+$ cells been associated with the innate immune response, a high cytotoxicity and have been proposed to cause virus mediated gastroenteritis [215]. These cells further seem to have an undetermined role in T memory cell activity and are regulated also by TGFβ that regulate mucosal T memory cells [216-218]. Zhou et al (2007) showed that despite severe injuries in the small intestine during virus infection and increased $CD8\alpha\alpha^+$ expression only mild effects were seen in the colon [215]. Recently, CD8αα cells was suggested to be present also among Rainbow trout IELs and correlated with a high expression of NK enhancement factors [219].

Combined effects of external stressors and infection

Additive effects of stressors

A healthy and unstressed fish with intact intestinal primary barriers will be able to withstand an infection even if exposed to different pathogens in the

surrounding water. That is, a fish in a state of good health and welfare will have the ability to cope with infectious and non-infectious stressors [8]. However, exposure of the fish to different environmental stressors will impose an allostatic load on the animal and make it less able to withstand additional infectious stressors. This will have consequences on several physiological functions even if plasma cortisol levels drop ([119], **II**). In paper **III** this is illustrated by a second increase in plasma cortisol levels when the fish, that was long-term exposed to hypoxia, were subjected to poly I:C or bacterial vaccine stimulation through injection. This acute, transient primary stress response was not observed in the fish that received PBS injections, indicating that the stress caused by immune challenge was much more severe than handling and injection stress (**III**).

Effects on the innate immune response

In paper **III** the effect of a secondary stress response caused by chronic hypoxic conditions was examined. In the proximal intestine the immune response to Poly I:C elicited by those fish that were exposed to long term hypoxic conditions suggested an inhibition of the anti-viral response (Mx and IFNγ). Mx was down regulated at day 1 and 3 after injection in the proximal intestine and at day 1 in the distal intestine (**III**). Also, in the distal intestine the up regulation of Mx was lower in the hypoxic group compared to control at day 2. This was supported by the lower level of response in head kidney macrophages in terms of IFNα and Mx mRNA expression (**III**). Hence, the anti-viral response is clearly suppressed by chronic hypoxic conditions in the intestine. Further, the trend suggests a decline in IFNγ mRNA expression in the intestine from day 1 to day 3 in the control group while there was a trend towards a peak in IFNγ at day 2 compared to day 1 and 3. This suggests a shift in immune status of the animals that results in a lowered ability to respond normally to an immune stimulation. As discussed above, this may be due to direct effects of cortisol on the glucocorticoid receptor [138]. When the receptor is activated it inhibits IκB cleavage by its kinase (IKK) and thereby suppresses the activation of NF-κB and further its downstream effects [138]. It can also inhibit the transcription process for e.g. IL-1β through inhibition of histone acetylation necessary to uncover the DNA for transcription [139]. Hence, long term stress leads to a delayed response to Poly I:C in the intestine. During long

term stress when a fish have had or have increased plasma cortisol levels the immune response to an actual pathogen, such as IPNV (**IV**), may be both decreased and delayed. Thus, the suppressing effects of cortisol may result in a continued viral propagation which can lead to higher mortality rates compared to an unstressed fish (**IV**). In the fish cohabitantly exposed to IPNV, the IFN type I expression is clearly suppressed in the presence of cortisol. However, Mx is instead up regulated under these conditions (**IV**). This may suggest an alternative pathway for induction of the anti-viral proteins. Further, the effect of IL-1β is potentiated in the cortisol treated group. As stated, the P1 domain of IL-1β has been shown to have anti-viral properties by inducing Mx expression [188]. Hence, the increase in Mx seen in the cortisol group can be a result of an alternative up-regulation of this gene. This is further supported by an increased expression of Mx protein in the cortisol treated group (**IV**). Also the response to bacteria is altered during stress. In head kidney macrophages the increase in IL-1β caused by LPS challenge was lower in chronically stressed fish compared to the control [195]. The cell mortality is also increased in stressed fish after *A. salmonicida* exposure [195]. In the presence of cortisol the up regulation of neutrophil chemotactic IL-8 by immune stimulation was suppressed in rainbow trout macrophage RTS-11 cell line [75]. This suggests an inhibition of neutrophil recruitment during stress. IL-10 was found to be synergistically up-regulated by cortisol under the same conditions [75].

