Molecular characterisation of key components of the mucosal immune system in Atlantic salmon (*Salmo salar* L) and transcriptome analysis of responses against the salmon louse (*Lepeophtheirus salmonis*)

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Tariku Markos Tadiso

Contents

Abstract

Mucosal immunity in mammals is mediated mainly by secretory immunoglobulin A (SIgA), which is produced by IgA plasma cells commonly located in the lamina propria, and a transport system involving the polymeric Ig receptor (pIgR). In teleost fish, IgM plays some roles associated with mucosal defence. Very recent findings indicate that IgT, an antibody exclusive to teleosts, might have a special role in mucosal immune responses, and a possible pIgR counterpart has been identified. The salmon louse (*Lepeophtheirus salmonis* Krøyer), an ectoparasitic copepod targeting the skin (and the gill to a lesser extent), has been a major challenge to the aquaculture industry. While the first line of defence against this parasite is crucial, equally important, in the context of vaccine development is the generation of information on the adaptive immune system. Based on this line of reasoning, Atlantic salmon IgT and pIgR were selected as targets for further characterization in the present study. Three distinct IgT heavy chain (τ) sub-variants, with an identity index of 76-80%, were described. The identity index between τ1 and μ1 (the first constant domains of the IgT and IgM heavy chains, respectively) in Atlantic salmon is 52%. It is plausible to assume that this relatively high similarity is a result of interactions with common light chains. The relative abundance of τ, μ, and δ transcripts in a series of tissues revealed an overall expression pattern of IgM >> IgT > IgD. Interestingly, challenge experiments with salmon louse showed 10 fold increase of IgM and IgT mRNA in skin samples, supporting the assumption that these antibodies are involved in mucosal immune responses. The search for pIgR homologues in Atlantic salmon resulted in two pIgR-like candidates: Salsal pIgR and Salsal pIgRL. Meanwhile, a comparative evaluation was made to a series of CD300-like molecules (CMRF-35 like molecules, CLM) reported to the databanks. Salsal pIgR and Salsal pIgRL were identified on the basis of similarity to homologous genes, and like the counterparts in other teleosts they are composed of two Ig superfamily (IgSF) V-like domains, a transmembrane region, a connecting peptide, and a cytoplasmic tail. Two CD300-like molecules in salmon (CLM1 and CLM7) also have the same domain structure, but their cytoplasmic region is predicted to contain putative immunoreceptor tyrosine based inhibition motifs (ITIM), which is a typical feature of CD300A and CD300F in humans. While Salsal pIgR and Salsal pIgRL were expressed in tissues of skin and gill respectively, their expression pattern is not restricted to mucosal tissues, but notably, their expression increased during infection with salmon louse. Further studies are needed to elucidate the transport mechanisms of mucosal antibodies in

salmon. To investigate how the salmon louse evades the immune system of the host, temporal immune gene expression changes in skin, spleen, and head kidney of Atlantic salmon were analysed using microarray and quantitative real-time PCR (qPCR) during the first 15 days post infection (dpi). This window represents the copepodid and chalimus stages of lice development. Transcriptomic responses, recorded already at 1 dpi, were highly complex and large by scale. Many genes showed bi-phasic expression profiles with abrupt changes taking place between 5 and 10 dpi (the copepodid-chalimus transitions). Large group of secretory splenic proteases with unknown roles showed the greatest fluctuations: up-regulated 1-5 dpi and markedly down regulated afterwards. T cell related transcripts showed a short term (1-5 dpi) increase. After 5 dpi, the magnitude of transcriptomic responses decreased markedly in skin. The findings provided an insight into the time windows in the development of the parasite which are critical to the host and where modulation of the host immune system might occur.

List of publications

Paper I

Tadiso T. M., Lie K. K. and Hordvik I. (2011). Molecular cloning of IgT from Atlantic salmon, and analysis of the relative expression of tau, mu, and delta in different tissues. *Vet. Immunol. Immunopathol*. **139**, 17-26.

Paper II

Tadiso, T. M., Sharma, A, Hordvik I. (2011). Analysis of polymeric immunoglobulin receptor- and CD300-like molecules from Atlantic salmon. *Mol. Immunol.* **49**, 462-473.

Paper III

Tadiso T. M., Krasnov A., Skugor S., Afanasyev S., Hordvik I. and Nilsen F. (2011). Gene expression analyses of immune responses in Atlantic salmon during early stages of infection by salmon louse (*Lepeophtheirus salmonis*) revealed bi-phasic responses coinciding with the copepod-chalimus transition. *BMC Genomics* **12**, 141.

Abbreviations

Ab	Antibody
Ag	Antigen
APC	Antigen-presenting cell
BCR	B cell receptor
CD	Cluster of differentiation
CDR	Complementarity determining region
CLM	CMRF-35 like molecule
CMS	Cardiomyopathy syndrome
CTL	Cytotoxic T-lymphocyte (s)
D	Diversity (-gene segment)
DEG	Differentially expressed genes
DNA	Deoxyribonucleic acid
Fc	Fragment crystallizable of Ig
FcR	Fc receptor
FαμR	Fc receptor for IgA and IgM
GALT	Gut-associated lymphoid tissue
H	Heavy chain (of Ig)
HSMI	Heart and skeletal muscle inflammation
hu	Human
ICAM	Intracellular adhesion molecule
IEL	Intraepithelial lymphocytes
IFN	Interferon
IMGT	International immunogenetics information system
Ig	Immunoglobulin
IL	Interleukin
ISA	Infectious salmon anemia
ITAM	Immunoreceptor tyrosine based activation motif
ITIM	Immunoreceptor tyrosine based inhibition motif
J	Joining (chain)
kDa	Kilodalton (mol. mass)
L	Light chain (of Ig)

LPS	Lipopolysaccharide
m	Membrane
MAb	Monoclonal antibody
MALT	Mucosa-associated lymphoid tissue
MDIR	Modular domain immune type receptor
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
n	Number in study or group
NA	Not available
NK	Natural killer (cell)
PCR	Polymerase chain reaction
pIgR	Polymeric immunoglobulin receptor
qPCR	Quantitative real-time PCR
RhoG	RAS homologue member G
RAG	Recombination-activating gene
RNA	Ribonucleic acid
RNAi	RNA interference
s	Secreted
S	Secretory
SC	Secretory component
SD	Standard deviation
SEM	Standard error of the mean
SYK	Spleen tyrosine kinase
TCR	T cell receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
V	Variable (region, gene segment)
V_H, VH	Variable region, heavy chain
V_L, VL	Variable region, light chain

1. Introduction

1.1. Background

The development of comparative genomics, particularly comparative immunology, enormously shaped the investigative approaches that we follow to study the immune system of fish. The field has developed rapidly during the era of gene technology, greatly benefitted from being organised through the international societies of Developmental and Comparative Immunology (ISDCI) and European Organisation of Fish Immunology (EOFFI). The completion of genome sequencing for several organisms has prompted a new era, as elegantly stated by the Nobel laureate Sydney Brenner as 'The End of the Beginning' (Brenner, 2000). This 'beginning', however, has not yet ended for some important fish species, and there is also a need for systematizing and characterizing immune genes.

Achieving effective protection against pathogens demands an understanding of the immune system of the host and the host pathogen interactions therein. This requires that one must be able to measure or quantify immune related molecules and cells. In the aquaculture industry, there is a need to develop methods for effective monitoring and control of pathogens at low cost to the environment; such as the development of effective vaccines to pathogens that can wholly or partially replace therapeutic treatments. Availability of well-characterised components of the immune system is thus a prerequisite to measure host responses (or to study any other physiological processes per se). Even though a wealth of knowledge is available on well characterised immune genes in fish, there is a need to broaden the spectrum of these elements and therefore one of the objectives of this project was in-parallel characterisation of relevant immune genes, with emphasis on mucosal immunity.

The mucosal immune system protects the body from first-encounter of pathogens, and holds a central place in protective immunity. In mammals, mucosal immune protection is mediated mainly by secretory immunoglobulin A (SIgA)[1] produced by IgA plasma cells commonly located in the lamina propria, and transported into the lumen by the polymeric immunoglobulin receptor (pIgR) (Brandtzaeg et al., 2008; Fagarasan et al., 2010; Kaetzel,

[1] To comply with mucosal immunology nomenclature (Brandtzaeg et al., 2008), a distinction has been made between secretory (denoted by capital letter S) and secreted (s) forms of Igs throughout this thesis.

2005; Woof and Mestecky, 2005). Whereas the intestine occupies a central place in fish mucosal immunology, as a non keratinised mucosal surface, the skin represents a vital defence element of teleost fish, as the aquatic habitat continuously exposes them to a broad spectrum of pathogens, compared to organisms living in the aerial environment (Rakers et al., 2010; Rombout and Joosten, 1998). Despite the fact that there is substantial knowledge in mucosal immunity in fish, much work awaits investigation especially with regard to the key molecules involved and their transport mechanisms. As an economically important species, insight into the mucosal immune system of Atlantic salmon is paramount, as it can aid in understanding and prevention of infection at mucosal surfaces. This study thus examined candidate genes in mucosal immunity and immune response to cutaneous mucosal infection.

1.2. Tools and current knowledge in the study of the fish immune system

A key to understanding the immune system of an organism is to be able to assess/measure it. This can be achieved for example by infection or vaccine experiments, utilizing tools that monitor changes in the expression of key molecules. Accordingly, availability of well-characterised genes is usually a prerequisite. Nevertheless, until now, not enough sequence data (genome sequencing and proper annotations) are available for most fish species and thus, current studies in parallel ought to clone and characterise immune relevant genes and provide a basis for future studies. One of the objectives of this PhD project was thus to broaden the spectrum of characterised immune genes in Atlantic salmon. In recent developments, a number of immune effector molecules have been described in teleosts in general and Atlantic salmon in particular. Researchers at our laboratory (including the present work) have characterised a panel of immune related genes, mainly of T cell receptors & co-receptors and immunoglobulins and associated molecules. However, what remains a bottleneck is the lack of established systems and tools corresponding to that of the mammalian systems, for functional studies of the fish immune system. There is lack of specific antibodies against suitable markers, for example, to distinguish sub-populations of lymphocytes. Another major concern is the fact that teleosts are very heterogeneous group, and knowledge obtained on a given species cannot be directly transferred to another: requiring species-specific research.

Study of the temporal and spatial gene expression patterns of biological samples in naïve conditions and in response to stimuli is becoming a routine and compulsory activity for monitoring disease state. To this end, microarrays and qPCR techniques have become appropriate technologies: by making use of multiple gene expression profiling capabilities of microarrays and quantitative performance of qPCR. Transcriptomic data, however, may not always be related to functional information (based on the mammalian model), as one may not know how a given transcript ends up being translated. In this study, an oligonucleotide microarray platform, called STARS (Salmon and Trout Annotated Reference Sequences) was used for gene expression profiling. This platform was specifically designed for the two important salmonid fish Atlantic salmon and rainbow trout, based on the Agilent Technology (Krasnov et al., 2011). While microarray has been used in **paper III** for global gene expression profiling of salmon responses, qPCR has been ubiquitously used in all studies: to determine the relative abundance of the various transcripts in different tissues (**Paper I**, and **Paper II**), to test the abundance of a given transcript in relation to uninfected control (**Paper II** and **Paper III**), and to validate microarray data (**Paper III**).

1.3. Atlantic salmon: a 3D motive to study its immune system

Atlantic salmon is a major contributor to the growing fisheries and aquaculture industry in many countries. In Norway, it takes the lion's share of seafood export. In 2009, it had an export value of 23.6 billion Norwegian kroner (NOK) that exceeded the combined value of all other marine products, which is 21 billion NOK (Norwegian Sea food Export Council, 2010). Moreover, Atlantic salmon has been recognised as an important model system in evolutionary and conservation biology (Garcia de Leaniz et al., 2007). The fact that this precious species has a unique place in the phylogeny makes it a suitable species for comparative studies: their relatively recent genome duplication (and duplicated Ig heavy chain loci) can be mentioned among others (Koop et al., 2008; Yasuike et al., 2010). Atlantic salmon has also been recognised as a potential model for medical research (Majalahti-Palviainen et al., 2000).

1.4. Innate and adaptive immune recognition

The ability of an organism to resist or minimise the impacts of pathogens depends on protective immune responses, which can be both innate and adaptive: the two major systems of immune recognition in mammals and other vertebrates. Immune cells (white blood cells or leukocytes) mediating these reactions are formed from precursor cells (called pluripotent hematopoietic stem cells) in the bone marrow (or most likely the head kidney in teleost fish), which give rise to the myeloid or lymphoid progenitor cells of the innate and adaptive immune system, respectively. Lymphocytes are further divided into B cells (immunoglobulin-positive) and T cells. Innate immunity constitutes the first line of defence against invaders, while adaptive immunity provides a more rigorous immune defence at later stages (and increases with repeated exposure to a particular antigen), as it remembers specific pathogens. A common feature of innate and adaptive immune responses is that they both involve immune receptors, which recognise pathogens. However, the receptor types used to recognise pathogens are distinct (Medzhitov, 2007).

The innate immune system senses pathogens via genetically pre-encoded receptors called pattern recognition receptors (PRR), which are evolved to recognise specific signals common to large groups of microorganisms (Janeway and Medzhitov, 2002). These signals are small molecular motifs conserved within a class of microbes and are referred to as pathogen associated molecular patterns (PAMPs). Toll-like receptors play a major role in pathogen recognition and initiation of inflammatory and immune responses (Janeway and Medzhitov, 2002). PRR activate conserved host defence signalling pathways that control the expression of a variety of immune response genes (Medzhitov and Janeway, 2000). In addition to the usual pathogen associated ligands, several damage-associated molecular patterns (DAMPs) have recently been shown to have immunological importance (Nace et al., 2012). Phagocytes respond to endogenous molecules derived from proteolytic degradation of substances such as collagen, which signal the presence of damage (alarm) via DAMPs. They are also referred to as alarmins (Oppenheim and Yang, 2005). They are part of the extracellular matrix (ECM), which, in addition to provision of structural support and adhesive substrates for the body tissues, has a key role in innate immunity and inflammation (Castillo-Briceno et al., 2009; Pacifici et al., 1991). The resulting proteolytic fragments of collagen after cleavage (for example by MMPs) will activate phagocytes. The role of ECM in serving as hazard signal

has been shown recently in teleost fish, where both collagen and gelatin increased the production of ROS by seabream (Castillo-Briceno et al., 2009). This is particularly important in anti-parasitic defence at cutaneous mucosal surfaces: e.g. cleaved transferrin during sea lice infection is implicated in NO mediated response of salmon macrophages (Easy and Ross, 2009). Inflammatory reactions can be cellular such as phagocytosis and phagocyte activity (including oxidative mechanisms), complement activity: are modulated by many fish parasites, including mainly ciliates, flagellates and myxozoans (Alvarez-Pellitero, 2008). Humoral immune factors (peroxidases, lysozyme, acute-phase proteins) are also implicated in the response to some parasites (Alvarez-Pellitero, 2008; Jones, 2001). B lymphocytes from fish also have potent phagocytic and microbicidal abilities (Li et al., 2006; Zhang et al., 2010).

In adaptive immune recognition, two types of antigen receptors are involved: T cell receptors and B cell receptors. The genes encoding for these receptors are assembled from variable and constant gene fragments in each T or B lymphoid cell through recombination-activating gene (RAG)-protein-mediated somatic recombination (Schatz et al., 1992). Combinatorial and junctional diversification leads to high number of different receptors (Danilova and Amemiya, 2009). Production of a diverse repertoire of receptors, with the potential to recognise almost any antigen, is a result of this process (Schatz et al., 1992).

Key components of the adaptive responses include B lymphocytes, T lymphocytes, and antigen presenting cells. Antibodies, produced by B cells, interact directly with antigens. The T cell receptor, the antigen binding molecule expressed by T cells, on the other hand, recognizes antigens processed and presented by the major histocompatibility complex (MHC) molecules. There are two subpopulations of T cells. T helper cells (which harbor CD4 membrane glycoprotein on their surface) play a crucial role by secreting cytokines and activating B cells; and cytotoxic T cells (displaying CD8) kill target cells (eg. virus infected cells). Clark and Ledbetter (1994) described how B and T cells interact for effective immune protection. Specific recognition of foreign antigen by cell surface Ig induces B cells to proliferate and differentiate into plasma cells (producing soluble Ig to fight infection) or memory B cells which function during repeated challenge with the same antigen (this constitutes the basis for vaccination). This process requires help from T cells: Once activated

by interaction with specific Ag presenting cells, T cells activate B cells by releasing cytokines such as interleukins (Clark and Ledbetter, 1994).

The basic mechanisms and molecular components of immunity in fish and mammals are similar especially in macrophage function, lymphocyte stimulation and characterised humoral factors such as antibodies (Rombout and Joosten, 1998). However, the structure and diversity of immunoglobulin (Ig) genes constitute a major source of variation. While IgG, IgE, and IgA are immunoglobulin classes present in mammals, but not in fish, the newly discovered IgT/Z isotype appear to be specific for teleosts (Danilova et al., 2005; Hansen et al., 2005). IgD and IgM are common in both. The absence of IgG and IgA and their functional equivalents in fish remains an immunological challenge. The biological function of IgD and IgT in teleosts is not clear, except for a recent study in rainbow trout, which claimed IgT to be a mucosal Ig, functioning similarly to the mammalian IgA (Zhang et al., 2010).

Innate and adaptive immune systems are not mutually exclusive, and both work together to enhance effective protection. Proper stimulation of innate effector molecules enhances generation of effective adaptive immunity. Most importantly, the innate immune system has an essential role in the clonal selection of lymphocytes and activation of the adaptive immune responses, whereas the adaptive immune system, in turn, activates innate effector mechanisms in an antigen specific manner (Medzhitov, 2007; Medzhitov and Janeway, 1998). In teleost fish, given the possibility that most fish pathogens can damage their hosts in relatively short period of time (before the building-up of adaptive immunity), the importance of innate defences become of crucial importance (Ellis, 2001; Jones, 2001). It has, however, been emphasised that the innate immune system in vertebrates has been evolved to depend, to some extent, on antigen-specific (adaptive) immunity (e.g. for pathogen clearance) (Medzhitov, 2007; Unanue, 1997).

1.5. The mucosal immune system

1.5.1. Mucosal surfaces

The body's mucosal surfaces are in a continuous direct contact with the environment. In mammals, the gastrointestinal, respiratory, genital, and urinary surfaces comprise major mucosal organs (Kraehenbuhl and Neutra, 1992). For example, the gut mucosa continuously interacts with food antigens, the gut commensal bacteria, and potential pathogens that enter the host through the intestine (Lambolez and Rocha, 2001). However, there are considerable morphological and functional differences between fish and mammals with regard to mucosal immunity (Bernard et al., 2006; Rombout et al., 2011). Little evidence is available on the presence of inductive sites (antigen capture and presentation) such as the Peyer's patches, sites of antibody production, and organised mucosa associated lymphoid tissues (O-MALT). Despite this, the fish possess a well functioning mucosal immune system (recently reviewed in (Rombout et al., 2011; Salinas et al., 2011)). Whereas the intestine holds a central place in both cases, equally important in bony fish are the gills and the skin as mucosal organs (Press and Evensen, 1999). In contrast to a keratinised skin of mammals, the fish skin is a mucosal organ (Fig. 1a-b), possessing a number of mucus producing goblet cells (Rakers et al., 2010). Compared to organisms living in the aerial environment, the aquatic habitat continuously exposes them (via their skin and gills) to a broad spectrum of pathogens (Rakers et al., 2010; Rombout and Joosten, 1998).

1.5.2. Immune mechanisms at mucosal surfaces of teleost fish

By forming a major barrier, the gut epithelium and its mucous layer trap invading pathogens which are then eliminated when the gut epithelium is shed (Lambolez and Rocha, 2001). Similarly, the skin mucus in fish entraps microorganisms and hinders their entry into the body; the mucus is continuously produced and eliminated carrying away the entrapped pathogens (Ourth, 1980). In the gut, local defence depends partly on T lymphocytes called intraepithelial lymphocytes (IELs), often generated locally, not in the thymus (Saito et al., 1998). The presence of putative T cells or their precursors in the fish gut, together with the RAG-1 expression of intestinal lymphoid cells (Rombout et al., 2011; Rombout et al., 2005), supports this phenomenon. These T cells modulate homeostasis of the gut epithelium through local production of cytokines (Rombout et al., 2011). The development of intestinal

inflammation (enteritis) in the antigen transporting second gut segment and the presence IEL and eosinophils/basophils seem to play a crucial role in fish (Rombout et al., 2011).

The fish skin is a multi-purpose organ. In addition to serving as anatomical and physiological barrier against the external environment, it is also an entry point for many bacteria and viruses, and an important immunological organ. Cutaneous mucus, secreted by goblet cells in the epidermis, contains a number of immune factors. Fish skin serves as a source of pro-inflammatory molecules as well as an active modulator of the local inflammation (Gonzalez et al., 2007a; Gonzalez et al., 2007b). Innate humoral immune components described in fish skin mucus include lysozyme, peroxidases, acute-phase proteins, trypsin like proteases, alkaline phosphatase, and esterases (Alvarez-Pellitero, 2008; Jones, 2001; Palaksha et al., 2008). Many of these immune factors are implicated in the response to some parasites (Alvarez-Pellitero, 2008). Increased expression of interleukin-1b (IL-1b), interferon-g (IFN-g), IL-10 and infiltration of CD3-positive cells in the tail fin epidermis are among characteristic responses of highly susceptible salmon against the ectoparasite *Gyrodactylus salaries* (Kania et al., 2010). The gill also represents a mucosal organ in fish. It is shown that Atlantic salmon gill tissues contain significant lymphoid accumulations (Haugarvoll et al., 2008; Koppang et al., 2010). Two studies indicated the presence of IgT positive cells in the epithelial lining of the gill lamellae, and IgT and IgM binding to surface structures of the parasite *Ichthyophthirius multifiliis* (Olsen et al., 2011; von Gersdorff Jorgensen et al., 2011).

Fish has a secretory immune system, and can mount cutaneous mucosal immune responses, irrespective of levels in serum (Dickerson and Clark, 1998; Lobb, 1987; Lobb and Clem, 1981a, b; Ourth, 1980; Rombout et al., 1993). Intracutaneous Ab secreting B-lymphocytes represent an integral component of the fish immune system and confer protection against parasitic infections (Dickerson and Clark, 1998; Zhao et al., 2008). In defence against the ectoparasite *I. multifiliis* it has been depicted that tetrameric Abs in skin/mucus bind to parasite surface i-antigens eventually leading to the removal of the parasite (Dickerson and Clark, 1998; Lin et al., 1996). Relatively, more lymphocyte accumulation is expected deeper within the epithelium (Lobb, 1987), which requires some kind of transport. Recent studies show the presence of teleost specific immunoglobulin (IgT) specialised on mucosal defences (Zhang et al., 2010), and expression of a possible transcytosis receptor (pIgR) in mucosal tissues (Hamuro et al., 2007; Rombout et al., 2008). Even though the mechanism how

antibodies from secondary lymphoid tissues are brought into the mucus is not known, it is apparent that antibodies and mainly intraepithelial IgM positive lymphocytes (IEL) are shown to exist. Most importantly, expression of Abs increase considerably with infection or immunization (Lobb, 1987; Maki and Dickerson, 2003; Zhang et al., 2010; Zhao et al., 2008).

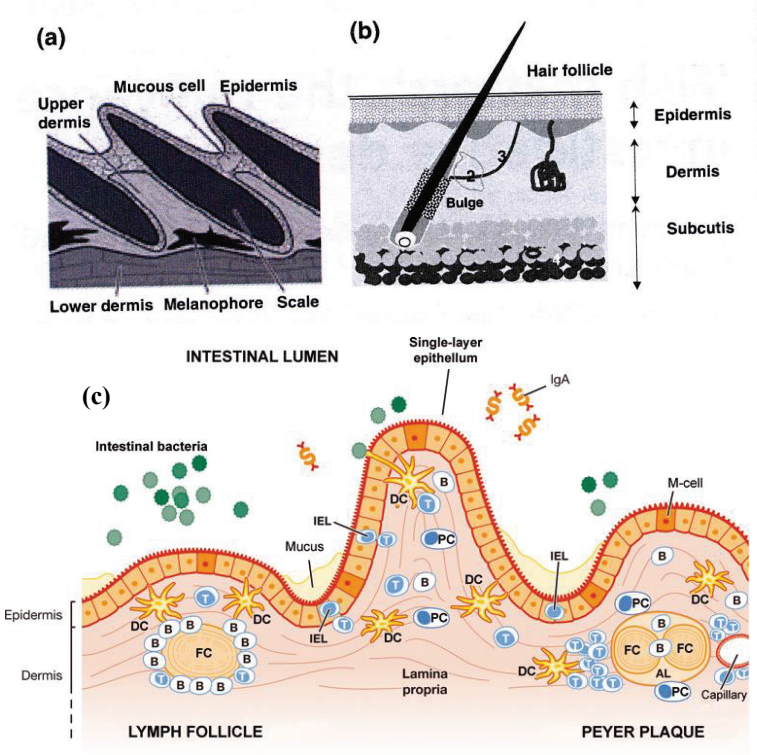


Fig. 1. (a) The structure of teleost skin with dermal scale (Original drawing by Matthias Emde, according to (Schempp et al., 2009)). b) Human skin. 1: sweat gland, 2: sebaceous gland, 3: arrector pili muscle, 4: blood vessel (Rakers et al., 2010). c) The immune system of the gut in human. Proliferative centers (lymph follicles, Peyer plaques) are located directly beneath the monolayer epithelium with high secretory function (production of mucus and IgA antibodies) (Original drawing: Matthias Emde, according to (Wolfle et al., 2009)).

1.5.3. Adaptive immunity at mucosal surfaces

When pathogens first attack mucosal surfaces, antigens (Ag) are sampled and transported across the mucosal epithelium. As lymphoid cells of mucosal tissues are separated from Ag by an epithelial barrier, Ag must be transported across the epithelium by specialised cells. In

mammals, this is accomplished by M-cells, residing at the O-MALT (Kraehenbuhl and Neutra, 1992) (Fig. 1c). B-lymphocytes, following Ag stimulation, leave the O-MALT and migrate to mucosal grandular sites where they differentiate and produce polymeric IgA (pIgA) antibodies. This follows a pIgR mediated transport of pIgA into the lumen. Even though this process has not been clearly described in fish, fish possess a shorter type pIgR and mucosal defence involving polymeric Ig and pIgR is possible. Fish do not have antigen sampling M-cells (Rombout et al., 2011). However, very recently, cells exhibiting the characteristics of M-cells have been reported (Fuglem et al., 2010).

1.5.4. Infections at surfaces of cutaneous mucosa: the case of *Lepeophtheirus salmonis*

The salmon louse (*Lepeophtheirus salmonis* Krøyer) is an economically important ectoparasitic copepod that infects both farmed and wild salmonids in Norway and throughout the northern hemisphere. During the last few years, there has been a dramatic increase in the number of *L. salmonis* in Norwegian fjords. Because of the potential economic and environmental impacts of *L. salmonis*, diverse research has been undertaken in the last three decades. Nevertheless, there are still large gaps in understanding of the host parasite interaction at the molecular level, particularly the mechanisms by which the host avoids the parasite, and how the parasite modulates the immune system of the host. Increased biological knowledge and competence in this field is important with regard to the management of the aquaculture industry and maintenance of wild populations of salmon. The need for new methods of parasite control requires a better understanding of the protective mechanisms. Limitations and decreasing efficiency of chemical treatment stimulate interest to immune responses, which are essential for the development of biological methods of protection. The target site for the salmon louse is the skin (and the gill to a lesser extent), and thus the mucosal immune system is of special interest. While the first line of defence against this parasite is crucial, equally important, in the context of vaccine development is the generation of information on the adaptive immune system, i.e. the part of the immune system that can ‘learn’, and improve its performance. For ectoparasites such as *I. multifiliis*, it has been shown that antibodies play a key role in immune exclusion of the parasite both from skin and gill tissues. In *L. salmonis*, however, the target antigens as well as the protective mechanisms which lead to the exclusion of the parasite are not known.

L. salmonis damage fish by feeding on their mucus, skin, and blood and the wounds increase the risk of secondary infections, and if persistent, the infections can lead to mortality. Overall, *L. salmonis* results in systemic stress and modulation of the immune system and physiological processes (Tully and Nolan, 2002; Wagner et al., 2008). Resistance against *L. salmonis* infections varies among species. Whereas pink (*Oncorhynchus gorbuscha*) and coho salmon (*O. kisutch*) seem to be the most resistant species, higher initial prevalence and intensity of infections are observed in Atlantic salmon and sea trout (*S. trutta*) followed by rainbow trout (*O. mykiss*), chum salmon (Oncorhynchus keta), and Chinook salmon (*O. tshawytscha*) (Fast et al., 2002; Jones et al., 2006; Jones et al., 2007; Wagner et al., 2008; Yazawa et al., 2008). It has been hypothesised that this variation is partly attributed to differences in Pacific and Atlantic forms of *L. salmonis* which are believed to be coevolved respectively with Pacific salmon (*Onchorhynchus* spp.) and Atlantic salmonids (*Salmo* spp.) (Yazawa et al., 2008).

2. Main goals of the PhD project

Broadly, this PhD project is aimed at molecular characterisation of key mucosal immune components of Atlantic salmon, and their transcriptional responses following infection by the salmon louse (*L. salmonis*).

Topics covered in the PhD project are:

1. Cloning and characterisation of immunoglobulin tau (IgT), and analysis of the relative expression of Ig isotypes in different tissues.
2. Molecular cloning and expression studies of pIgR and related molecules from Atlantic salmon, and revealing their possible roles in mucosal immune defence.
3. Analysis of immune gene expression (and possible immune modulation) on Atlantic salmon during early infection by the salmon louse.

3. Summary of papers

Paper I

Tadiso T. M., Lie K. K. and Hordvik I. (2011). Molecular cloning of IgT from Atlantic salmon, and analysis of the relative expression of tau, mu, and delta in different tissues. *Vet. Immunol. Immunopathol*. **139**, 17-26.

The aim of this study was to clone and characterise a key candidate of mucosal immunoglobulins, IgT. In this study, three distinct IgT heavy chain sub-variants, with an identity index of 76-80%, have been described. This is in contrast to IgM and IgD for which two sub variants were reported previously. The similarity between τ1 and μ1 in Atlantic salmon is relatively high (identity index of 52%) when compared to the remaining part of the molecules, showing that non-random processes are involved in the evolution of these genes. qPCR assays were designed and evaluated to compare the relative abundance of τ, μ, and δ transcripts in different tissues (head kidney, thymus, spleen, gill, skin, hind gut, brain and muscle) of Atlantic salmon. The analysis revealed relatively high expression of IgM (up to 200 fold more than IgD) followed by IgT (up to 20 fold more than IgD) in most tissues. Head kidney and spleen contained the highest transcript abundance compared to other tissues.

Paper II

Tadiso, T. M., Sharma, A, Hordvik I. (2011). Analysis of polymeric immunoglobulin receptor- and CD300-like molecules from Atlantic salmon. *Mol. Immunol.* **49**, 462-473.

In this study, two pIgR-like molecules of Atlantic salmon (Salsal pIgR and Salsal pIgRL) were cloned and characterised, and a comparative evaluation was made to CD300-like molecules submitted to GenBank (submitted in salmon as CMRF35-like molecules (CLM1, CLM7 and CLM8)). Salsal pIgR and Salsal pIgRL were identified on the basis of similarity to homologous genes in other teleosts. pIgR-like molecules in salmon, like counterparts in other teleosts, are composed of two IgSF V-like domains, a transmembrane region, a connecting peptide, and a cytoplasmic tail. CLM1 and CLM7 in salmon also have the same domain structure, but their cytoplasmic region is predicted to contain putative immunoreceptor tyrosine based inhibition motifs (ITIM), which is a typical feature of CD300A and CD300F proteins in humans. The two V domains of Salsal pIgR and Salsal

pIgRL correspond to the mammalian [D1] and [D5], however, they show the same disulphide bridge topology only with [D1]. While Salsal pIgR and Salsal pIgRL were expressed in tissues of skin and gill respectively, their expression pattern is not restricted to mucosal tissues.

Paper III

Tadiso T. M., Krasnov A., Skugor S., Afanasyev S., Hordvik I. and Nilsen F. (2011). Gene expression analyses of immune responses in Atlantic salmon during early stages of infection by salmon louse (*Lepeophtheirus salmonis*) revealed bi-phasic responses coinciding with the copepod-chalimus transition. *BMC Genomics* **12**, 141.

This study addressed the question of what immune transcripts are involved in response to mucosal infections by the salmon louse (*Lepeophtheirus salmonis* Krøyer), an economically important ectoparasitic copepod that infects both farmed and wild salmonids throughout the northern hemisphere. In this study a 21 k oligonucleotide microarray and qPCR were used to examine the temporal immune gene expression changes in skin, spleen, and head kidney of Atlantic salmon during the first 15 days post infection (dpi), representing the copepod and chalimus stages of lice development. Transcriptomic responses, recorded already at 1 dpi, were highly complex and large by scale. Many genes showed bi-phasic expression profiles with abrupt changes taking place between 5 and 10 dpi (the copepod-chalimus transitions). Large group of secretory splenic proteases with unknown roles showed the greatest fluctuations: up regulated 1-5 dpi and markedly down regulated afterwards. T cell related transcripts (T cell receptor alpha, CD4-1, and possible regulators of lymphocyte differentiation) showed a short term (1-5 dpi) up-regulation, suggesting recruitment of T cells of unidentified lineage to the skin probably with innate immune roles. After 5 dpi, the magnitude of transcriptomic responses decreased markedly in skin. Matrix metalloproteinases in all studied organs showed an increase in the second phase. Increase of IgM and IgT transcripts in skin indicated an onset of adaptive humoral immune responses, while MHCI and related transcripts appeared to be down-regulated. The findings gave an insight of the time windows in the development of the parasite which are critical to the host and where modulation of the host immune system might occur.

4. General discussion

The aim of the present work was to identify and characterise key components of mucosal immunity in Atlantic salmon and analyse immune responses of Atlantic salmon to infection by *L. salmonis*. When this study was started, there were not enough data on characterised (mucosal) immune components. Due to the requirement that the PhD should be finished in a three years period, experiments for the papers to be included in this thesis started somewhat in parallel. The papers presented in the thesis are thus organised such that in **paper I** and **II**, the key candidates for mucosal immunity: IgT and pIgR-like molecules were described, including assay design for qPCR studies of all Ig isotypes, pIgR, and CD300–like molecules. **Paper III** explored global gene expression changes due to an ectoparasite invading cutaneous mucosa of salmonids.

When the cloning of a salmon pIgR homolog was initiated, data from other teleosts had just been published, indicating that the mechanisms involved are similar to those in mammals. Thus, it was somewhat unexpected to find an expression pattern of Salsal pIgR and Salsal pIgRL that might point to important differences with regard to possible antibody transport mechanisms in salmon. Regarding the discovery of IgT as a mucosal antibody, which was done in parallel with the present study by another research group, it must be emphasised that the concentration of antibodies in the mucus of fish is still only a fraction of that in mammals. However, the present work has provided important data that further studies can be based on, and identified a possible time window during infection which appear to be essential with regard to immune modulation.

4.1. Sampling and individual variation

One important lesson obtained in this study is that expression levels of Igs and related transcripts is tissue specific. Moreover, there is an obvious individual variation. This variation is again dependent on the tissue type. Whereas the spleen is relatively homogenous and shows little variation, the hind gut, followed by the head kidney (the foremost part of the kidney), show the greatest variation in transcript levels of immunoglobulins and pIgR. This is possibly because of variation in the exact tissue section sampled, as the tissues are heterogeneous with regard to lymphoid accumulations. The head kidney is a lympho-myeloid

compartment serving as both hematopoietic and secondary lymphoid organ (Press and Evensen, 1999).

4.2. Quantitative real-time PCR: The most ubiquitously used method

The most ubiquitously used method in this study was quantitative real-time PCR **(**qPCR). The important steps to be followed when performing such experiments, among others, are: selection and validation of an internal reference gene, and verifying that the amplification efficiencies of the reference gene and the target genes are similar, followed by statistical analysis of the data. In this study, except for few practical limitations, attempts were made to stick to the MIQE (minimum information for publication of quantitative real-time PCR experiments) guideline (Bustin et al., 2009): Good quality RNA was used, a reference gene was chosen as mentioned in the papers, PCR efficiencies were calculated for selected transcripts, followed by proper statistical analysis. Of particular importance was the design of PCR assays that take into consideration immunoglobulin diversity. The aim of qPCR analysis was to show the relative abundance of the various transcripts in different tissues (**Paper I**, and **Paper II**), and to test the abundance of a given transcript in relation to uninfected control (**Paper II** and **Paper III**).

Statistical analysis of qPCR data is done, in most cases, based on -ΔCt or -ΔΔCt values [ΔCt = Ct of target gene (gene of interest) - Ct of reference gene (internal control), while ΔΔCt = ΔCt of sample - ΔCt of calibrator]. However, since relative expression is given as $2^{-\Delta Ct}$, or as $2^{-\Delta\Delta Ct}$, the statistical data should be converted into the linear form, rather than the raw values (Livak and Schmittgen, 2001), i.e., gene expression gives sense if shown as copy number instead of delta Ct values). For example, in the final calculation, the error is estimated by evaluating the $2^{-\Delta\Delta Ct}$ (or $2^{-\Delta Ct}$) term using ΔΔCt plus or minus the standard deviation (Livak and Schmittgen, 2001). This, however, leads to a range of values that are asymmetrically distributed relative to the average value, a result of converting the results of an exponential process into a linear comparison (Livak and Schmittgen, 2001). To avoid this discrepancy and for simplicity, both figures (obtained using the raw -ΔΔCt (or -ΔCt) values as well as $2^{-\Delta Ct}$) were used for comparative purposes in this thesis.

The $-\Delta\Delta Ct$ or $2^{-\Delta\Delta Ct}$ method is often used where there are two sets of samples to be compared (e g. a test and a control sample), or by the use of a calibrator (that show the highest or the lowest expression from the groups). When the objective was direct comparison of gene expression in different tissues, as in **Paper I**, and partly **Paper II**, this method has not been utilised because it is not always easy choosing which tissue to use as a calibrator (in this case, simply because the different Ig, pIgR-like, and CLM genes show tissue specific variation). Moreover, the use of a calibrator sample does not always give a biologically meaningful result (Livak and Schmittgen, 2001). Thus, all analyses were based on the $2^{-\Delta Ct}$ method, without a calibrator.

In this study, pair-wise comparison of transcript abundance (based on percentage copy # relative to EF1A or $2^{-\Delta Ct}$) of τ, μ, and δ within the various tissues was done by calculating the probability associated with the student's t-test (2 tailed) using the Microsoft Excel spreadsheet program (**Paper I**). Based on this test, the most abundant Ig transcript in all tissues examined was IgM followed by IgT. In all tissues tested, IgD (= mIgD) transcripts were minimal. Similarly, a t-test for the overall Ig transcript abundance in various tissues revealed that relative Ig transcript levels were significantly higher in head kidney and spleen when compared to all other tissues ($P<0.05$). Transcript levels of each gene was compared between tissues by one way ANOVA, and individual 95% CIs for mean based on pooled StDev of $-\Delta Ct$ values (**Paper I,** Appendix 1) and using $2^{-\Delta Ct}$ values **(Paper II)** was calculated using Minitab 14 software. From this it was possible to see the individual variation of transcripts studied (mainly IgM) especially in the hind gut and head kidney. **In paper III**, qPCR was used for validation of microarray results and analyses of additional transcripts and tissues not included in the microarray. In **paper II** (partly) and **Paper III**, to test for difference between control and test samples at each sampling point, the data were presented as mean $-\Delta\Delta Ct \pm SE$. $-\Delta\Delta Ct$ was calculated as: $-\Delta\Delta Ct = -(\Delta Ct_{Test} - \Delta Ct_{Control})$, where $\Delta Ct = (Ct_{Target} - Ct_{EF1A})$. The probability related to student's t-test (2 sample) was calculated for each transcript.