Effects on the acquired immune response

Thus, the decreased expression of IFN type I during IPNV challenge in the presence of cortisol suggests a suppression of the innate immune function (IV). Suppression of the immune response is also seen in the latent phase of IPNV viral propagation [206]. In this suppression, IL-10 is important and may act through inhibition of macrophage signaling to result in a decreased activity in T_h1 cell like processes. IL-10 further inhibits $CD8^+$ cell proliferation and impairs antigen presentation in mammals [199]. This is in line with the results in the proximal intestine during IPNV infection and high circulating levels of cortisol in the present thesis (**IV**). The protein expression of $CD8^+$ cells is significantly decreased in the cortisol treated group. In surviving fish, macrophages that have already taken up the virus may function as a viral memory cell that propagates the virus at low levels

[207, 208]. Further, an impaired antigen presentation and suppressed chemokine expression could result in a low transfer rate of virus and suppressed immune response to the antigen [199, 209]. IPNV can be encountered at different life stages and may become activated during stressful periods. This would even further increase the damaging effects on the intestinal mucosa and further account for the decrease in $CD8^+$ cells in the inflamed areas, similar to what is seen in the damaged area of the skin during sea lice infection [213].

Crosstalk between the intestinal immune response and the epithelium

There is a constant and intricate communication between the physical epithelial barrier and the underlying immune barrier. In propagation of a chronic intestinal inflammation the two systems affect each other, but the cause relationship of this interaction is yet to be elucidated. Both infectious and non-infectious stressors impair the physical intestinal epithelial barrier and make this barrier more leaky for different antigens (**I**, **II**, **III**, [119]).

Effects of recombinant cytokines on the intestinal epithelium

The results presented in paper **V** shows that the intestinal mucosal immune system of salmonids contributes to the changes in intestinal permeability that are seen during stressful conditions as well as during an infection (**I-IV**). The immune system of the intestine together with the epithelia allows antigen sampling and presentation during normal conditions. This function may lead to negative consequences in a case of multiple stressors by increasing the permeability of the physical barrier and thereby making it too leaky to immune stimulating antigens. Increased understanding of the effect of the local immune response on the physical barrier is therefore essential. As the TJs are composed of several possible combinations of the multiple claudin isoforms found in both fish and mammals, the differential expression of claudins is a main key to the understanding of TJ regulation.

The most abundantly expressed claudins in the intestine of Atlantic salmon are claudin15 and 25b [181]. These two claudin isoforms can be examined in fish using the mammalian anti bodies for claudin 10 and 4 respectively as these human claudins show sequence similarities and cross reactivity with the fish claudin 15 and 25b [181, 182].

The cytokine IL-1β, have proven to be affected by all experimental protocols investigated in the present thesis (**I-IV**) and studies on mammals show that this cytokine is affecting the TJs and the intestinal epithelial permeability [56]. Therefore this cytokine was prioritized in order to examine possible effects of pro-inflammatory cytokines on the intestinal integrity and expression of intestinal TJ claudins and ZO-1. The results of exposing rainbow trout intestinal epithelium to recombinant IL-1β show a an increased permeability across the physical barrier concomitant with a down regulation of two claudin isoforms (mammalian Cdl 4 and 10) as well as a redistribution of ZO-1 (**V**; Figure 12). Of the two parameters measuring paracellular permeability IL-1β exposure resulted in an increased P_{app}, whereas the TER was unaffected by the cytokine (**V**). This suggests that the permeability for uncharged molecules were increased while the paracellular permeability to charged molecules was less affected. The permeability of the TJs to different types of molecules is to a large extent dictated by the characteristics of the pore forming claudin isoforms [183]. Increased expression of Na selective claudins like the mammalien Cld 10 (similar to fish Cld 15) will favour permeability of negatively charged ions and small molcules. Whereas the general barrier forming mammalian claudin 4 (similar to fish Cld 25b) restrict the permeability for the passing moleules dependent on both size and charge. After IL-1β exposure of the intestinal arterial circulation for 90 minutes there was a clear down regulation of Cld 4, suggesting a decrease in the tightening claudin isoform which thus resulted in an increased permeability to the uncharged molecule mannitol (**V**). Further, the actin connected protein ZO-1 was re-distributed to show a more even distribution in the epithelia, with localization close to the apical membrane and away from the lateral sides of the enterocyte. This is in accordance with effects of Il-1β on human corneal epithelial cells were the ZO-1 protein were found to be redistributed from the neighboring cells after treatment with the cytokine [184]. Thus, the expression pattern of the TJ proteins investigated in paper **V** suggest that IL-1β affects epithelial permeability though regulation of claudin isoform expression in the TJs together with regulation of the ZO-1 protein distribution. This results in increased transepithelial permability mainly to uncharged, hydrophilic molecules. Discrepancies between the TER and the Papp may also relate to the fact that TER is measured in milliseconds while Papp are measured over a longer time and therefore takes the TJ dynamics into account [110].