4.3. Antibody isotypes

Still its basic components being conserved in many species, the adaptive immune system involving immunoglobulins is considerably dynamic in terms of Ig loci organisation, Ig structure, and means to generate diversity (Danilova and Amemiya, 2009).

Generally, antibodies comprise Ig heavy (IgH) and light (IgL) chains. Whereas the variable region of the IgH chain (VH) determines the antigenic specificity, differences in the IgH constant regions (IgC) determine the antibody isotypes. Most mammals harbor gene segments encoding for five antibody isotypes: IgM, IgD, IgG, IgA, and IgE. In teleosts, however, only IgM, IgD, and IgT gene segments have been found. The latter is a teleost specific antibody described very recently in many fish species, including Atlantic salmon (**Paper I**). In addition, variable numbers of antibody subclasses exists in different species. Amphibians possess IgX (which seems to be an IgA equivalent), and IgY (analogous to mammalian IgG) (Hadge and Ambrosius, 1984; Mussmann et al., 1996). Cartilaginous fish (sharks, skates and rays), in addition to two forms of IgM, have two isotypes of IgNAR, a dimer which does not associate with IgL chain; and IgW, an isotype phylogenetically related to IgD (Berstein et al., 1996; Dooley and Flajnik, 2006; Hordvik et al., 1999). IgH loci are organised in two major ways, named cluster (in cartilaginous fish) and translocon. The latter is a typical feature of IgH loci in tetrapods (amphibians, reptiles, birds, and mammals) where an array of VH genomic segments is followed by diversity (DH) and joining (JH) segments. Located downstream of these regions are segments encoding the CH. Antibodies are then generated by VH-DH-JH-CH rearrangements. In bony fish, the heavy chains are in translocon configuration (Warr, 1995), whereas the pattern varies among species. Except for minor differences in Cζ/τ gene locations and channel catfish which is devoid of the IgT/Z genes, generally, the VH segments lie together in tandem upstream of the constant region in the following manner: [VH-DH-JH-Cζ/τ-(VH)-DH-JH-Cμ-Cδ] (recently reviewed in (Hikima et al., 2011)). While IgT in rainbow trout is located within the VH gene region (Hansen et al., 2005); in zebrafish (*Danio rerio*), fugu (*Takifugu rubripes*), and three-spined stickleback (*Gasterosteus aculeatus*), the IgT/Z genes are sandwiched between the VH and DH-JH-Cμ-Cδ regions: IgT/Z, IgM, and IgD all share the same VH regions, while IgT/Z has its own DH and JH segments (Danilova et al., 2005; Deza et al., 2009; Savan et al., 2005) (Fig. 2).

In Atlantic salmon, there are two highly similar IgM and IgD transcripts, corresponding to the two duplicated IgH loci (A and B) (Hordvik et al., 2002), whereas three IgT sub-variants with an identity index of 76-80% have been found (**Paper I**). A more detailed study indicated the presence of multiple Cτ genes upstream of the Cμ region, with three of them (two in locus A, and one in locus B) being functional (Yasuike et al., 2010) (Fig. 2). The study also indicated that the IgT sub-variants exhibit some differences in their expression patterns. In Atlantic salmon and brown trout (*Salmo trutta*), the two IgM subpopulations were shown to vary in their reaction to a monoclonal antibody (mAb): the two sub-variants differ with respect to an additional cysteine at the C-terminal of the fourth constant domain (μ4), whereas the mAb reacts with the third constant domain (Hordvik, 1998; Hordvik et al., 2002; Kamil et al., 2011). But so far differential expression of these genes has not been reported.

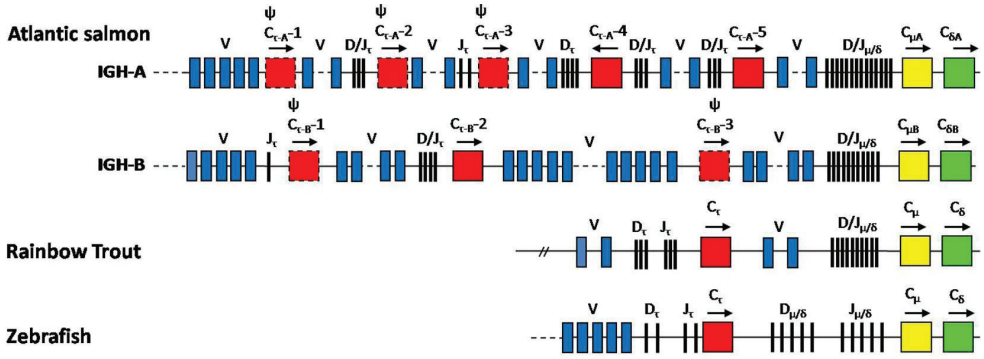


Fig. 2 IgH loci organisation of Atlantic salmon, rainbow trout and zebrafish. The Atlantic salmon duplicated IgH loci, IGH-A (670 kb) and IGH-B (710 kb), were completely sequenced by (Yasuike et al., 2010). Dashed-line boxes indicate the CH pseudogenes (ψ). Transcription directions are shown by arrowhead. Adapted from (Yasuike et al., 2010).

Ig isotypes in mammals occur solely in a monomeric (IgG, IgD, and IgE) or polymeric form (IgM), while IgA is found in both forms (Woof and Mestecky, 2005). In teleosts, IgM predominantly occurs as a tetramer, and its monomeric units are composed of two identical light chains (~25KDa) and two identical heavy chains (~75KDa) (Pilstrom and Bengten, 1996).

Immunoglobulins have two basic forms which determine their function. Membrane bound (m) Igs serve as antigen receptors on the surface of B cells, while secreted (s) Igs (antibodies secreted into blood and other body fluids) function as immune effector molecules (Pilstrom and Bengten, 1996). Teleost B lymphocytes express mIgM as a monomer and secrete soluble IgM as a tetramer (Miller et al., 1998). Lymphocytes in fish also express transcripts encoding IgT, which may represent a novel BCR. IgT is shown to be a monomer (in serum), and a polymer (in gut) (Zhang et al., 2010). Both membrane and secreted transcripts are generated from the IgT and IgM genes, while most teleosts appear to express primarily (or only) membrane bound IgD (discussed in **Paper I**; Fig. 3). Channel catfish, however, do not have the IgT genes, while both secreted and membrane forms of IgD have been reported (Bengten et al., 2002). The heavy chains of both secreted and membrane anchored forms of salmon IgT include four constant Ig domains, τ1-τ4, like the μ chains of other vertebrates. However, the membrane form of IgM includes only three constant Ig domains, μ1-μ3. A typical feature of IgD of most teleosts is a chimeric Cμ1–Cδ structure, where μ1 is incorporated between the variable Ig domain and the δ chain. Internal duplications of the Cδ exons generates a longer IgD molecule. In mammals, secreted IgD is involved in mucosal immunity (Chen and Cerutti, 2010, 2011), and in channel catfish $IgM^{-}IgD^{+}$ cells were described (Chen et al., 2009; Edholm et al., 2010). These cells show variations among individuals. Very recent studies show special exon usage of IgD in zebrafish (Zimmerman et al., 2011), and splicing patterns resulting in a secreted form of IgD (sIgD) in rainbow trout (Ramirez-Gomez et al., 2011). Conventionally, secreted Igs are formed by splicing to the transmembrane exon, whereas trout sIgD is produced via transcription through the splice site at the end of the δ7 domain and continuing into the intron until a stop codon is reached, resulting in an 11 amino acid secretory tail (Ramirez-Gomez et al., 2011). This sIgD arrangement differs from the one observed in channel catfish, which is generated from a separate sIgD terminal exon (Bengten et al., 2002; Wilson et al., 1997).

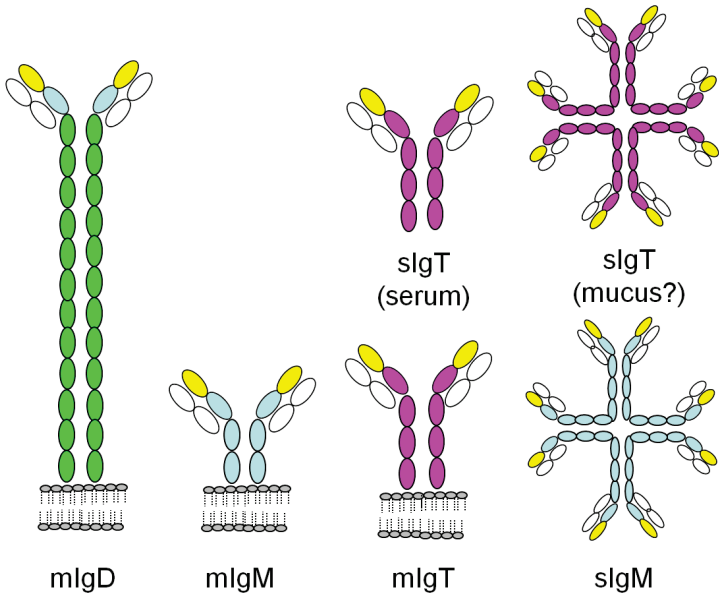


Fig 3. Secreted and membrane forms of immunoglobulins in teleost fish. Both membrane and secreted transcripts are generated from the IgT and IgM genes, while most teleosts appear to express only membrane bound IgD. The heavy chains of both secreted and membrane anchored forms of salmon IgT include four constant Ig domains, τ1-τ4, like the μ chains of other vertebrates. However, the membrane form of IgM includes only three constant Ig domains, μ1-μ3. Internal duplications of the Cδ exons generates a longer IgD molecule. μ1 is incorporated into the IgD heavy chain, between the variable Ig domain and the δ chain. IgT is monomeric in serum but in gut mucus, it has been indicated to be polymeric, similar to IgM. The heavy chain variable regions are shown by yellow ovals, and constant regions (CH domains) are represented by colored ovals: baby blue, bright pink and bright green, respectively for μ, τ, and δ.

4.4. Expression of Ig isotypes in different tissues

The relative abundance of τ, μ, and δ transcripts in a series of tissues revealed an overall expression pattern of IgM >> IgT > IgD (**Paper I**). This trend has also been maintained in many fish species e.g. (Zimmerman et al., 2011). This asserts the fact that IgM is the predominant antibody class in most teleosts. Even though the abundance of IgT transcripts in samples of mucosal tissues (skin, gill, and hind gut) is relatively weak, IgT enormously responds against infection, supporting the assumption that these antibodies are involved in mucosal immune responses. The expression level of IgD is somewhat similar (or lower) to membrane IgM (mIgM), while secreted IgM (sIgM) transcripts are abundant in most tissues.

The spleen and the head kidney, serving as major hematopoietic tissues (Pilstrom and Bengten, 1996; Press and Evensen, 1999), contain the highest transcript levels of immunoglobulins. However in the hindgut and head kidney, there is a large individual variation in contrast to the spleen which shows the least variability in Ig transcript levels among individuals (Appendix 1). Even though the transcriptional control of Ig isotypes leading to tissue specific expression is not clearly described in fish (Hikima et al., 2011; Pilstrom and Bengten, 1996), in mammals, tissue specificity of Ig gene expression is regulated atleast by three DNA sequence elements: the enhancer, the promoter, and undefined intragenic sequences (Grosschedl and Baltimore, 1985).

4.5. pIgR-like and similar molecules in Atlantic salmon

The polymeric immunoglobulin receptor (pIgR) is a transmembrane glycoprotein mediating transport of polymeric immunoglobulins (IgA, and to a lesser extent IgM) across mucosal epithelium (Kaetzel, 2005). In **paper II**, two pIgR-like molecules of Atlantic salmon (Salsal pIgR and Salsal pIgRL) were cloned and characterised (Appendix 2 A and B), and a comparative evaluation was made to CD300-like molecules submitted to GenBank (submitted in salmon as CMRF35-like molecules (CLM1, CLM7 and CLM8)). Whereas the extracellular region of higher vertebrate's pIgR is composed of five or four immunoglobulin superfamily (IgSF) variable-like (V) domains (Braathen et al., 2007; Krajci et al., 1992; Kulseth et al., 1995; Piskurich et al., 1995; Wieland et al., 2004), teleost pIgR (including Atlantic salmon) is a shorter type with two domains (**Paper II**). In both cases, the V domains are followed by a transmembrane region, a connecting peptide, and a cytoplasmic tail. The two domains in teleost pIgR correspond to [D1] and [D5] of the mammalian counterparts. Teleost pIgR polypeptides were identified on the basis of homology to the mammalian counterparts, and also share some functional properties, even though their expression is not restricted to mucosal tissues (discussed in **paper II**). However, there is difficulty in establishing homology because of differences in the domain structures. Teleost pIgR, in addition to being shorter, share very low amino acid sequence identity to pIgR of other vertebrates. Moreover, **Paper II** identified structurally and phylogenetically similar protein products in Atlantic salmon. Teleost pIgR also share substantial percent identities to the V domains of CD300A molecules, despite sharing a very low overall amino acid sequence identity. Searching by the IMGT/DomainGapAlign (http://www.imgt.org/3Dstructure-

DB/cgi/DomainGapAlign.cgi) tool (Ehrenmann et al., 2010) using [D1] and [D2] of Salsal pIgR and Salsal pIgRL also results in CD300A hits on top. There is also some similarity in the connecting region (eg. *O*-glycosylation). However, BLAST search clearly indicated that the best hits are pIgRs. Two things must be clear here. Firstly, as CD300 molecules contain only one domain, their overall identity is low and they don't appear in BLAST searches. Secondly, pIgR do not possess activating or inhibitory motifs which is a typical feature of CD300 molecules (Clark et al., 2009). However, teleost pIgR V domains have characteristic disulphide bridge topology (Cx_7C motif) that makes them resemble the mammalian pIgR [D1], and V domains of salmon CLM (partly), human CD300A, FcαμR, and NKp44 (Fig. 4).

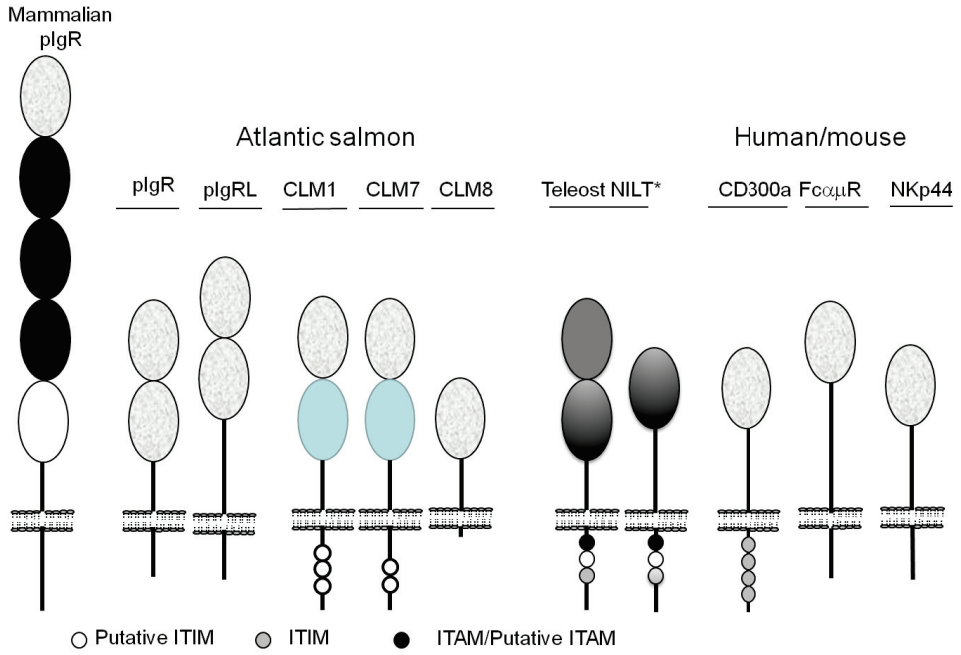


Fig. 4. Schematic representation comparing the main features of pIgR, pIgRL, and CLM V domains with other molecules. Mammalian pIgR contains 5 extracellular domains, with [D1] and [D5] being the corresponding domains of teleost counterparts. Domains with Cx_7C motifs are shown in light gray texture. Salmon pIgR, pIgRL, CLM (partly), human CD300, FcαμR, and NKp44 V domains have Cx_7C motifs which make them resemble the mammalian [D1]. Cytoplasmic signaling regions are indicated. *There are diverse NILT genes in teleosts with varying number and composition of V-like domains and signaling motifs.

Spleen tissue was used to isolate Salsal pIgR and Salsal pIgRL cDNA. To check if cDNA of pIgR varies among different tissues (spleen, skin, gut or liver), EST sequences of Atlantic salmon pIgR as well as trout pIgR were compared for the presence of single nucleotide polymorphisms (SNPs) or deletions and insertions. No differences in pIgR sequences from muscle, spleen, thymus, and 'mixed tissue' were found. However, a tandem repeat in the connecting region differs in pIgR cDNA from Norway and Canada, as well as between salmon and trout pIgR.

CMRF35-like molecules (CLM), which are designated as CD300, are a family of proteins having 7 members in humans and 9 members in mouse (Clark et al., 2009). In Atlantic salmon, they have been reported to GenBank as CLM1, CLM7, and CLM8 (Leong et al. 2010), and their in silico analysis has been presented in **Paper II** (and Appendix 3 A-C). CLM1 and CLM7 have a similar domain structure to that of teleost pIgR, but owing to the presence of putative ITIM in their cytoplasmic region, it is likely that they belong to the inhibitory CD300 members (CD300A and CD300F) in humans (Cantoni et al., 1999; Clark et al., 2009; Shi et al., 2006). BLAST search showed that CLM8 in salmon is most similar to CLM8 in rats but doesn't have the inhibitory motifs of rat CLM8: this indicates the possibility of splice variants or domain deletions. Moreover, Clark et al. (2009) emphasised the fact that these molecules (CD300 versus CLM) are not necessarily functional orthologues; for example, the CD300c gene orthologue is CLM6 but CD300c is more functionally similar to CLM4 (Reviewed in (Clark et al., 2009)). It should be noted that these molecules are complex and their naming is not yet clearly standardised.

The two pIgR-like protein sequences contain a connecting region rich in polar amino acids serine and threonine (comprising ~28% of the connecting region) which are very prone to posttranslational modifications such as *O*-linked glycosylation and phosphorylation. The two sequences, however, show some differences; this region is restricted to the connecting region and considerably longer in Salsal pIgRL (**Paper II**). The connecting region in salmon CLM7 (21% of the region) and CLM8 (27%) also contain these residues. Interestingly, the presence of these amino acids is correlated with a longer connecting region (Appendix 3 A-C), supporting the idea that these *O*-glycosylation residues help maintain a longer stalk structure. In contrast, in silico analysis revealed that mammalian pIgR barely contains these residues. This also poses a question because *O*-glycosylation causes resistance against proteolytic

cleavage (Kim et al., 1994), which is a crucial step for delivering secretory Igs to mucosal surfaces (Asano et al., 2004; Kaetzel, 2005). The above observations, added with differences in the expression patterns of mammalian and teleost (putative) pIgRs, implies important differences with regard to possible antibody transport mechanisms between mammals and teleosts.