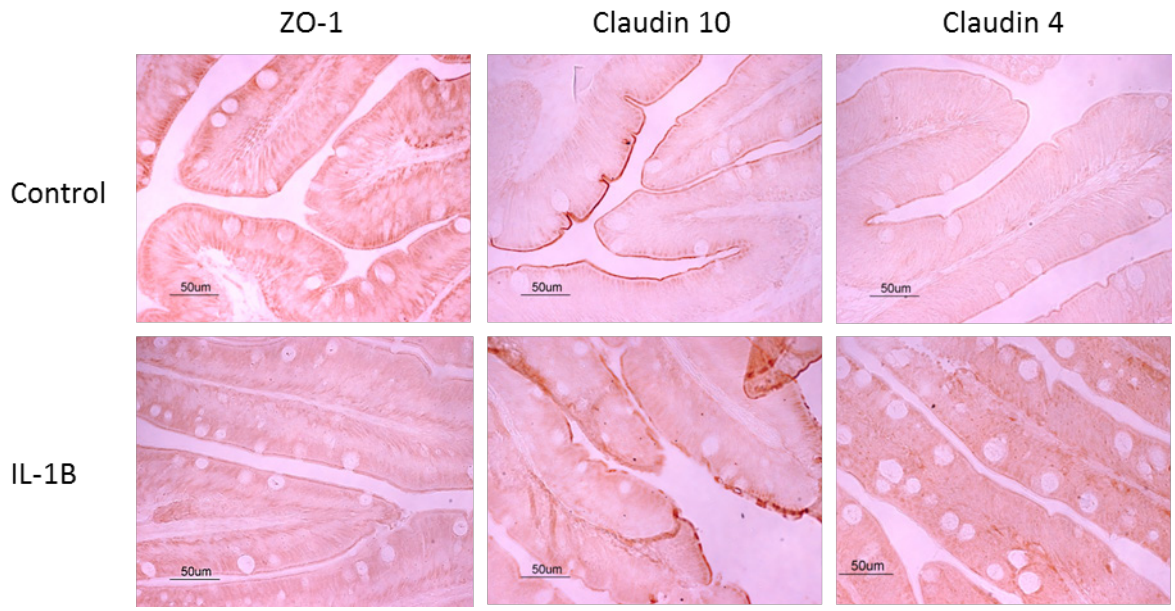

Figure 11.
Presence of (in darker color) ZO-1, Clauding-10 and claudin 4 in control fish and fish exposed to IL-1β. Marked effect on claudin 10 that is more localized in the tight junction between the epithelial cells in the control group compared to the IL-1β group.

IL-6 has in recent years been suggested as important in the intestinal control of inflammation and epithelial integrity [67]. In line with this, the effect of IL-6 on the physical barrier was almost identical to that seen for IL-1β in the rainbow trout intestine (**V**). A decreased Papp was seen when administrating rIFNγ at a low dose but there was no effect on permeability by rTNFα. However, in mammals these two cytokines are known to increase permeability when administrated together. Therefore, the studies of combined effects of cytokines are important for future studies in order to evaluate the effects of the immune system on the physical intestinal epithelia.