4.6. Mucosal antibodies and pIgR

In mammals, mucosal immunity is mediated mainly by secretory IgA, and it is accomplished by the mucosal B cell system and secretory component (SC) of polymeric Ig receptor (pIgR) (Brandtzaeg, 1995). In teleost fish, IgM is the predominant antibody present in mucosal immune tissues. Very recent findings indicate that IgT, an antibody exclusive to teleosts, might have a special role in mucosal immune responses (Zhang et al., 2010; Zhang et al., 2011). In common carp, IgZ2 (a chimera molecule composed of μ1 and ζ4) shows preferential expression at mucosal sites (Ryo et al., 2010). In cutaneous mucus of teleosts, B cells and antibody secreting cells have been identified (Dickerson and Clark, 1998; Hatten et al., 2001; Zhao et al., 2008). In gut mucus, however, little data is available on the presence of Igs (Hatten et al., 2001; Rombout et al., 2011). The strong proteolytic environment in the gut mucus may account for this (Hatten et al., 2001). In this regard, it is worth noting that pIgR at mucosal surfaces of mammals has a great role of protecting Igs from proteolytic degradation (Kaetzel, 2005). In mammals, secreted IgD is also involved in mucosal immunity, and in channel catfish $IgM^{-}IgD^{+}$ cells were described at mucosal surfaces suggesting that catfish secreted IgD might have mucosal immune functions (Edholm et al., 2010).

In mammals, following the binding of IgA to pIgR, most of the IgA involved in immunity, produced locally at submucosal sites, is transported into external secretions as a pIgR-IgA complex (Kaetzel, 2005). Then, the secretory component is cleaved. Free SC and IgA play here a protective role (Fig 5). Likewise, the presence of IgM and IgT on mucosal surfaces of teleosts depicts local production of Igs or the presence of associated transcytosis receptors, mainly of pIgR. A possible pIgR counterpart has been identified in different fish species, including Atlantic salmon (Feng et al., 2009; Hamuro et al., 2007; Rombout et al., 2008; Zhang et al., 2010). Even though the functional aspects of teleost pIgR particularly the

association of the secretory component (SC) with poly Igs has not been clearly described, pIgR and IgT/IgM mediated mucosal defence is possible.

Studies show that mucosal antibodies are locally produced, which is evidenced partly because production of mucosal antibodies is independent of that of levels in serum (Lobb and Clem, 1981a; Rombout et al., 1993). Structural and functional differences (e.g. differences in immunoreactivity to a MAb) between serum and mucus Igs in carp depicted that they are independent (Rombout et al., 1993). Migration of antibodies is one possibility, but this has not been confirmed by *in vivo* functional studies, which is partly hampered by the availability of antibodies that can identify relevant Abs. Due to possible physiological barriers, polymeric high molecular weight Abs such as IgM may not easily migrate into the skin mucus, which make immunisation difficult (Dickerson and Clark, 1998; Lin et al., 1996).

Even though the levels of antibodies at mucosal tissues of teleost fish are low compared to the mammalian counterparts, this magnitude is dynamic and increases enormously with infection or immunization (Zhang et al., 2010; Zhao et al., 2008). For some ectoparasites such as *I. multifiliis*, it has been shown that antibodies play a crucial role in immune exclusion of the parasite both from skin and gill tissues. Presence of IgT positive cells in the epithelial lining of the gill lamellae of rainbow trout was documented in one study (Olsen et al., 2011). Another study documented IgT and IgM binding to surface structures of the parasite *I. multifiliis* early during infection in the gills of immune rainbow trout (von Gersdorff Jorgensen et al., 2011). The target antigens of this parasite are surface proteins called immobilizing antigens or i-antigens to which mucosal antibodies will bind eventually leading to removal of the parasite (Dickerson and Clark, 1998; Lin et al., 1996). In *L. salmonis*, however, the target antigens as well as the protective mechanisms which lead to the exclusion of the parasite are not known. Transcriptomic data in **paper III** suggested involvement of IgT and IgM antibodies. Zhang et al. (2010) also showed the role of IgT in anti-parasitic defence against gut mucosal parasites. It is thus plausible to assume that antibodies are good candidates of immune effector molecules against parasites. However, involvement of other immune factors is possible (see Section 4.7), and this is something to be dealt with in detail in future studies.

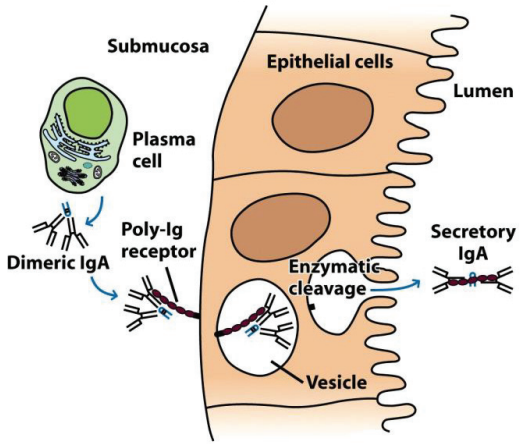


Fig. 5. Formation of secretory IgA in mammals. Following the binding of IgA to pIgR, most of the IgA involved in immunity, produced locally at submucosal sites, is transported into external secretions as a pIgR-IgA complex (Kaetzel, 2005). Then, the secretory component is cleaved at the lumen. Here, Free SC and IgA play innate and adaptive immune functions. Source: Kindt et al. (2007)

4.7. Host parasite interactions at mucosal surfaces: the case of *L. salmonis*

L. salmonis are host specific ectoparasitic copepods known to affect salmonid fish, and cause significant losses. However, there is considerable inter-species variation in the immunological responses and ability of salmonids to reject the parasite. **Paper III** addressed transcriptomic (microarray and qPCR) immune responses of Atlantic salmon during the time window encompassing the copepod and chalimus stages of lice development.

The fact that Atlantic salmon is more susceptible to infections by *L. salmonis*, compared to closely related species, such as sockeye and coho salmon, is well established. A possible evidence for this is the failure of Atlantic salmon to clear infections, while resistant salmonids get rid of lice few days after challenge. Weak inflammation at the site of attachment was regarded as a plausible explanation for this (reviewed in (Wagner et al., 2008)). Susceptibility of Atlantic salmon to *L. salmonis* infections may also be due to the composition of skin mucosal agents that block or stimulate the release of proteases and other agents to the skin (Fast et al., 2003). Lice produce prostaglandin E synthase (PGE2), trypsin-like proteases, and other products that suppress the immune system of Atlantic salmon (Firth

et al., 2000). Significant reduction of oxidative and phagocytic activities of macrophages (Mustafa et al., 2000), and reduced transcription of IL-1β and COX-2 in lice infected salmon has been reported (Fast et al., 2007; Fast et al., 2006). Experiments conducted on LPS stimulated Atlantic salmon macrophage-like SHK-1 cells have shown that PGE(2) inhibits the expression of MHC I and II and pro-inflammatory cytokine interleukin-1 beta (Fast et al., 2005). PGE2 is a potent vasodilator found on most parasite secretions and has a significant effect on immunity; for example, prostaglandin dependent suppression of lymphocyte proliferation is documented (Papadogiannakis et al., 1985). The aforementioned factors indicate that early responses seem to be critical in this host parasite system. In addition, as the louse depends on the host after its attachment, clearing infections by immobilising the parasites at early stages would be a great advantage to the host, which barely happens in Atlantic salmon. **Paper III** thus addressed early responses 1-15 days post challenge.

Despite the high lice load found by the end of the experiment (15 dpi), transcriptomic analyses showed dramatic gene expression changes immediately after infection in the target sites and in the spleen, indicative of rapid local and systemic sensing. This suggested that low resistance of Atlantic salmon to lice appears to be accounted for by the character of immune response rather than the scale of the response. Interestingly, most of the differentially expressed genes are not those that are commonly included in studies of salmon immunity. Thus, it was difficult to detect these changes based on the candidate genes approach. An unexpected finding was involvement of splenic proteases. Dramatic expression changes of a group of genes encoding functionally related proteins implied their important role, which remains completely unknown.

4.7.1. The copepodid-chalimus transition is a critical stage in host immune gene regulation

Salmon louse develops through ten stages. Even though the pre-adult and adult louse can move unrestricted on the host surface, and subsequently result in an increased virulence, the copepodid is the infectious stage of *L. salmonis* and is able to settle and recognise a relevant host. During the copepodid-chalimus transition, the louse penetrates the salmon epidermis, and the response at this stage appears to be very critical. Interestingly, the transcriptome changes observed at this transition supported this phenomenon (**Paper III**). The findings clearly depicted early sensing at day one post infection and subsequent regulation of a diverse

array of genes. A remarkable finding was the presence of groups of genes with similar profiles: early up (or down)-regulation followed by strong down (or up)-regulation between days 5 and 10 both in skin and spleen. The large switch during the copepodid-chalimus transition (5 and 10 dpi) suggested that this is a possible time-window where modulation of the host immune response occurs.

4.7.2. Early expression of putative T cell (-like) transcripts and their regulators

Transcriptomic responses to lice were also characterised by an early increase of T cell specific transcripts in the skin, which totally ceases after day 5. Their functional roles remain to be identified, while a number of questions were raised including from where they originate (head kidney or the thymus) or if they are locally produced. The former possibility is plausible because a number of T cell related transcripts have shown depletion in parallel. On the other hand, activation of RAG and other regulators of transcription of T cells suggest that they can be locally produced. The next question is what happened to those transcripts after 5 dpi? One possibility is that they are blocked by the parasite. A good example here is the parasite *Trypanosoma evansi* induces tyrosine phosphatase mediated killing of lymphocytes (Antoine-Moussiaux et al., 2009). Due to the enormous abilities of parasites to trigger highly polarized CD4+ T cell subset responses, which depends on the host genetic background, T lymphocyte-parasite interactions have been regarded as crucial subjects of research (Jankovic et al., 2001; Sher and Coffman, 1992).

4.7.3. Splenic proteases: important anti-louse strategies or vice-versa?

Somewhat unexpectedly, in the spleen, a group of related transcripts encoding for secretory proteases showed an early (1-5 dpi) up-regulation followed by strong down-regulation in the second phase (10-15 dpi) (**Paper III,** Appendix 4). In parallel with the up-regulation of proteases day 1-5 dpi in the spleen, some of their precursor molecules also increased in the skin, which go off completely after day 10. Furthermore, a large number of transcripts for secretory proteins were down-regulated in parallel with proteases at 10 dpi. It is tempting to speculate that the first phase can be stimulation where proteolytic enzymes are produced, which migrate to the target site in the second phase. Nevertheless, there are important questions which remain unanswered, including where these enzymes reside and what their roles are, and where they migrate to? It can be deduced, however, that production of

extracellular proteases in spleen is an important anti-louse strategy. In invertebrates, a serine protease cascade (analogous to the complement system in mammals) is responsible for initiating an innate immune response following parasitic infection (Kanost et al., 2004; Volz et al., 2005). Conversely, the observation in *L. salmonis* can also be an anti-host strategy by the parasite. Apart from producing these proteases themselves, it is suspected that salmon louse have the ability to stimulate the release of such enzymes from the host (in this case the spleen) which possibly are transported to the skin/mucus surface. Studies show that in response to infection with salmon louse, low molecular weight (18-25 kDa) trypsin-like proteases were detected in the skin mucus of infected fish, but not in healthy fish (Ross et al., 2000). These are suggested to be produced by louse to aid feeding and suppress the immune system of Atlantic salmon (Firth et al., 2000). However, it is not possible to rule out the possibilities that these proteases can be of host origin, migrated from the spleen (or other organs) to the skin/mucus. Nevertheless, this deserves a thorough biochemical investigation at protein level including immunohistochemistry and enzyme assays.

It is worth mentioning here that the target antigens for many parasites are (cysteine) proteases: e.g. in *T. cruzi* causing Chagas disease (Cazorla et al., 2008; Doyle et al., 2007; Stempin et al., 2008), and in *Schistosomiasis mansoni* which causes human schistosomiasis (Abdulla et al., 2007). These immunomodulatory peptides inhibit a number of immune mechanisms including macrophage functions. Cysteine proteases also represent potential antigens in anti-parasitic DNA vaccines (Jorgensen and Buchmann, 2011). Proteases thus seem to occupy a central role in future host-parasite interaction studies and thus deserve a closer investigation.

The overall pattern of transcriptomic responses (**paper III**) indicated regulation of group of genes, such as transcripts encoding for splenic proteases, secretory proteins, ECM proteins, pro-inflammatory molecules, etc., all indicative of a holistic response rather than a few special genes. Besides, an opposite regulation in virus responsive genes suggested a connection between louse and viral infection, which requires further studies including a co-challenge experiment.

Finally, the question of how louse modulates the mucosal immune components of Atlantic salmon has been postulated. 1) *By hampering local production/recruitment of T lymphocytes*.

Transient (1-5 dpi) increase of T cell receptor alpha, CD4-1, and possible regulators of lymphocyte differentiation didn't last after 5 dpi indicating possible blockage of lymphocytes by the parasite. 2) *Decreased abundance of transcripts involved in antigen presentation*. This is perhaps an important indication of immune modulation observed especially in head kidney and skin, as witnessed by decreased transcript levels of MHCI and B2M consistently throughout the experiment. In addition, a number of heat shock proteins (HSPs) are down regulated early in the skin and the spleen. In humans, it has been shown that HSPs play a role in delivering antigens to antigen presenting cells (Nishikawa et al., 2008). The down regulation of these transcripts may thus imply that the fish might not be able to mount the necessary responses. 3) *Stimulation of production and or depletion of splenic proteases* as discussed above. In parallel examination of host immune responses both in resistant and susceptible species is thus crucial to analyse host immune modulation in terms of the above parameters.

5. Conclusions and future perspectives

Enhancing effective protection against pathogens is a crucial but challenging task, and requires a deeper understanding of the immune system of the host, and the host-pathogen interactions therein. In this study, information on components of the mucosal immune system in Atlantic salmon has been gathered including design of qPCR assays that consider immunoglobulin diversity in salmon; this can greatly assist monitoring of Ig gene expression and immune responses. Secondly, pIgR like molecules and their possible role in mucosal immunity have been revealed. Thirdly, transcriptional responses of Atlantic salmon to an economically important ectoparasite *L. salmonis* were determined.

In *L. salmonis*, even though the target antigens as well as the protective mechanisms which lead to the exclusion of the parasite are not known, from other ectoparasites such as *I. multifiliis*, it can be deduced that antibodies can have potential roles. Transcriptomic data also suggested involvement of IgT and IgM antibodies. The role of IgT in anti-parasitic defence against gut mucosal parasites has also been documented. Nevertheless, it must be emphasised that the concentration of mucosal antibodies in fish is still only a fraction of that in mammals. This magnitude, however, is dynamic and increases enormously with infection or immunization. On the other hand, due to a potential anti-host effects of proteolytic enzymes, coupled with the unexpected transcriptomic fluctuations of splenic proteases observed in response to *L. salmonis* infection, it is not possible to exclude involvement of other immune factors as well, and this is something to be dealt with in detail in future studies. An important aspect being investigated in parallel is regulation of genes in *L. salmonis* during the same time-window of infection. This will enable the identification of possible antigens of the louse that can be targeted for vaccination. A recent study in related species *Caligus rogercresseyi* documented that a novel gene named my32 (having a high identity to *L. salmonis* akirin-2) has been found to be a promising target for vaccination against sea lice (Carpio et al., 2011). Study of the immune response of Atlantic salmon in the absence of immunomodulatory substances can also be done by blocking the responsible gene/s (gene silencing) by using molecular technologies such as RNA interference (RNAi). This method has been found working in *L. salmonis* where knock down of putative PGE synthase has been tested

(Campbell et al., 2009). A compilation of genomic resources is now available to study the biology, immunology, and other aspects of sea lice (Yasuike et al., 2011).

The completion of genome sequencing for many species now allows the use of modern techniques within proteomics, and functional studies which previously were restricted to human immunology: for systematizing and characterizing immune genes, and developing tools to study the immune system in different fish species such as in (http://www.umass.edu/vetimm/catfish/index.html).

The present work has provided important data that further studies can be based on, and identified a possible time window during infection which appear to be essential with regard to immune modulation. Altogether, the information gathered in this thesis is believed to add to the existing knowledge in the immunology of infection of Atlantic salmon that will assist further research to the effective control of pathogens in general, and *L. salmonis* in particular.

References

Abdulla, M.H., Lim, K.C., Sajid, M., McKerrow, J.H., and Caffrey, C.R. (2007). Schistosomiasis mansoni: Novel chemotherapy using a cysteine protease inhibitor. Plos Medicine *4*, 130-138.

Alvarez-Pellitero, P. (2008). Fish immunity and parasite infections: from innate immunity to immunoprophylactic prospects. Vet. Immunol. Immunopathol. *126*, 171-198.

Antoine-Moussiaux, N., Cornet, A., Cornet, F., Glineur, S., Dermine, M., and Desmecht, D. (2009). A non-cytosolic protein of Trypanosoma evansi induces CD45-dependent lymphocyte death. PLoS One *4*, e5728.

Asano, M., Takenouchi-Ohkubo, N., Matsumoto, N., Ogura, Y., Nomura, H., Suguro, H., and Moro, I. (2004). Multiple cleavage sites for polymeric immunoglobulin receptor. Immunology *112*, 583-589.

Bengten, E., Quiniou, S.M.A., Stuge, T.B., Katagiri, T., Miller, N.W., Clem, L.W., Warr, G.W., and Wilson, M. (2002). The IgH locus of the channel catfish, Ictalurus punctatus, contains multiple constant region gene sequences: Different genes encode heavy chains of membrane and secreted IgD. J. Immunol. *169*, 2488-2497.

Bernard, D., Six, A., Rigottier-Gois, L., Messiaen, S., Chilmonczyk, S., Quillet, E., Boudinot, P., and Benmansour, A. (2006). Phenotypic and functional similarity of gut intraepithelial and systemic T cells in a teleost fish. J. Immunol. *176*, 3942-3949.

Berstein, R.M., Schluter, S.F., Shen, S., and Marchalonis, J.J. (1996). A new high molecular weight immunoglobulin class from the carcharhine shark: implications for the properties of the primordial immunoglobulin. Proc. Natl. Acad. Sci. U. S. A. *93*, 3289-3293.

Braathen, R., Hohman, V.S., Brandtzaeg, P., and Johansen, F.E. (2007). Secretory antibody formation: conserved binding interactions between J chain and polymeric Ig receptor from humans and amphibians. J. Immunol. *178*, 1589-1597.

Brandtzaeg, P. (1995). Basic Mechanisms of mucosal immunity-a major adaptive defense system. The immunologist *3*, 89-96.

Brandtzaeg, P., Kiyono, H., Pabst, R., and Russell, M.W. (2008). Terminology: nomenclature of mucosa-associated lymphoid tissue. Mucosal Immunol. *1*, 31-37.

Brenner, S. (2000). Genomics. The end of the beginning. Science *287*, 2173-2174.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L*., et al.* (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. *55*, 611-622.

Campbell, E.M., Pert, C.C., and Bowman, A.S. (2009). RNA-interference methods for gene-knockdown in the sea louse, Lepeophtheirus salmonis: studies on a putative prostaglandin E synthase. Parasitology *136*, 867-874.

Cantoni, C., Bottino, C., Augugliaro, R., Morelli, L., Marcenaro, E., Castriconi, R., Vitale, M., Pende, D., Sivori, S., Millo, R*., et al.* (1999). Molecular and functional characterization of IRp60, a member of the immunoglobulin superfamily that functions as an inhibitory receptor in human NK cells. Eur. J. Immunol. *29*, 3148-3159.

Carpio, Y., Basabe, L., Acosta, J., Rodriguez, A., Mendoza, A., Lisperger, A., Zamorano, E., Gonzalez, M., Rivas, M., Contreras, S*., et al.* (2011). Novel gene isolated from Caligus rogercresseyi: a promising target for vaccine development against sea lice. Vaccine *29*, 2810-2820.

Castillo-Briceno, P., Sepulcre, M.P., Chaves-Pozo, E., Meseguer, J., Garcia-Ayala, A., and Mulero, V. (2009). Collagen regulates the activation of professional phagocytes of the teleost fish gilthead seabream. Mol. Immunol. *46*, 1409-1415.

Cazorla, S.I., Frank, F.M., Becker, P.D., Corral, R.S., Guzman, C.A., and Malchiodi, E.L. (2008). Prime-boost immunization with cruzipain co-administered with MALP-2 triggers a protective immune response able to decrease parasite burden and tissue injury in an experimental Trypanosoma cruzi infection model. Vaccine *26*, 1999-2009.

Chen, K., and Cerutti, A. (2010). New insights into the enigma of immunoglobulin D. Immunol. Rev. *237*, 160-179.

Chen, K., and Cerutti, A. (2011). The function and regulation of immunoglobulin D. Curr. Opin. Immunol.

Chen, K., Xu, W., Wilson, M., He, B., Miller, N.W., Bengten, E., Edholm, E.S., Santini, P.A., Rath, P., Chiu, A.*, et al.* (2009). Immunoglobulin D enhances immune surveillance by activating antimicrobial, proinflammatory and B cell-stimulating programs in basophils. Nat. Immunol. *10*, 889-898.

Clark, E.A., and Ledbetter, J.A. (1994). How B and T cells talk to each other. Nature *367*, 425-428.

Clark, G.J., Ju, X., Tate, C., and Hart, D.N. (2009). The CD300 family of molecules are evolutionarily significant regulators of leukocyte functions. Trends Immunol. *30*, 209-217.

Danilova, N., and Amemiya, C.T. (2009). Going adaptive: the saga of antibodies. Ann. N. Y. Acad. Sci. *1168*, 130-155.

Danilova, N., Bussmann, J., Jekosch, K., and Steiner, L.A. (2005). The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z Nat. Immunol. *6*, 295-302.

Deza, F.G., Espinel, C.S., and Mompo, S.M. (2009). Presence of a unique IgT on the IGH locus in Gasterosteus aculeatus and the very recent generation of a repertoire of VH genes. Dev. Comp. Immunol. *34*, 114-122.

Dickerson, H., and Clark, T. (1998). Ichthyophthirius multifiliis: a model of cutaneous infection and immunity in fishes. Immunol. Rev. *166*, 377-384.

Dooley, H., and Flajnik, M.F. (2006). Antibody repertoire development in cartilaginous fish. Dev. Comp. Immunol. *30*, 43-56.

Doyle, P.S., Zhou, Y.M., Engel, J.C., and McKerrow, J.H. (2007). A cysteine protease inhibitor cures Chagas' disease in an immunodeficient-mouse model of infection. Antimicrob. Agents Chemother. *51*, 3932-3939.

Easy, R.H., and Ross, N.W. (2009). Changes in Atlantic salmon (Salmo salar) epidermal mucus protein composition profiles following infection with sea lice (Lepeophtheirus salmonis). Comp. Biochem. Physiol. D. Genomics Proteomics *4*, 159-167.

Edholm, E.S., Bengten, E., Stafford, J.L., Sahoo, M., Taylor, E.B., Miller, N.W., and Wilson, M. (2010). Identification of two IgD+ B cell populations in channel catfish, Ictalurus punctatus. J. Immunol. *185*, 4082-4094.

Ehrenmann, F., Kaas, Q., and Lefranc, M.P. (2010). IMGT/3Dstructure-DB and IMGT/DomainGapAlign: a database and a tool for immunoglobulins or antibodies, T cell receptors, MHC, IgSF and MhcSF. Nucleic Acids Res. *38*, D301-307.

Ellis, A.E. (2001). Innate host defense mechanisms of fish against viruses and bacteria. Dev Comp Immunol *25*, 827-839.

Fagarasan, S., Kawamoto, S., Kanagawa, O., and Suzuki, K. (2010). Adaptive immune regulation in the gut: T cell-dependent and T cell-independent IgA synthesis. Annu. Rev. Immunol. *28*, 243-273.

Fast, M.D., Burka, J.F., Johnson, S.C., and Ross, N.W. (2003). Enzymes released from *Lepeophtheirus salmonis* in response to mucus from different salmonids. J. Parasitol. *89*, 7-13.

Fast, M.D., Johnson, S.C., Eddy, T.D., Pinto, D., and Ross, N.W. (2007). *Lepeophtheirus salmonis* secretory/excretory products and their effects on Atlantic salmon immune gene regulation. Parasite Immunol. *29*, 179-189.

Fast, M.D., Muise, D.M., Easy, R.E., Ross, N.W., and Johnson, S.C. (2006). The effects of *Lepeophtheirus salmonis* infections on the stress response and immunological status of Atlantic salmon (*Salmo salar*). Fish Shellfish Immunol. *21*, 228-241.

Fast, M.D., Ross, N.W., and Johnson, S.C. (2005). Prostaglandin E(2) modulation of gene expression in an Atlantic salmon (Salmo salar) macrophage-like cell line (SHK-1). Dev. Comp. Immunol. *29*, 951-963.

Fast, M.D., Ross, N.W., Mustafa, A., Sims, D.E., Johnson, S.C., Conboy, G.A., Speare, D.J., Johnson, G., and Burka, J.F. (2002). Susceptibility of rainbow trout *Oncorhynchus mykiss*, Atlantic salmon *Salmo salar* and coho salmon *Oncorhynchus kisutch* to experimental infection with sea lice *Lepeophtheirus salmonis*. Dis. Aquat. Organ. *52*, 57-68.

Feng, L.N., Lu, D.Q., Bei, J.X., Chen, J.L., Liu, Y., Zhang, Y., Liu, X.C., Meng, Z.N., Wang, L., and Lin, H.R. (2009). Molecular cloning and functional analysis of polymeric immunoglobulin receptor gene in orange-spotted grouper (Epinephelus coioides). Comp. Biochem. Physiol. B. Biochem. Mol. Biol. *154*, 282-289.

Firth, K.J., Johnson, S.C., and Ross, N.W. (2000). Characterization of proteases in the skin mucus of Atlantic salmon (*Salmo salar*) infected with the salmon louse (*Lepeophtheirus salmonis*) and in whole-body louse homogenate. J. Parasitol. *86*, 1199-1205.

Fuglem, B., Jirillo, E., Bjerkas, I., Kiyono, H., Nochi, T., Yuki, Y., Raida, M., Fischer, U., and Koppang, E.O. (2010). Antigen-sampling cells in the salmonid intestinal epithelium. Dev. Comp. Immunol. *34*, 768-774.

Garcia de Leaniz, C., Fleming, I.A., Einum, S., Verspoor, E., Jordan, W.C., Consuegra, S., Aubin-Horth, N., Lajus, D., Letcher, B.H., Youngson, A.F*., et al.* (2007). A critical review of adaptive genetic variation in Atlantic salmon: implications for conservation. Biol. Rev. Camb. Philos. Soc. *82*, 173-211.

Gonzalez, S.F., Buchmann, K., and Nielsen, M.E. (2007a). Real-time gene expression analysis in carp (Cyprinus carpio L.) skin: inflammatory responses caused by the ectoparasite Ichthyophthirius multifiliis. Fish Shellfish Immunol. *22*, 641-650.

Gonzalez, S.F., Huising, M.O., Stakauskas, R., Forlenza, M., Lidy Verburg-van Kemenade, B.M., Buchmann, K., Nielsen, M.E., and Wiegertjes, G.F. (2007b). Real-time gene expression analysis in carp (Cyprinus carpio L.) skin: inflammatory responses to injury mimicking infection with ectoparasites. Dev. Comp. Immunol. *31*, 244-254.

Grosschedl, R., and Baltimore, D. (1985). Cell-type specificity of immunoglobulin gene expression is regulated by at least three DNA sequence elements. Cell *41*, 885-897.

Hadge, D., and Ambrosius, H. (1984). Evolution of low molecular weight immunoglobulins--IV. IgY-like immunoglobulins of birds, reptiles and amphibians, precursors of mammalian IgA. Mol. Immunol. *21*, 699-707.

Hamuro, K., Suetake, H., Saha, N.R., Kikuchi, K., and Suzuki, Y. (2007). A teleost polymeric Ig receptor exhibiting two Ig-like domains transports tetrameric IgM into the skin. J. Immunol. *178*, 5682-5689.

Hansen, J.D., Landis, E.D., and Phillips, R.B. (2005). Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. Proc. Natl. Acad. Sci. U. S. A. *102*, 6919-6924.

Hatten, F., Fredriksen, A., Hordvik, I., and Endresen, C. (2001). Presence of IgM in cutaneous mucus, but not in gut mucus of Atlantic salmon, Salmo salar. Serum IgM is rapidly degraded when added to gut mucus. Fish Shellfish Immunol. *11*, 257-268.

Haugarvoll, E., Bjerkas, I., Nowak, B.F., Hordvik, I., and Koppang, E.O. (2008). Identification and characterization of a novel intraepithelial lymphoid tissue in the gills of Atlantic salmon. J. Anat. *213*, 202-209.

Hikima, J., Jung, T.S., and Aoki, T. (2011). Immunoglobulin genes and their transcriptional control in teleosts. Dev. Comp. Immunol. *35*, 924-936.

Hordvik, I. (1998). The impact of ancestral tetraploidy on antibody heterogeneity in salmonid fishes. Immunol. Rev. *166*, 153-157.

Hordvik, I., Berven, F.S., Solem, S.T., Hatten, F., and Endresen, C. (2002). Analysis of two IgM isotypes in Atlantic salmon and brown trout. Mol. Immunol. *39*, 313-321.

Hordvik, I., Thevarajan, J., Samdal, I., Bastani, N., and Krossoy, B. (1999). Molecular cloning and phylogenetic analysis of the Atlantic salmon immunoglobulin D gene. Scand. J. Immunol. *50*, 202-210.

Janeway, C.A., Jr., and Medzhitov, R. (2002). Innate immune recognition. Annu. Rev. Immunol. *20*, 197-216.

Jankovic, D., Sher, A., and Yap, G. (2001). Th1/Th2 effector choice in parasitic infection: decision making by committee. Curr. Opin. Immunol. *13*, 403-409.

Jones, S., Kim, E., and Dawe, S. (2006). Experimental infections with Lepeophtheirus salmonis (Kroyer) on threespine sticklebacks, Gasterosteus aculeatus L., and juvenile Pacific salmon, Oncorhynchus spp. J Fish Dis *29*, 489-495.

Jones, S.R. (2001). The occurrence and mechanisms of innate immunity against parasites in fish. Dev. Comp. Immunol. *25*, 841-852.

Jones, S.R., Fast, M.D., Johnson, S.C., and Groman, D.B. (2007). Differential rejection of salmon lice by pink and chum salmon: disease consequences and expression of proinflammatory genes. Dis. Aquat. Organ. *75*, 229-238.

Jorgensen, L.G., and Buchmann, K. (2011). Cysteine proteases as potential antigens in antiparasitic DNA vaccines. Vaccine *29*, 5575-5583.

Kaetzel, C.S. (2005). The polymeric immunoglobulin receptor: bridging innate and adaptive immune responses at mucosal surfaces. Immunol. Rev. *206*, 83-99.

Kamil, A., Falk, K., Sharma, A., Raae, A., Berven, F., Koppang, E.O., and Hordvik, I. (2011). A monoclonal antibody distinguishes between two IgM heavy chain isotypes in Atlantic salmon and brown trout: protein characterization, 3D modeling and epitope mapping. Mol. Immunol. *48*, 1859-1867.

Kania, P.W., Evensen, O., Larsen, T.B., and Buchmann, K. (2010). Molecular and immunohistochemical studies on epidermal responses in Atlantic salmon Salmo salar L. induced by Gyrodactylus salaris Malmberg, 1957. J. Helminthol. *84*, 166-172.

Kanost, M.R., Jiang, H., and Yu, X.Q. (2004). Innate immune responses of a lepidopteran insect, Manduca sexta. Immunol. Rev. *198*, 97-105.

Kim, H., Yamaguchi, Y., Masuda, K., Matsunaga, C., Yamamoto, K., Irimura, T., Takahashi, N., Kato, K., and Arata, Y. (1994). O-glycosylation in hinge region of mouse immunoglobulin G2b. J. Biol. Chem. *269*, 12345-12350.

Kindt, T.J., Goldsby, R.A., Osborne, B.A., and Kuby, J. (2007). Kuby immunology, 6th edn (New York: W.H. Freeman).

Koop, B.F., von Schalburg, K.R., Leong, J., Walker, N., Lieph, R., Cooper, G.A., Robb, A., Beetz-Sargent, M., Holt, R.A., Moore, R.*, et al.* (2008). A salmonid EST genomic study: genes, duplications, phylogeny and microarrays. BMC Genomics *9*, 545.

Koppang, E.O., Fischer, U., Moore, L., Tranulis, M.A., Dijkstra, J.M., Kollner, B., Aune, L., Jirillo, E., and Hordvik, I. (2010). Salmonid T cells assemble in the thymus, spleen and in novel interbranchial lymphoid tissue. J. Anat. *217*, 728-739.

Kraehenbuhl, J.P., and Neutra, M.R. (1992). Molecular and cellular basis of immune protection of mucosal surfaces. Physiol. Rev. *72*, 853-879.

Krajci, P., Kvale, D., Tasken, K., and Brandtzaeg, P. (1992). Molecular-Cloning and Exon-Intron Mapping of the Gene Encoding Human Transmembrane Secretory Component (the Poly-Ig Receptor). Eur. J. Immunol. *22*, 2309-2315.

Krasnov, A., Timmerhaus, G., Afanasyev, S., and Jorgensen, S.M. (2011). Development and assessment of oligonucleotide microarrays for Atlantic salmon (Salmo salar L.). Comp. Biochem. Physiol. D. Genomics Proteomics *6*, 31-38.

Kulseth, M.A., Krajci, P., Myklebost, O., and Rogne, S. (1995). Cloning and characterization of two forms of bovine polymeric immunoglobulin receptor cDNA. DNA Cell Biol. *14*, 251-256.

Lambolez, F., and Rocha, B. (2001). Immunology. A molecular gut reaction. Science *294*, 1848-1849.

Li, J., Barreda, D.R., Zhang, Y.A., Boshra, H., Gelman, A.E., LaPatra, S., Tort, L., and Sunyer, J.O. (2006). B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. Nat. Immunol. *7*, 1116-1124.

Lin, T.L., Clark, T.G., and Dickerson, H. (1996). Passive immunization of channel catfish (Ictalurus punctatus) against the ciliated protozoan parasite Ichthyophthirius multifiliis by use of murine monoclonal antibodies. Infect. Immun. *64*, 4085-4090.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods *25*, 402-408.

Lobb, C.J. (1987). Secretory Immunity Induced in Catfish, Ictalurus-Punctatus, Following Bath Immunization. Dev. Comp. Immunol. *11*, 727-738.

Lobb, C.J., and Clem, L.W. (1981a). The metabolic relationships of the immunoglobulins in fish serum, cutaneous mucus, and bile. J. Immunol. *127*, 1525-1529.

Lobb, C.J., and Clem, L.W. (1981b). Phylogeny of immunoglobulin structure and function. XI. Secretory immunoglobulins in the cutaneous mucus of the sheepshead, Archosargus probatocephalus. Dev. Comp. Immunol. *5*, 587-596.

Majalahti-Palviainen, T., Hirvinen, M., Tervonen, V., Ilves, M., Ruskoaho, H., and Vuolteenaho, O. (2000). Gene structure of a new cardiac peptide hormone: a model for heart-specific gene expression. Endocrinology *141*, 731-740.

Maki, J.L., and Dickerson, H.W. (2003). Systemic and cutaneous mucus antibody responses of channel catfish immunized against the protozoan parasite Ichthyophthirius multifiliis. Clin. Diagn. Lab. Immunol. *10*, 876-881.

Medzhitov, R. (2007). Recognition of microorganisms and activation of the immune response. Nature *449*, 819-826.

Medzhitov, R., and Janeway, C., Jr. (2000). Innate immune recognition: mechanisms and pathways. Immunol. Rev. *173*, 89-97.

Medzhitov, R., and Janeway, C.A., Jr. (1998). Innate immune recognition and control of adaptive immune responses. Semin. Immunol. *10*, 351-353.

Miller, N., Wilson, M., Bengten, E., Stuge, T., Warr, G., and Clem, W. (1998). Functional and molecular characterization of teleost leukocytes. Immunol. Rev. *166*, 187-197.

Mussmann, R., Du Pasquier, L., and Hsu, E. (1996). Is Xenopus IgX an analog of IgA? Eur. J. Immunol. *26*, 2823-2830.

Mustafa, A., MacWilliams, C., Fernandez, N., Matchett, K., Conboy, G.A., and Burka, J.F. (2000). Effects of sea lice (Lepeophtheirus salmonis Kroyer, 1837) infestation on

macrophage functions in Atlantic salmon (Salmo salar L.). Fish Shellfish Immunol. *10*, 47-59.

Nace, G., Evankovich, J., Eid, R., and Tsung, A. (2012). Dendritic cells and damage-associated molecular patterns: endogenous danger signals linking innate and adaptive immunity. J Innate Immun *4*, 6-15.

Nishikawa, M., Takemoto, S., and Takakura, Y. (2008). Heat shock protein derivatives for delivery of antigens to antigen presenting cells. Int J Pharm *354*, 23-27.

Norwegian Sea food Export Council (2010). Facts and figures for Norwegian sea food exports for 2009 (in Norwegian).

Olsen, M.M., Kania, P.W., Heinecke, R.D., Skjoedt, K., Rasmussen, K.J., and Buchmann, K. (2011). Cellular and humoral factors involved in the response of rainbow trout gills to Ichthyophthirius multifiliis infections: molecular and immunohistochemical studies. Fish Shellfish Immunol. *30*, 859-869.

Oppenheim, J.J., and Yang, D. (2005). Alarmins: chemotactic activators of immune responses. Curr Opin Immunol *17*, 359-365.

Ourth, D.D. (1980). Secretory IgM, lysozyme and lymphocytes in the skin mucus of the channel catfish, Ictalurus punctatus. Dev. Comp. Immunol. *4*, 65-74.

Pacifici, R., Carano, A., Santoro, S.A., Rifas, L., Jeffrey, J.J., Malone, J.D., McCracken, R., and Avioli, L.V. (1991). Bone matrix constituents stimulate interleukin-1 release from human blood mononuclear cells. J. Clin. Invest. *87*, 221-228.

Palaksha, K.J., Shin, G.W., Kim, Y.R., and Jung, T.S. (2008). Evaluation of non-specific immune components from the skin mucus of olive flounder (Paralichthys olivaceus). Fish & Shellfish Immunology *24*, 479-488.

Papadogiannakis, N., Johnsen, S.A., and Olding, L.B. (1985). Strong prostaglandin associated suppression of the proliferation of human maternal lymphocytes by neonatal lymphocytes linked to T versus T cell interactions and differential PGE2 sensitivity. Clin. Exp. Immunol. *61*, 125-134.

Pilstrom, L., and Bengten, E. (1996). Immunoglobulin in fish - Genes, expression and structure. Fish Shellfish Immunol. *6*, 243-262.

Piskurich, J.F., Blanchard, M.H., Youngman, K.R., France, J.A., and Kaetzel, C.S. (1995). Molecular cloning of the mouse polymeric Ig receptor. Functional regions of the molecule are conserved among five mammalian species. J. Immunol. *154*, 1735-1747.

Press, C.M., and Evensen, Ø. (1999). The morphology of the immune system in teleost fishes. Fish Shellfish Immunol. *9*, 309–318.

Rakers, S., Gebert, M., Uppalapati, S., Meyer, W., Maderson, P., Sell, A.F., Kruse, C., and Paus, R. (2010). 'Fish matters': the relevance of fish skin biology to investigative dermatology. Exp. Dermatol. *19*, 313-324.