Implications for the use of DNA constructs to study mucosal immune function

To follow up the effects of IL-1β on intestinal epithelial integrity, in more long term perspectives a plasmid construct containing the IL-1β sequence was injected into rainbow trout (**V**). This construct is shown to self-induce expression of IL-1β in different cell-lines examined (Secombes and Martin

personal communication) and have also been shown to result in increased IL-1β expression when injected intra muscularly into fish. Gene expression analysis of IL-1β in head kidney samples, 7 days post-injection, failed to verify a successful transfection. The sampling time was selected due to the probability of a quick innate response. Studies using an IL-8 construct, however, have shown a delayed effect in the head kidney compared to the for example spleen [71, 72]. In these studies the IL-8 construct was used to evaluate its effect on cytokine and CC chemokine induction and the modulation of the response induced by the chemokine during a viral infection with VHSV. For IL-1β and TNFα the effect on the head kidney and spleen was shown to differ with an increased expression day 3 and 7 after transfection in the spleen whereas the expression in the head kidney was increased day 10. Further, the effect on chemokine expression was apparent at day 3 in both tissues. IL-8 was also found to modulate the response induced by a VHSV derived construct. Hence, despite the absent effect of IL-1β in head kidney samples after 7 days the experiment performed needs further analyses to properly evaluate the possible effect of the injections. Also, other immune markers, down-stream to IL-1β, should to be assessed as IL-1β is a diverse and strong inducer of several inflammatory markers.

CONCLUDING REMARKS

Conclusions

The present thesis clearly shows the existence of a basal expression of immune markers in the intestinal mucosa of salmonids. This induction of immune markers contributes to the intestinal responses to environmental changes as well as pathogens (Figure 12; **I-V**). The changes in immune status during long term stress are seen concomitant with endocrine changes in terms of increased plasma levels of cortisol as well as physiological changes in terms of increased intestinal permeability (**I**-**IV**). The infiltration of neutrophils dramatically increases in response to environmental stress, indicating an increased risk of developing intestinal inflammations due to stress (**II**). However, neutrophils are found in the epithelia also without immune stimulation suggesting that chemotactic

processes are present in the tissue also during non-challenged conditions (**I**). Anti-viral markers as well as inflammatory markers are up regulated during infection (**III**, **IV**). There is also evidence for the presence of T cells as well as antigen presentation in the intestine which suggests a functional antigen presentation to $CD8^+$ cells (**IV**).

A differential expression of inflammatory, anti-viral and immune cell markers in the proximal intestine compared to the distal intestine is seen

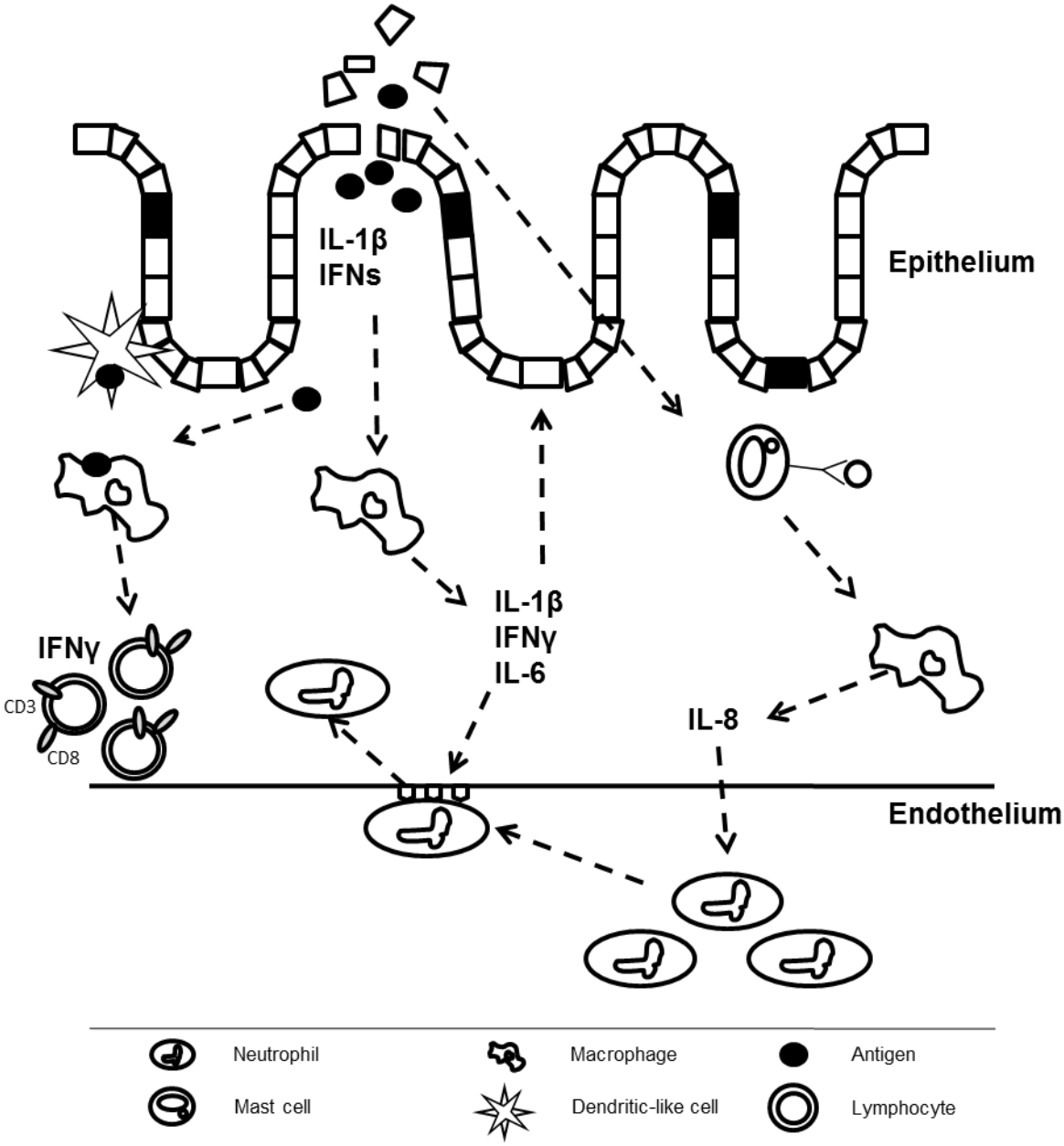

Figure 12.
Schematic picture of the intestinal mucosal immune response based on the results from the present thesis. An influx of antigen causes an immune response. IFNs, IL-1β as well as CD3 mRNA expression is up regulated during infection. Presence of neutrophils and CD8+ cells can be found in the epithelia. IL-1β, IL-6 and possibly IFNγ affects the intestinal permeability.

during normal conditions as well as during stress and infection (**I-IV**). The function of such a difference is in agreement with what is seen in the small intestine and colon in mammals.

These differential expression patterns reflect the differences in anatomy and physiology between the segments, but it also suggests separate functions for the mucosal immune system in the two regions. This is evident in paper **IV** where there is a clear response to IPNV in the proximal intestine while the distal intestine is unaffected by the viral challenge. The proximal intestine is probably more exposed to particles and microorganisms due to this region being the first part in contact with digested antigens. However, the lower response seen in the distal intestine could also be due to a more pronounced mucus secretion protecting the epithelia in that region. The functional differences of the intestinal tract may have resulted in different needs for immune activity and can therefore explain differential responses to antigen. It can be speculated, based on the presented results, that the immune system facilitates antigen sampling over the epithelia through rearrangement of epithelial tight junction proteins in a similar way as have been shown for mammals (**V**). From the present work it can be concluded that the intestine is a tissue with a clear and separate response to immune stimulation compared to the systemic immune tissues such as the head kidney and the spleen. Immune function is constantly altered to match the present situation.