Ramirez-Gomez, F., Greene, W., Rego, K., Hansen, J.D., Costa, G., Kataria, P., and Bromage, E.S. (2011). Discovery and Characterization of Secretory IgD in Rainbow Trout: Secretory IgD Is Produced through a Novel Splicing Mechanism. J. Immunol.

Rombout, J.H., Abelli, L., Picchietti, S., Scapigliati, G., and Kiron, V. (2011). Teleost intestinal immunology. Fish Shellfish Immunol. *31*, 616-626.

Rombout, J.H., Huttenhuis, H.B., Picchietti, S., and Scapigliati, G. (2005). Phylogeny and ontogeny of fish leucocytes. Fish Shellfish Immunol. *19*, 441-455.

Rombout, J.H., Taverne, N., van de Kamp, M., and Taverne-Thiele, A.J. (1993). Differences in mucus and serum immunoglobulin of carp (Cyprinus carpio L.). Dev. Comp. Immunol. *17*, 309-317.

Rombout, J.H., van der Tuin, S.J., Yang, G., Schopman, N., Mroczek, A., Hermsen, T., and Taverne-Thiele, J.J. (2008). Expression of the polymeric Immunoglobulin Receptor

(pIgR) in mucosal tissues of common carp (Cyprinus carpio L.). Fish Shellfish Immunol. *24*, 620-628.

Rombout, J.H.W.M., and Joosten, E.P.E.M. (1998). Immunology of fishes. In Handbook of Vertebrate Immunology, P.P. Pastoret, P. Griebel, H. Bazin, and A. Govaerts, eds. (London: Academic Press), pp. 1-43.

Ross, N.W., Firth, K.J., Wang, A., Burka, J.F., and Johnson, S.C. (2000). Changes in hydrolytic enzyme activities of naive Atlantic salmon Salmo salar skin mucus due to infection with the salmon louse Lepeophtheirus salmonis and cortisol implantation. Dis Aquat Organ *41*, 43-51.

Ryo, S., Wijdeven, R.H., Tyagi, A., Hermsen, T., Kono, T., Karunasagar, I., Rombout, J.H., Sakai, M., Verburg-van Kemenade, B.M., and Savan, R. (2010). Common carp have two subclasses of bonyfish specific antibody IgZ showing differential expression in response to infection. Dev. Comp. Immunol. *34*, 1183-1190.

Saito, H., Kanamori, Y., Takemori, T., Nariuchi, H., Kubota, E., Takahashi-Iwanaga, H., Iwanaga, T., and Ishikawa, H. (1998). Generation of intestinal T cells from progenitors residing in gut cryptopatches. Science *280*, 275-278.

Salinas, I., Zhang, Y.A., and Sunyer, J.O. (2011). Mucosal immunoglobulins and B cells of teleost fish. Dev. Comp. Immunol. *35*, 1346-1365.

Savan, R., Aman, A., Sato, K., Yamaguchi, R., and Sakai, M. (2005). Discovery of a new class of immunoglobulin heavy chain from fugu. Eur. J. Immunol. *35*, 3320-3331.

Schatz, D.G., Oettinger, M.A., and Schlissel, M.S. (1992). V(D)J recombination: molecular biology and regulation. Annu. Rev. Immunol. *10*, 359-383.

Schempp, C., Emde, M., and Wolfle, U. (2009). Dermatology in the Darwin anniversary. Part 1: Evolution of the integument. J Dtsch Dermatol Ges *7*, 750-757.

Sher, A., and Coffman, R.L. (1992). Regulation of immunity to parasites by T cells and T cell-derived cytokines. Annu. Rev. Immunol. *10*, 385-409.

Shi, L., Luo, K., Xia, D., Chen, T., Chen, G., Jiang, Y., Li, N., and Cao, X. (2006). DIgR2, dendritic cell-derived immunoglobulin receptor 2, is one representative of a family of IgSF inhibitory receptors and mediates negative regulation of dendritic cell-initiated antigen-specific T-cell responses. Blood *108*, 2678-2686.

Stempin, C.C., Garrido, V.V., Dulgerian, L.R., and Cerban, F.M. (2008). Cruzipain and SP600125 induce p38 activation, alter NO/arginase balance and favor the survival of Trypanosoma cruzi in macrophages. Acta Trop. *106*, 119-127.

Tully, O., and Nolan, D.T. (2002). A review of the population biology and host-parasite interactions of the sea louse Lepeophtheirus salmonis (Copepoda: Caligidae). Parasitology *124 Suppl*, S165-182.

Unanue, E.R. (1997). Studies in listeriosis show the strong symbiosis between the innate cellular system and the T-cell response. Immunol. Rev. *158*, 11-25.

Volz, J., Osta, M.A., Kafatos, F.C., and Muller, H.M. (2005). The roles of two clip domain serine proteases in innate immune responses of the malaria vector Anopheles gambiae. J. Biol. Chem. *280*, 40161-40168.

von Gersdorff Jorgensen, L., Heinecke, R.D., Skjodt, K., Rasmussen, K.J., and Buchmann, K. (2011). Experimental evidence for direct in situ binding of IgM and IgT to early trophonts of Ichthyophthirius multifiliis (Fouquet) in the gills of rainbow trout, Oncorhynchus mykiss (Walbaum). J Fish Dis *34*, 749-755.

Wagner, G.N., Fast, M.D., and Johnson, S.C. (2008). Physiology and immunology of *Lepeophtheirus salmonis* infections of salmonids. Trends Parasitol *24*, 176-183.

Warr, G.W. (1995). The immunoglobulin genes of fish. Dev. Comp. Immunol. *19*, 1-12.

Wieland, W.H., Orzaez, D., Lammers, A., Parmentier, H.K., Verstegen, M.W., and Schots, A. (2004). A functional polymeric immunoglobulin receptor in chicken (Gallus gallus) indicates ancient role of secretory IgA in mucosal immunity. Biochem. J. *380*, 669-676.

Wilson, M., Bengten, E., Miller, N.W., Clem, L.W., Du Pasquier, L., and Warr, G.W. (1997). A novel chimeric Ig heavy chain from a teleost fish shares similarities to IgD. Proc. Natl. Acad. Sci. U. S. A. *94*, 4593-4597.

Wolfle, U., Martin, S., Emde, M., and Schempp, C. (2009). Dermatology in the Darwin anniversary. Part 2: Evolution of the skin-associated immune system. J Dtsch Dermatol Ges *7*, 862-869.

Woof, J.M., and Mestecky, J. (2005). Mucosal immunoglobulins. Immunol. Rev. *206*, 64-82.

Yasuike, M., de Boer, J., von Schalburg, K.R., Cooper, G.A., McKinnel, L., Messmer, A., So, S., Davidson, W.S., and Koop, B.F. (2010). Evolution of duplicated IgH loci in Atlantic salmon, Salmo salar. BMC Genomics *11*, 486.

Yasuike, M., Leong, J., Jantzen, S.G., von Schalburg, K.R., Nilsen, F., Jones, S.R., and Koop, B.F. (2011). Genomic Resources for Sea Lice: Analysis of ESTs and Mitochondrial Genomes. Mar Biotechnol (NY).

Yazawa, R., Yasuike, M., Leong, J., von Schalburg, K.R., Cooper, G.A., Beetz-Sargent, M., Robb, A., Davidson, W.S., Jones, S.R., and Koop, B.F. (2008). EST and mitochondrial DNA sequences support a distinct Pacific form of salmon louse, *Lepeophtheirus salmonis*. Mar Biotechnol (NY) *10*, 741-749.

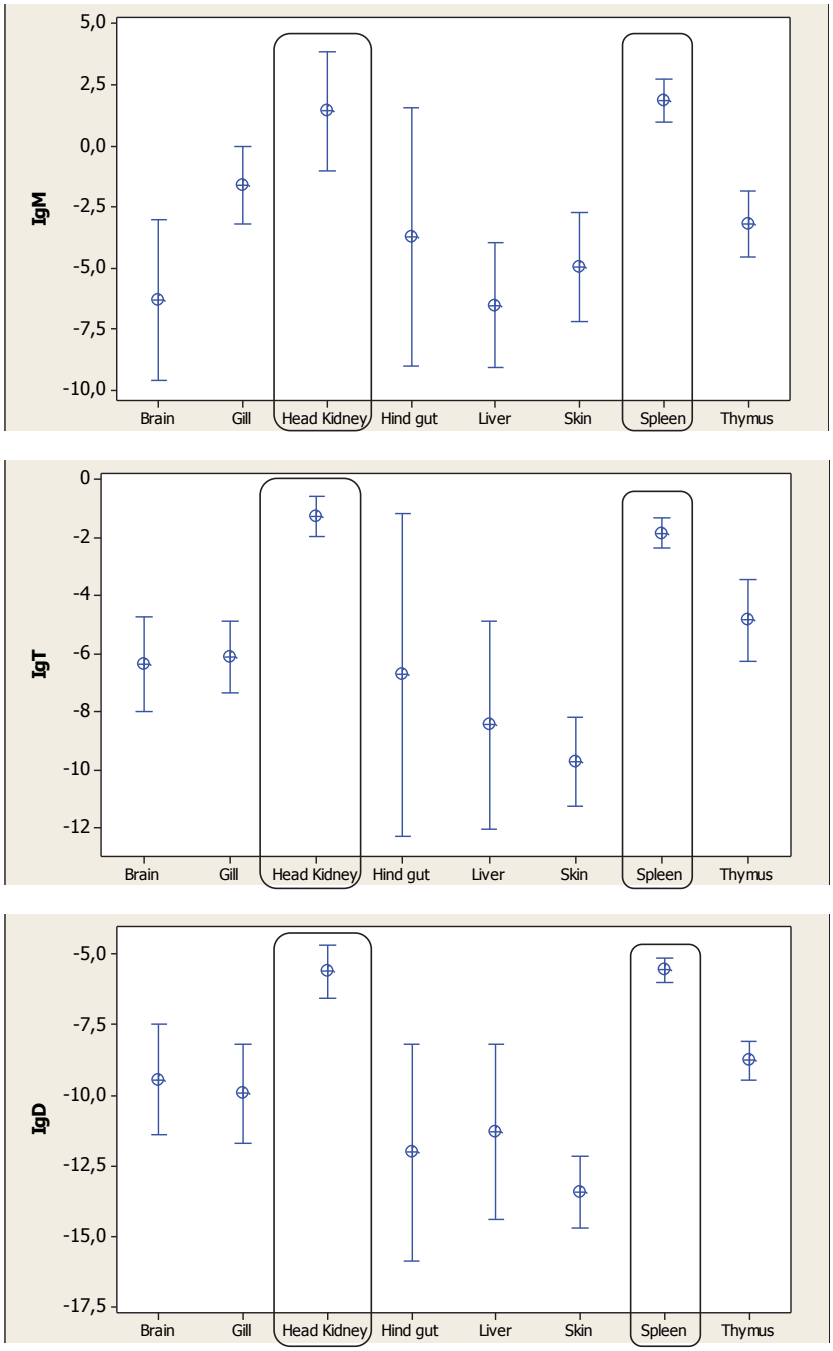
Zhang, Y.A., Salinas, I., Li, J., Parra, D., Bjork, S., Xu, Z., LaPatra, S.E., Bartholomew, J., and Sunyer, J.O. (2010). IgT, a primitive immunoglobulin class specialized in mucosal immunity. Nat. Immunol. *11*, 827-835.

Zhang, Y.A., Salinas, I., and Oriol Sunyer, J. (2011). Recent findings on the structure and function of teleost IgT. Fish Shellfish Immunol.

Zhao, X., Findly, R.C., and Dickerson, H.W. (2008). Cutaneous antibody-secreting cells and B cells in a teleost fish. Dev. Comp. Immunol. *32*, 500-508.

Zimmerman, A.M., Moustafa, F.M., Romanowski, K.E., and Steiner, L.A. (2011). Zebrafish immunoglobulin IgD: unusual exon usage and quantitative expression profiles with IgM and IgZ/T heavy chain isotypes. Mol. Immunol. *48*, 2220-2223.

Appendix



Appendix 1. Interval plot of IgM, IgT, and IgD expression in different tissues. 95% confidence interval for the mean (based on –ΔCt values normalized against EF1A) is shown. Tissues with highest expression (spleen and head kindey) are boxed. Note also individual variation.

```
atagcctcacatagtgacagtctggaacATGACTCCTCTACTCTTCTTCGCCTTCCTCCTC    61
                             M  T  P  L  L  F  F  A  F  L  L      11
CTCTCTCATCTGCCAGGCTCTCTCTGTAGGGTGACCACCGTGGGTGACCTGGCGGTCCTG    121
 L  S  H  L  P  G  S  L  C  R  V  T  T  V  G  D  L  A  V  L     31
GAAGGTCGGTCGGTGATGATCCCGTGCCATTACGGGCCGCAATACGCCAGTTATGTGAAG    181
 E  G  R  S  V  M  I  P  C  H  Y  G  P  Q  Y  A  S  Y  V  K     51
TACTGGTGCCATGGCAGTGTAAAGGACCTCTGTACCAGTCTGGTTCGCAGCGATGCCCCA    241
 Y  W  C  H  G  S  V  K  D  L  C  T  S  L  V  R  S  D  A  P     71
CGGGGGCAGGCTGCACCTGGAGAAGACAAGGTGGCCATGTTTGATGACCCGGTACAGCAG    301
 R  G  Q  A  A  P  G  E  D  K  V  A  M  F  D  D  P  V  Q  Q     91
GTGTTCACAGTAACAATGACAGAGCTGCAGAAGGAGGACTCTGGATGGTACTGGTGTGGT    361
 V  F  T  V  T  M  T  E  L  Q  K  E  D  S  G  W  Y  W  C  G    111
GTGGAGGTGGGAGGAGTCTGGAGTGCTGATGTCACAGCCTCCCTTCACATCAATGTTATC    421
 V  E  V  G  G  V  W  S  A  D  V  T  A  S  L  H  I  N  V  I    131
CAAGGTATGTCTGTGGTGAACAGCATGGTGAGCGGAGAGGAAGGGACCAGCGTTACTGTC    481
 Q  G  M  S  V  V  N  S  M  V  S  G  E  E  G  T  S  V  T  V    151
CAGTGTCTCTACAGCCAGGGATACAGGCAGCATGAGAAGCGTTGGTGTCGTAGTGGTGAC    541
 Q  C  L  Y  S  Q  G  Y  R  Q  H  E  K  R  W  C  R  S  G  D    171
TGGAGCTCCTGTCTAGTGACAGATGGTGAAGGGCGGTATGAAGACCAGGCAGTAGAGATC    601
 W  S  S  C  L  V  T  D  G  E  G  R  Y  E  D  Q  A  V  E  I    191
AGAGATGACCTGACCAAGGCTTTCACTGTCACCCTGAAGGGACTGGCCCGGAGAGATACA    661
 R  D  D  L  T  K  A  F  T  V  T  L  K  G  L  A  R  R  D  T    211
GGCTGGTACTGGTGTGCTGCAGGACAACAACAAGTGGCTGTCTACATCCTGGTCACTCCT    721
 G  W  Y  W  C  A  A  G  Q  Q  Q  V  A  V  Y  I  L  V  T  P    231
CCATCCACAACAGCACCTGCTCCTACAGTGACGTCCCCACCAGAAGAAAGCCCCCAGTCT    781
 P  S  T  T  A  P  A  P  T  V  T  S  P  P  E  E  S  P  Q  S    251
GTTCCTGTGTCTCCGTCTGTGTCTCCGTCTGTGTCTCCGTCTGTGTCTCCTCTTCCCAGA    841
 V  P  V  S  P  S  V  S  P  S  V  S  P  S  V  S  P  L  P  R    271
CACGTAGCTAAAGGAGCAGACCACCACAGGCCACTATGGGAGTTTCCTCTGATGGTGTGT    901
 H  V  A  K  G  A  D  H  H  R  P  L  W  E  F  P  L  M  V  C    291
GGAGTTCTGTTCATCTTGATGGTTCTGGTGCTGCTGCCATGGAAGATACTGGACCAATAC    961
 G  V  L  F  I  L  M  V  L  V  L  L  P  W  K  I  L  D  Q  Y    311
AACAAAACACACAGGACAAGACAGGCAGAGCTGGAAGCCAGACTCAGTGACCCCCCAGGT    1021
 N  K  T  H  R  T  R  Q  A  E  L  E  A  R  L  S  D  P  P  G    331
GACGACTGGCAGAACACCTCTGTCGTCTTCCTCAACTCCGCCTCACAGAAGGTGTACGGT    1081
 D  D  W  Q  N  T  S  V  V  F  L  N  S  A  S  Q  K  V  Y  G    351
TTCTGAccccgtccaccctccagtcacctctgacctctaacgtccaccctctatctctgg    1141
 F  *                                                           352
ccacctttcatcagagctgggcgccactaaatattccatccatccaacgatattttcagt    1201
accatgttgtatgatccatcattaccacaaatgttattgtgatcattacctgacatgtta    1261
aataatgttaccatgtttactgaaatcagtgtgtatataatctactgcaactatttgttt    1321
gtattacatatttacatttctattaaaaaaaaaacatttt                        1361
```