Future perspectives

Cell studies

To fully understand the interplay between the mucosal immune system, the intestinal epithelia and the surrounding, there is a need to develop cell based tools to break the mechanisms down to the core. Primary cell cultures have been used and recently one cell lines from the intestine of rainbow trout have been developed [222]. This type of based tools would be valuable in future stimulation experiments as well as in cultured epithelia-based Ussing experiments. Further, the recent increasing antibody repertoire for IHC and flow cytometry purposes are valuable tools in order to enhance our understanding of the local immune system in the gut as well as of the immune system as a whole.

Macrophages are believed to be the foundation of a functioning immune system and by some proposed as the first immune competent cell from which other subsets have evolved [223]. It is intriguing to speculate that an ancient macrophage cell line had what was needed for the organism to cope with its environment and that evolving specialization and proliferation of this cell type led to new immune cell populations emerging. In mammals, macrophage cell lines are established in the tissues prior to birth and increasing evidence suggest that they thereafter are self-maintaining [224]. This property would be expected of a first immune competent cell population and a similar pattern is seen in fish suggesting a common ancient origin of these cells [225]. Assessment of intestinal derived macrophages would be a way to investigate the intestinal immune function in comparison to more studied subsets e.g. from head kidney. It is not surprising that classification of fish immune cells have proven difficult since classification is based on functions that in fish immune cells are more diverse. The presences of mast cells have been a matter of debate in fish. In fish, the cells stain eosinophil when water based fixative is used and has therefore been called eosinophil granular cells (EGC) by some. However, since the granules in some species stain metachromatic with alcohol based fixative, which is the definition of mast cells in mammals, the same cells has also been called mast cells. However, the presence of a cell type with similar function as mammalian mast cells has been found [226]. These cells are also an interesting subject for future studies on salmonid intestine. The debate weather fish possess dendritic cells have not yet been given a clear answer. However, the function that such a cell type would have is certainly in place also in fish. Enterocytes and macrophages have both been suggested as functional equivalents and this is therefore also an interesting future focus for fish immune system research. In mammals, CD83 is readily expressed on dendritic cells that connect innate and specific immune function. CD83 in fish is dependent on IFN regulating factors and NF-κB [227]. There is a correlated increase in expression in response to rhabdovirus in rainbow trout. This indicates the presence of functional dendritic cells in fish. Using immunohistochemistry, dendritic cell like cells have been spotted in e.g. the intestine. For all these examples, the function is there but the cell types might not be as clearly defined as in mammals.

The commensals

The bacteria present in the intestinal mucus layer has an important function in processing luminal content but also to trim the immune system as well as keeping the habitat occupied from more infectious counterparts. As discussed previously, bacterial infection can occur through the intestinal route [133]. There is a lack of multifunctional studies of the intestinal mucosal immune system including the community of non-pathogenic commensal bacteria. This is also an intriguing field that has been studied in mammals but so far only separate from the epithelial immune system in fish. In this respect also the mucus layer of the salmonid intestinal mucosa needs further focus. An interest in research on antimicrobial peptides and secretory immune globulins has increased recently due to the discovery of the IgT/IgZ [13].

Cytokines

The use of gene expression analysis in the present thesis was proven to be a viable way to assess the immune response in the intestine. By combining mRNA detection with protein detection we saw a correlation in terms of T cell activity as well as anti-viral markers. As the available tools in salmonids are restricted the best way forward would be to combine the two techniques using corresponding mRNA-protein assays. In terms of mRNA expression studies on the intestine, future experiments would benefit from measuring cytokines involved in a suggested T_h system as well as a general T cell directed approach. Assays based on sequences for IL-4/13, IL-2 and IL-12 (Fig 2) should be included in this assessment. Further, transcription factors as well as downstream effector genes of the different cytokines should be assessed to unmask the immune response during different challenges. Future studies would also benefit from using cell based or tissue based Ussing chambers to further investigate the effect of the immune response on the epithelia.

Combining techniques to assess the spectrum of effects elicited by cytokines is necessary to understand the functionality of the immune system. The use of Ussing chamber technique, protein expression and mRNA expression is a way forward for mucosal immunology in salmonids.

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