Appendix 2A. Nucleotide and deduced amino acid sequences of Salsal pIgR (GQ892057). The start and stop codons are in red font. The predicted regions shown are: Leader (L) (underlined), domain 1 (shaded light gray), domain 2 (white font in dark gray background), transmembrane (highlighted red). Each potential *O*-glycosylation site is shown in green. Translated nucleotides are in upper case, and 5' and 3' untranslated regions are in lower case letters. The stating position of each exon is double underlined.

```
actgtgagcagacacactgatgcggattgcaccagctcacccatctacatattactttga     60
catgctgtacacgtctatttgactaaggagagagtagactaacattggtattgacacaat     120
tagaacaaataagtacacATGGTTTCTCTTTATCTTCTCATCCTTGTCTTTGTCCATGGA     180
                     M  V  S  L  Y  L  L  I  L  V  F  V  H  G      14
CCATCAGGTTGCAACAGCCTGTGGACTGTGACAAAAAGTACTGCAAAGAGTGGCGGAGCC     240
 P  S  G  C  N  S  L  W  T  V  T  K  S  T  A  K  S  G  G  A      34
ATCACGATCCCCTGCCACTACCACCGCATGTTCAGAGACCATGCCAAATACTGGTGTAAG     300
 I  T  I  P  C  H  Y  H  R  M  F  R  D  H  A  K  Y  W  C  K      54
GGCAGAACCTGGCCCTTATGTACTGTGATGGCAAGCACCGACCCAAGGCGGAACAGAGGA     360
 G  R  T  W  P  L  C  T  V  M  A  S  T  D  P  R  R  N  R  G      74
GGCATGTCAATCACAGACATCCCAGAGGAGCTGGTCTTCACTGTGACCATGAAGAACCTA     420
 G  M  S  I  T  D  I  P  E  E  L  V  F  T  V  T  M  K  N  L      94
CAGGAGACTGACACCAACAGGTACTGGTGTGCTCTGAAAGTGGGTGGGATAGGCAAGCCA     480
 Q  E  T  D  T  N  R  Y  W  C  A  L  K  V  G  G  I  G  K  P     114
GATGTCAAAGTATCCGTGGACCTCACAGTCACCCAAGGCTCTCCTGATCTGTCAGTGGTT     540
 D  V  K  V  S  V  D  L  T  V  T  Q  G  S  P  D  L  S  V  V     134
GATGAGCTGGTATCTGGTGAGGAGGGGGGCAGTGTCAGTGTACAGTGTCTCTACAGTGAC     600
 D  E  L  V  S  G  E  E  G  G  S  V  S  V  Q  C  L  Y  S  D     154
ACACTCAGAGGCAAAGAGAAGAAGTGGTGCAGGAGTGGGGACCGGCATTCCTGTCAGACA     660
 T  L  R  G  K  E  K  K  W  C  R  S  G  D  R  H  S  C  Q  T     174
CAGACAGATACTAGCCAGAATGCATCTGTTGTGATCAGTGATGGCAAGAGGGGAGTGTTC     720
 Q  T  D  T  S  Q  N  A  S  V  V  I  S  D  G  K  R  G  V  F     194
AATGTGACAATGAAACAACTAGAGAAGAAAGATGCAGGCTGGTATTGGTGCTCTGTTGGA     780
 N  V  T  M  K  Q  L  E  K  K  D  A  G  W  Y  W  C  S  V  G     214
GACCTACAGGCTGCAGTTCATATCAACGTCACTCAGAGATCCACAGCACACAGGAACACT     840
 D  L  Q  A  A  V  H  I  N  V  T  Q  R  S  T  A  H  R  N  T     234
GCAGAAGCAGTGACTACTCCAACGCTGCCCATGTTACACTCCGCATTCTCGACATTTGCA     900
 A  E  A  V  T  T  P  T  L  P  M  L  H  S  A  F  S  T  F  A     254
GACTCCACAGCTACCTCTACCTCAAAGACAAACCCTTCCGCCACAGTAGACTCTGCTCTG     960
 D  S  T  A  T  S  T  S  K  T  N  P  S  A  T  V  D  S  A  L     274
ACAACATCCTCATATGGCTCTGCAACTCCCTATCCATCTGTCTCAGTTCCATACATCACA    1020
 T  T  S  S  Y  G  S  A  T  P  Y  P  S  V  S  V  P  Y  I  T     294
ATCCATTCTTCCACACTGACTTCTTCACTAACAACAGTACAACCTACATGCTGTCCTTCT    1080
 I  H  S  S  T  L  T  S  S  L  T  T  V  Q  P  T  C  C  P  S     314
ACTGAATCTACAGCTACTTACAAGAAAATCAGCAACCTGCCCTGGCATGCTCTTATTCTC    1140
 T  E  S  T  A  T  Y  K  K  I  S  N  L  P  W  H  A  L  I  L     334
ATTGTGTTGGCCATGGTGTTGTTGGTTATTGTTGTTATGGCTGCAGTTAACATCTATAGA    1200
 I  V  L  A  M  V  L  L  V  I  V  V  M  A  A  V  N  I  Y  R     354
TATTCCAGGAATAATATCAGACCTGTGGAAGGAGAGATGACTGAGCTGGTGATAAATCAA    1260
 Y  S  R  N  N  I  R  P  V  E  G  E  M  T  E  L  V  I  N  Q     374
GATCAATAAtatcaattaaaagagcctaaaaataaatttgaatcataaaaattactgtaa    1320
 D  Q  *                                                        376
gtttgctttaatatcaccaaaataatcacatgtacagtaaatggattgtagcaataacac    1380
Aattagctagagaacagagaaaaaaa                                      1406
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Appendix 2B. Nucleotide and deduced amino acid sequences of Salsal pIgRL (HM452379). Each potential *O*-glycosylation site, located in the connecting region, is highlighted green. Description of predicted regions and color codes are as in Appendix 2A.

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ctgacacacaccagtgcacccattcaaacataaaagctaccagagaatttctctctcatcta     62
atcATGGCTCCATATCTCCTCAGTGTCCTGCACATCCTTTTCTTCCTCACTGGACTGTCA     122
    M  A  P  Y  L  L  S  V  L  H  I  L  F  F  L  T  G  L  S      19
GGTATTCACGGTGTGTCCACAGTGAGTCATGTGTCTGTAAGTGAAGGAGGCTCCATCACC    182
 G  I  H  G  V  S  T  V  S  H  V  S  V  S  E  G  G  S  I  T      39
ATCCCATGTCTCTATCATCATGGCTCTGAAAAACATGTGAAATACTGGTGTAGTGGATAT    242
 I  P  C  L  Y  H  H  G  S  E  K  H  V  K  Y  W  C  S  G  Y      59
TTTTTCCATTTTTGCTCTACTCTAATACGTACTGACTCCCAGAGTGCATCTAATTGGTTG    302
 F  F  H  F  C  S  T  L  I  R  T  D  S  Q  S  A  S  N  W  L      79
TCCATCGCTGATAATGTTACTACAAGAGTCTTCACTGTGACCATGAAGAATCTGCAACTA    362
 S  I  A  D  N  V  T  T  R  V  F  T  V  T  M  K  N  L  Q  L      99
GGGGTCTCTGGATATTTCTGGGGTGCTGTGGAGAAAGGTGACACCACACATCTATATCTG    422
 G  V  S  G  Y  F  W  G  A  V  E  K  G  D  T  T  H  L  Y  L     119
TCAGTTTCCACAGGTACTGCAGGACTCTATGTGGACCAACAACATGTGACTGGAGTTGAA    482
 S  V  S  T  G  T  A  G  L  Y  V  D  Q  Q  H  V  T  G  V  E     139
GGACAGAGTGTCACTGTCAATTGTAACTATAGTAACTCTGGAGGTTTCTGCTGGTGCAGG    542
 G  Q  S  V  T  V  N  C  N  Y  S  N  S  G  G  F  C  W  C  R     159
CTGGGTGGCTCTTGCATGGAGAGGAGTGTTGGGAATTTAGATGGAGCATCAGTAGAAATA    602
 L  G  G  S  C  M  E  R  S  V  G  N  L  D  G  A  S  V  E  I     179
AAGCGGATTCTTGCCAATGGAAAAAAAGTCATGATCGTAACAATGAGTCAACTAAAAATT    662
 K  R  I  L  A  N  G  K  K  V  M  I  V  T  M  S  Q  L  K  I     199
AAGAACACTGGCTGGTACTGGTGTGCAGTTGGAGATCTACAGATACCTGTTCATATCACT    722
 K  N  T  G  W  Y  W  C  A  V  G  D  L  Q  I  P  V  H  I  T     219
GTTAGTCAACCAACCACAACACAGAGTAACACCACCATCACAAACCAGAACAATGGGGGA    782
 V  S  Q  P  T  T  T  Q  S  N  T  T  I  T  N  Q  N  N  G  G     239
ACTGACGAGAAGCGACAGGAAAGGATCCAGAGTTTGCTGGTAGTCCTGGTCATTCCTCTG    842
 T  D  E  K  R  Q  E  R  I  Q  S  L  L  V  V  L  V  I  P  L     259
AGCCTGTTGGTGGTGTTGATAGTTGGTACCTTGGTCACATTGAAGATATTCAGAAAACAT    902
 S  L  L  V  V  L  I  V  G  T  L  V  T  L  K  I  F  R  K  H     279
AAGGACAAGAAGGCAAAGGACCAACCACCAAACACCCCAGTACAGTCTGCTGACTCTGAG    962
 K  D  K  K  A  K  D  Q  P  P  N  T  P  V  Q  S  A  D  S  E     299
CAGAACATTACCTACAGCACTGTGAGTCACACCAGAAGAACAGCACAACAGGACCCATTA   1022
 Q  N  I  T  Y  S  T  V  S  H  T  R  R  T  A  Q  Q  D  P  L     319
CCTGATGATGCAGTGACATACAGCACCGTGGTCACCAAGAACAAGACCCAACCAAATGCA   1082
 P  D  D  A  V  T  Y  S  T  V  V  T  K  N  K  T  Q  P  N  A     339
GCAAAACCAGATGTGGTCTACAGCACAGTGGCCGCACACCAAAGATAGcaggaggcaaca   1142
 A  K  P  D  V  V  Y  S  T  V  A  A  H  Q  R  *                 354
gaacagactgattgaagtccctccaaattaaaaataggccttgttccattttactcccta   1202
gctcttacctctttgggggttactggttactaatgcctgtctattacagtcttgttcttt   1262
tccattgttcaggctctgctggcttaattatgccagtgattgtgtattctagacaaaggc   1322
agattgagtgtaatttttgatttctgaagtttaatgtacacactgatgtgtatttcaat    1382
gcttcatatctgaaataaactactttaagaaactt                            1417
```

Appendix 3A. Nucleotide and deduced amino acid sequences of Salsal CLM1 (NM_001140948). Three putative ITIM motifs (positions 302-307, 324-329, 344-349) are shaded light blue. Description of predicted regions and color codes are as in Appendix 2A.No potential *O*-glycosylation residues were found.

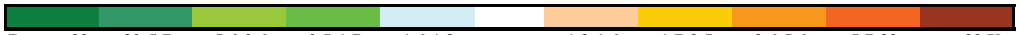
```
cccatttaacgtaacaggcatttaacgtaactctcATGGCTCTTCATCTCTCCCTCCTCCTC    62
                                   M  A  L  H  L  S  L  L  L     9
CTCCTCCTCCTCATCTTCTACAGACTCTCAGCAGGGAGGCATGTGTCTGTGAAGACAGGA    122
 L  L  L  L  I  F  Y  R  L  S  A  G  R  H  V  S  V  K  T  G    29
GGCTCCATCACCTTCCCATGTAGCTATGATCTGAATCACATCAACCATGTGAAATACTGG    182
 G  S  I  T  F  P  C  S  Y  D  L  N  H  I  N  H  V  K  Y  W    49
TGTAAAGGATTAGGTTGGGATGTATGTTCTTATGTAGTACGCACTGACTATCCTAAGAGC    242
 C  K  G  L  G  W  D  V  C  S  Y  V  V  R  T  D  Y  P  K  S    69
AGTGGTAAAAACTCAATCTCTGATGACATCAACAAGAGAATCTTCACTGTGACCATGACT    302
 S  G  K  N  S  I  S  D  D  I  N  K  R  I  F  T  V  T  M  T    89
GACCTGGAGCCAGAGGATTCTGAGAATTACAGGTGTGTTGTGGAGATCAATAGAGGACCA    362
 D  L  E  P  E  D  S  E  N  Y  R  C  V  V  E  I  N  R  G  P   109
GATATCAGGATACAATGGTTGTACCTATCTGTCACTCCAGGTACTCCAGAACTCTATGTG    422
 D  I  R  I  Q  W  L  Y  L  S  V  T  P  G  T  P  E  L  Y  V   129
GACCAACAGGAAGTGACTGGAGTAGAAGGAGGGAGTGTCACTGTCCGTTGTTACTATAGT    482
 D  Q  Q  E  V  T  G  V  E  G  G  S  V  T  V  R  C  Y  Y  S   149
ACCTCTGGAGATATGAAGTGGTGCAGGATGGGTGGTGATTGTGTGAGGGGGTATTCTGGG    542
 T  S  G  D  M  K  W  C  R  M  G  G  D  C  V  R  G  Y  S  G   169
ACTTTTCATGGAACATCAGTCACATTAATGCGGACTAGTGATGCCAACAACAGAAAAGTC    602
 T  F  H  G  T  S  V  T  L  M  R  T  S  D  A  N  N  R  K  V   189
TTAACAGTGACTATGAGTGGACTGAAGATGGAGAACACTGACTGGTATTGGTGTAGAGTG    662
 L  T  V  T  M  S  G  L  K  M  E  N  T  D  W  Y  W  C  R  V   209
GGAGAACTAGAGATGCCTGTTCACATCACTGTCAGTCAACAAACTGCAACACAGAGAAGC    722
 G  E  L  E  M  P  V  H  I  T  V  S  Q  Q  T  A  T  Q  R  S   229
TCTAAGATGACCTCAACAACCCAAGATCCAACCACTCAACAACCCTCTGCCTCTCCAACT    782
 S  K  M  T  S  T  T  Q  D  P  T  T  Q  Q  P  S  A  S  P  T   249
GCTGAGCCTGTTCAGAATGACAACACAAGTCAAGGAGCTGAGGGGAACATGGAGGAAGTC    842
 A  E  P  V  Q  N  D  N  T  S  Q  G  A  E  G  N  M  E  E  V   269
CACCAGAGGCCCATAGATGTGAAAGTCCTACTCATCTCTCTGGGCATGTTGGTGGTGGTG    902
 H  Q  R  P  I  D  V  K  V  L  L  I  S  L  G  M  L  V  V  V   289
ACAGCTGGTATCCTAGTGGCATGGAAGATGTGGAGAAAGCACAAAGACAATAAAGCCGAG    962
 T  A  G  I  L  V  A  W  K  M  W  R  K  H  K  D  N  K  A  E   309
AACCAGCCAATAATCACCTCAGCGGACCGATTTCCTGACAACGATGATGTAACGTACAGT    1022
 N  Q  P  I  I  T  S  A  D  R  F  P  D  N  D  D  V  T  Y  S   329
ACTGTCGTTCTAAAGAGGAAGACCCAGCAAAAGTTACAGACCAAGTCAGCAGAACCAGAT    1082
 T  V  V  L  K  R  K  T  Q  Q  K  L  Q  T  K  S  A  E  P  D   349
GATAATGTGTTCTACAGCTCACTAGCTCTACAGGTGACCACACAGCATAGGGCAGCAGCA    1142
 D  N  V  F  Y  S  S  L  A  L  Q  V  T  T  Q  H  R  A  A  A   369
GCACAGCAGTTACTGACTGAGAGAAGACCTTGGTCGAGTCCTTAAcctggaaggacagaa    1202
 A  Q  Q  L  L  T  E  R  R  P  W  S  S  P  *                  383
caattcctctcctgtgtcacaacatctatctgcctccaatatgcttctgtcatacctctt    1262
aacccacccccagaaagacccctctatcctgttgtgtaataaaccactgaagaccgt 1318
```

Appendix 3B. Nucleotide and deduced amino acid sequences of Salsal CLM7 (NM_001146562). Each potential *O*-glycosylation residue is highlighted green. Two putative ITIM motifs (positions 326-331, 352-357) are highlighted light blue. Description of predicted regions and color codes are as in Appendix 2A.

```
aggggaccacaaacaaagtttccatttccataacctttaaagtcatagatttcagataa     60
agattgagtttaagcagaaagATGACTACTAAGATTTGGATCAATCTCATCATCTTATGT    120
                       M  T  T  K  I  W  I  N  L  I  I  L  C     13
TTACTAACAGCCTCATCCCTCTCCGGACCCTCAGAGGTGAAGAGTGCAGTTGGTGAAACG    180
 L  L  T  A  S  S  L  S  G  P  S  E  V  K  S  A  V  G  E  T     33
GTCCACATCTCCTGCCAGTATCACCAGTTCAACAGGGACAAGGTCAAATTCTGGTGCAGG    240
 V  H  I  S  C  Q  Y  H  Q  F  N  R  D  K  V  K  F  W  C  R     53
GGTTATCACTGGTACTTCTGCACAGTTATCATTCGATCTGATCATCCTAAATATCTGACC    300
 G  Y  H  W  Y  F  C  T  V  I  I  R  S  D  H  P  K  Y  L  T     73
AGTGATGTTCAGATATTGGATGATAAAAACCTTGGGTTATTCACAGTCAGTATGAAAGGA    360
 S  D  V  Q  I  L  D  D  K  N  L  G  L  F  T  V  S  M  K  G     93
GCAACAGCCGAGGACAGTGGCTGGTACTGGTGTGCAATTGAAAGAGCCAGCAGAACTTTG    420
 A  T  A  E  D  S  G  W  Y  W  C  A  I  E  R  A  S  R  T  L    113
GCATTTCGTCTTCAGCTGACTGTCTCTGAGTGGCTTGTACCTCGGCTCAAACCAGAAACC    480
 A  F  R  L  Q  L  T  V  S  E  W  L  V  P  R  L  K  P  E  T    133
ACTGAACAGTACAACGAGACCTCAACTTCCCCAACAACGTTACCTCATCTTGAGACAACA    540
 T  E  Q  Y  N  E  T  S  T  S  P  T  T  L  P  H  L  E  T  T    153
ACACGGTCCACACCTTGTATGACTCAAACAACAAGTCTCATATCCGCTACAAGCAATTCA    600
 T  R  S  T  P  C  M  T  Q  T  T  S  L  I  S  A  T  S  N  S    173
ACAAGTGTGACAGTGTCCTTGGATCAAGATGATCTGGTGTGGAAAGTATGGAGAGTACTG    660
 T  S  V  T  V  S  L  D  Q  D  D  L  V  W  K  V  W  R  V  L    193
CGCTGGATGCTCTTCCTCTTCCTGTGTCTGTTCCTTGTTCTCTTCAGTATACAATGCCAT    720
 R  W  M  L  F  L  F  L  C  L  F  L  V  L  F  S  I  Q  C  H    213
CGCTGAtatttgtaccattcctgccagtgtatcgcttacctcaatgtgtgctacattttc    780
 R  *                                                           214
Cccaaatcaaaatcgttgagaatattgtttgtattaattaataaaagtaaaatattctaa    840
Catgcacatatattaataataagcaaataaattgaggtcacaacatgttctgagagatat    900
Taatgtaaagagaaacaatactaaagatgtaggatctttatttgaaccggtttgctacag    960
Caggaaaataatcctgcagcaacagaaaatgtgaaatccatagattagggtctaatacat   1020
Ttacttaaattggctgatttccttatatgaactgtaactcagtaaaatctttgaaattgt   1080
Tgcatgtcacatgtatatctttaataaacccaaccagatt                       1120
```

Appendix 3C. Nucleotide and deduced amino acid sequences of Salsal CLM8 (NM_001140890). CLM8 has one extracellular domain and the cytoplasmic region is encoded by only four codons. Description of predicted regions and color codes are as in Appendix 2A.

Gene	Day 1	Day 3	Day 5	Day 10	Day 15
tryspin [Oncorhynchus masou]	0.12	1.12	0.64	-2.30	-2.08
alpha amylase [Salmo salar]	1.39	1.57	-0.01	-2.53	-2.93
anionic trypsin [Oncorhynchus keta]	1.01	1.21	0.61	-2.36	-1.95
carboxylic ester hydrolase	1.12	1.55	1.65	-2.07	-2.06
Carboxypeptidase A1 precursor [Salmo salar]	1.13	0.91	0.80	-4.81	-2.96
Carboxypeptidase A2 precursor [Salmo salar]	0.35	1.56	0.58	-2.84	-2.77
Carboxypeptidase B precursor [Salmo salar]	0.64	0.98	0.22	-2.42	-2.11
Chymotrypsin B [Salmo salar]	1.10	1.65	0.80	-4.15	-2.79
Chymotrypsin B [Salmo salar]	1.06	1.48	0.26	-2.16	-2.04
Chymotrypsin-like protease CTRL-1 precursor [Salmo salar]	0.75	1.56	0.97	-3.38	-2.66
Elastase-1	1.40	1.30	0.71	-4.32	-2.80
Endoplasmic reticulum resident protein ERp27 [Salmo salar]	1.05	0.84	1.27	-3.09	-2.91
Guanidinoacetate N-methyltransferase [Salmo salar]	1.20	0.50	0.63	-3.04	-2.59
Guanidinoacetate N-methyltransferase [Salmo salar]	1.51	0.27	0.81	-3.10	-2.43
High choriolytic enzyme 1 precursor [Salmo salar]	1.25	0.73	0.87	-3.20	-2.42
Proproteinase E precursor [Salmo salar]	0.10	1.19	0.74	-4.78	-3.56
Proproteinase E precursor [Salmo salar]	0.37	0.97	0.62	-3.66	-2.84
syncollin	1.01	1.40	0.84	-3.93	-2.84
Trypsin II	1.78	1.48	0.93	-2.46	-1.95
Trypsin II	1.42	1.19	0.44	-2.22	-1.68
Trypsin III	1.28	1.25	0.96	-3.15	-2.47
Trypsin precursor [Salmo salar]	0.54	0.99	0.81	-1.18	-1.77
Trypsin-1 precursor [Salmo salar]	1.14	1.36	0.89	-1.67	-1.49
Trypsin-1 precursor [Salmo salar]	0.33	1.44	1.04	-2.42	-1.92



Appendix 4. Proteases with differential expression in spleen, microarray results. Data are log2-ER. Fold change (up or down regulation) is indicated by colour scale.

Papers

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Research paper

Molecular cloning of IgT from Atlantic salmon, and analysis of the relative expression of τ, μ and δ in different tissues

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ABSTRACT

In the present study, IgT genes of Atlantic salmon were cloned and characterised. Analysis of our sequence data as well as ESTs reported to the databases revealed three distinct IgT heavy chain sub-variants in salmon, as opposed to two of IgM and IgD. The IgT sub-variants in salmon are 76–80% identical to each other, and 75–82% identical to the reported rainbow trout sequences, whereas the similarity to the orthologous molecules in zebrafish, grass carp, mandarin fish, and grouper is 25–41%. The heavy chains of both secreted and membrane anchored forms of salmon IgT include four constant Ig domains, τ1–τ4. This parallels the IgM heavy chains in elasmobranch fish and higher vertebrates, but differs from IgM in teleost fish where the membrane anchored form include only three constant Ig domains, μ1–μ3. The similarity between τ1 and μ1 in salmon is relatively high (52%) when compared to the remaining part of the molecules (τ2–τ4 and μ2–μ4 are 13–24% similar). To compare τ, μ and δ expressions in different tissues (head kidney, thymus, spleen, gill, skin, hind gut, brain and muscle) of Atlantic salmon, RT-qPCR assays were designed and evaluated. The analyses revealed that IgM transcripts are most abundant (up to 200 times more than IgD) followed by IgT (up to 20 times more than IgD) in most tissues. Highest expression of IgM, IgT, and IgD was in head kidney and spleen.

1. Introduction

The teleost Ig heavy chain gene complex typically encodes three antibody classes: IgM, IgD and IgT (or IgZ). While transcripts of both membrane and secreted forms are generated from the IgT and IgM genes, most teleosts appear to express primarily (or only) membrane bound IgD (Hordvik et al., 1999; Stenvik and Jorgensen, 2000; Stenvik et al., 2001; Hordvik, 2002; Hirono et al., 2003; Saha et al., 2004), except in channel catfish which possesses both secreted and membrane forms of IgD, and lacks IgT (Bengten et al., 2002). Whereas IgD, and to some degree IgT, have been subjected to major structural divergence (Hordvik et al., 1999; Stenvik and Jorgensen, 2000; Zhao et al., 2002; Danilova et al., 2005; Hansen et al., 2005; Savan et al., 2005a; Rogers et al., 2006; Deza et al., 2009; Hu et al., 2010), the IgM monomer has been evolutionary stable; with four constant Ig heavy chain domains, μ1–μ4. However, IgM make up tetramers in teleost fish, in contrast to mammals and elasmobranch fish where IgM typically form pentamers (Rombout and Joosten, 1998; Bromage et al., 2006).

IgT of rainbow trout was reported simultaneously as the orthologous molecule in zebrafish, named IgZ (Danilova et al., 2005; Hansen et al., 2005). The secreted form of both of these molecules possesses four Ig domains, τ1–τ4 (ζ1–ζ4). As in mammalian IgM, τ4/ζ4 is included in the membrane anchored form of IgT/IgZ. This is in contrast to teleost IgM, where μ3 is fused directly to the membrane anchoring part. The special splicing pattern that leads to the exclusion

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Table 1
PCR assays used to distinguish between A and B sub-variants of IgT, IgM, and IgD.

Amplicon	Forward primer	Reverse primer	Product A	Product B
IgT: τ3–τ4	GAACAAAGTCCAGTGTTCTG	GGTGGTGGTGAAGACGCTGG	629 bp	826 bp
IgM: μ4–μTM1	GCCAAGCCAAGTAGAAGTTG	GACAGTCAGTCAACACGAGA	1058 bp	951 bp
IgD: δTM1–δTM2	GAACTGGAACATGGCCTGC	TGCAATGTTGTAAAGAAACG	564 bp	611 bp

of the entire fourth constant Ig domain from membrane anchored IgM has been introduced after bony fish branched off the phylogenetic tree: Membrane bound IgM in elasmobranch fish includes the fourth constant domain as in mammals (Kokubu et al., 1988), whereas both μ3-TM1 and μ4-TM1 splice variants are present in some primitive bony fish (Wilson et al., 1995).

In contrast to the IgM genes which consistently encode four constant Ig domains, the overall structure of the IgT/Z genes varies to some degree. While the rainbow trout IgT and zebrafish IgZ contain four constant Ig domains (Danilova et al., 2005; Hansen et al., 2005), the IgT genes in stickleback encode three constant Ig domains (Deza et al., 2009; Bao et al., 2010). In fugu, the IgZ gene encodes only two Ig domains, corresponding to zebrafish ζ1 and ζ4 (Savan et al., 2005a), and the ζ gene of common carp is a chimera of IgM–IgZ with two constant region domains made up of μ1 of carp and a domain similar to ζ4 of zebrafish (Savan et al., 2005b). Very recently, a membrane bound zebrafish IgZ homologue (named IgZ-2), was reported (Hu et al., 2010). This is an independent homologue (with 53.5% identity to IgZ), indicating that IgZ variants are encoded by different genes, in addition to alternative splicing (Hu et al., 2010).

In the present study, IgT genes of Atlantic salmon were cloned and characterised. Cognizant to this, we designed RT-qPCR assays and analysed the relative expression of IgT, IgM, and IgD in a series of tissues (head kidney, thymus, spleen, gill, skin, hind gut, brain, muscle, and liver). Designing gene expression assays for Atlantic salmon is challenging because of a whole genome duplication event that most likely occurred in the ancestor of salmonids; which subsequently resulted in two distinct heavy chain loci (Hordvik, 1998). Thus, to achieve optimum gene quantification, qPCR assays must be carefully designed and tested. This gets even trickier when it comes to designing assays that amplify the membrane and secreted forms of the Igs. Thus, the present work provides a basis for further studies involving Ig gene expression in Atlantic salmon.

2. Materials and methods

2.1. Fish

Atlantic salmon (*Salmo salar*) were obtained from the wet lab at the Institute of Marine Research, and from the Industrial and Aquatic Laboratory at the High Technology Center in Bergen, Norway. Fish were killed by a blow to the head, and tissues of head kidney, thymus, spleen, gill, skin, hind gut, brain, muscle, and liver were collected immediately, frozen in liquid nitrogen, and stored at −80 °C until use.

2.2. Preparation of RNA and cDNA

RNA was isolated using the iPrep™ TRIzol® Plus kit (Invitrogen). The quantity and quality of the total RNA was assessed using a NanoDrop Spectrophotometer (NanoDrop Technologies) on all samples and an Agilent 2100 Bioanalyzer (Agilent Technologies), on random samples. When required, cDNA was synthesized by reverse transcription of RNA using MMLV–RT according to the manufacturer's protocol (Invitrogen). Total RNA and cDNA were stored at −80 and −20 °C, respectively, until use.

2.3. Sequencing and analysis of two IgT genes in Atlantic salmon

A salmon IgM cDNA-fragment was amplified by PCR from clone 7.3 (Hordvik et al., 1992). Subsequently, it was radioactive labelled with α^{32}P-CTP and used as probe to screen a BAC library of Atlantic salmon as described by Harstad et al. (2008). Using PCR primers designed to distinguish between IgM and IgD sub-variants (Table 1), 73 different clones were sorted into two groups, corresponding to the previously characterized A and B loci in salmon (Hordvik et al., 1992, 2002). The number of clones which were double positive for IgM-A and IgD-A was 48, whereas 25 clones were of B-type. Two B clones did not contain the IgD gene, and end-sequencing of these

Table 2
QPCR primers and probes.

Gene	Forward primer 5′3′	Exon	Reverse primer 5′3′	Exon	Probe 5′3′	Exon
IgM	TGAGGAGAACTGTGGGCTACACT	μ2	TGTTAATGACCACTGAATGTGCAT	μ3	CATCAGATGCAGGTCC	μ2/μ3
sIgM	GGTCCTTGGTAAAGAAACCCTACAA	μ3	GCCAGCAGAAAGACAGATGGA	μ4	AGACCGGAGGAGATC	μ3/μ4
mIgM	GGTCCTTGGTAAAGAAACCCTACAA	μ3	CTGCATGGACAGTCAGTCAACAC	μTM1	AGGGAGACCGAGTGTC	μ3/μTM1
IgD	CCAGGTCCGAGTGGGATCA	δ6	TGGAGCAGGGTTGCTGTTG	δ7	CACTGAGCCAAATAC	δ6/δ7
spIgD	CCAGGTCCGAGTGGGATCA	δ6	TTCTGTCATATGGAAGTGTCCTGAA	δTM2	AGAATGTATTTGACAAAA	δ6/δTM2
IgT	CAACACTGACTGGAACAACAAGGT	τ2	CGTCAGCGGTTCTGTTTTGGA	τ2/τ3	AGTACAGCTGTGTGGTGCA	τ2
EF1A-A	CCCCTCCAGGACGTTTACAAA	E5	CACACGGCCCACAGGTACA	E6	ATCGGTGGTATTGGAAC	E5/E6

The exons from which the primer/probes were designed are shown in the last column. Note that primers and probes between τ2 and τ3, μ2 and μ3 amplify both secreted and membrane forms of IgT/M.

showed that the inserts of these clones were restricted by an *Eco*RI site between μ4 and TM1. Using primer pairs designed to anneal to IgT genes in salmon, IgT-A (GenBank acc. no. GQ907003) was cloned from BAC clone 252_G20, whereas IgT-B (GenBank acc. no. GQ907004) was cloned from genomic DNA.

Sequences were translated using Expasy translate tool (http://us.expasy.org/tools/dna.html) and aligned with ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) softwares. Phylogenetic trees were constructed using ClustalW Multiple Alignment tool (http://www.bioinformatics.nl/tools/clustalw.html). Prediction of overall Domain structure, SP and TM regions was confirmed by the online tools SignalP (http://www.cbs.dtu.dk/services/SignalP/), SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html/), and InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/) respectively. N-linked glycosylation sites were predicted using the online tool NETNGLYC (http://www.cbs.dtu.dk/services/NetNGlyc/).

2.4. Real-time quantitative PCR

For gene expression analysis, TaqMan® probe-based real-time PCR assays were designed using Primer express 3.0 software (Applied Biosystems). The assays were made in such a way that either the primer or probe should span between two exons, to avoid detection of genomic DNA (Table 2). As shown in Fig. 1, two assays were used to quantify transcripts encoding secreted and membrane IgM (sIgM and mIgM), respectively, and a third assay was used to quantify both forms of both sub-variants. Conserved regions in τ2 and τ3 were chosen to make a single assay detecting all sub-variants of both sIgT and mIgT. One assay was used to quantify both sub-variants of IgD, and another was designed to detect a rare splice variant of IgD (spIgD).

Quantitative PCR was performed using 7500 Fast Real-Time PCR System (Applied Biosystems), and RNA was quantified in a single step assay (both RT and PCR steps carried out in the same tube) using the Verso™ 1-step QRT-PCR low ROX kit (Thermo Scientific). The PCR reaction mix contained 1-step QPCR low ROX mix (2×), enzyme mix, RT enhancer, 900 nM of each primer, 200 nM of TaqMan probe and 2 μl (50–100 ng) of RNA in a final volume of 12.5 μl. Thermal cycling was carried out according to the manufacturers protocol (Thermo Scientific) as follows: cDNA synthesis at 50 °C for 15 min was followed by an enzyme activation step of 95 °C for 15 min, and 40 cycles of 95 °C for 15 s (denaturation) and 60 °C for 60 s (annealing/extension). All samples were run in duplicate (or triplicates) with non-template controls (NTC) on the same plate. Prior to the 1-step RT-qPCR analysis (and before we had IgT sequence data), a comprehensive 2-step RT-qPCR analysis of sIgM, mIgM, IgD, and spIgD was performed on 10 tissues from up to 12 individuals of Atlantic salmon. To evaluate the assays, a series of RNA dilutions (0.2, 0.1, 0.05, 0.025, 0.0125, and 0.00625 μg/μl) from each tissue of one individual was reverse transcribed and subjected to real time PCR as described previously (Moore et al., 2005).

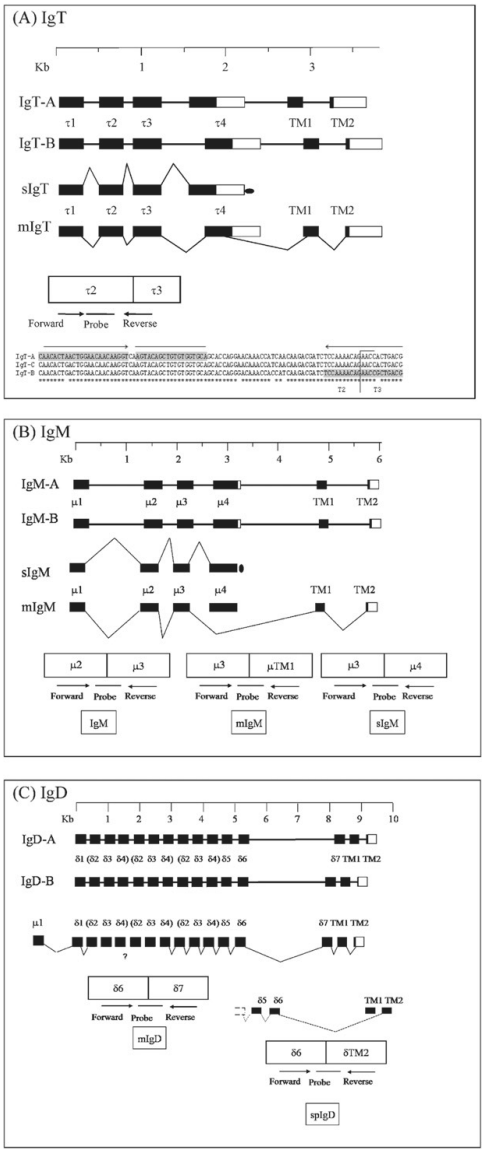

Fig. 1. Diagram showing the gene structure of two IgT genes (A), two IgM genes (B) and two IgD genes (C) in Atlantic salmon. The splicing pattern as well as positions of primers and probes are indicated. White boxes represent 5′- and/or 3′-UTR, black boxes represent translated regions while the introns are indicated with a line.

2.5. Statistical analysis

Relative expression of mRNA was calculated using the $2^{-\Delta Ct}$ method with elongation factor 1alpha (EF1A-A) as

Table 3
Amino acid identity of the Ig domains of salmon IgT (based on IgT-A) compared to the other four- Ig domain containing IgT/Z genes, and salmon IgM.

Salmon IgT	Grouper IgZ				Mandarin fish IgT				Zebrafish IgZ				Grass carp IgZ				Salmon IgM			
	ζ1	ζ2	ζ3	ζ4	τ1	τ2	τ3	τ4	ζ1	ζ2	ζ3	ζ4	ζ1	ζ2	ζ3	ζ4	μ1	μ2	μ3	μ4
Salmon τ1	45	20	12	14	43	18	8	13	20	13	4	14	20	15	3	7	52	18	13	19
Salmon τ2	17	41	7	11	16	35	14	13	16	30	9	15	14	32	5	16	22	21	18	35
Salmon τ3	10	17	47	7	8	25	44	6	3	8	24	1	18	12	24	12	12	6	14	2
Salmon τ4	21	24	11	43	21	19	9	40	9	18	3	26	8	12	5	29	16	17	17	24

an internal reference gene (Olsvik et al., 2005). The data are presented in percentage as mean ± SEM. Pair-wise comparison of transcript abundance (based on $2^{-\Delta Ct}$) of the Ig genes in each individuals tested within the various tissues was done by calculating the probability associated with the student's *t*-test (2-tailed) using the Microsoft Excel spreadsheet program. The transcript level of each Ig gene was compared between tissues by one-way ANOVA. Individual 95% CIs for mean based on pooled StDev of $-\Delta$Ct values was calculated using Minitab 14 software. Additionally, an interval plot of the Ig genes versus tissues, based on $-\Delta$Ct values was constructed using MINITAB 14. The level of significance was set to $P < 0.10$ for all analyses (supplementary data).

3. Results

3.1. IgT sequence analysis

Previous studies have shown that there are two highly similar IgM and IgD genes in Atlantic salmon, most probably related to ancestral tetraploidy (Hordvik, 1998; Hordvik et al., 2002). In accordance with this pattern, the present work revealed two IgT genes as well. However, when IgT-A and IgT-B were used as queries in BLAST searches, a third group of IgT sequences appeared (named IgT-C). The ESTs showed identity indices of approximately 89% to both IgT-A and IgT-B, i.e. a similar frequency of substitutions as between IgT-A and IgT-B. All three groups were represented by approximately equal numbers of ESTs. This could indicate that there is more than one IgT gene in each of the A and B Ig heavy chain gene complexes (see Section 5). A PCR assay that generates two differently sized fragments of the IgT-A and IgT-B genes (Table 1) did not generate additional bands when it was used on genomic DNA. Thus, from our analysis we could not conclude whether the third variant we identified among the ESTs was an allelic or isotypic variant.

The translated sequences of the IgT sub-variants in salmon are 76–80% identical to each other, and 75–82% identical to the reported rainbow trout sequences, whereas the similarity to the orthologous molecules in zebrafish, grass carp, mandarin fish, and grouper is 25–41%. Domain wise, the first domain of IgT (τ1) showed the highest identity to μ1 (52%), followed by grouper ζ1 (45%), mandarin fish τ1 (43%), eel μ1 (41%), zebrafish μ1 (36%), and human μ1 (32%). The remaining IgT domains (τ2–τ4) showed a similar correspondence with grouper and mandarin fish τ2–τ4 (Table 3).

An alignment of IgT, IgZ and IgM sequences is shown in Fig. 2. Two N-linked glycosylation sites were predicted to be present in salmon IgT (in τ2 and τ4) (Fig. 2). The number and locations of conserved cysteine residues in IgT and IgM are similar to other species, except in zebrafish and grass carp IgZ where the distance between the two cysteines in ζ3 is 16 codons shorter. IgT/Z appears to be shorter than IgM of teleosts and other species, particularly in τ2 as indicated by the gaps in Fig. 2.

Despite low amino acid identity indices to IgT, a BLAST search using human IgM against the salmon database resulted in top scores with IgT. The CART motifs (residues 15, 8, 1, 12, 5, 16 on the helical wheel diagram on Fig. 3) are fully conserved in human, mouse, and shark IgM and teleost IgT/Z; and differ in two codons in salmon/trout IgM, and in one residue in bovine IgM: possibly indicating signaling similarities of IgT/Z with mammalian IgM.

3.2. Differential expression of IgT, IgM, and IgD

The RT-qPCR analyses revealed that IgM transcripts are most abundant (up to 200 times more than IgD, $P < 0.001$) followed by IgT (up to 20 times more than IgD, $P < 0.001$)

Table 4
Mean ± SEM values of transcript levels (%) normalized against EF1A-A, obtained by a 2-step quantitative real-time PCR.

Tissue	*N*	sIgM		mIgM		mIgD		spIgD
		Mean	SEM	Mean	SEM	Mean	SEM	
Head kidney	12	874.36	553	51.9	26.20	17.80	12.30	0.003
Spleen	12	774.80	197	26.12	7.12	10.42	3.10	0.001
Hind gut	12	253.97	95.3	1.592	0.57	0.2658	0.08	Undet.
Thymus	12[a]	39.45	16.4	1.517	0.63	0.5900	0.36	Undet.
Gill	7	4.66	2.6	0.816	0.21	0.2550	0.04	Undet.
Skin from side	7	1.99	0.783	0.284	0.23	0.0584	0.03	Undet.
Skin from head	6	0.40	0.362	0.0425	0.01	0.0212	0.01	Undet.
Brain	6	8.01	4.34	0.901	0.25	0.1493	0.03	Undet.
Liver	7	1.31	0.86	0.0659	0.02	0.0261	0.00	Undet.

[a] $N = 11$ for mIgD.

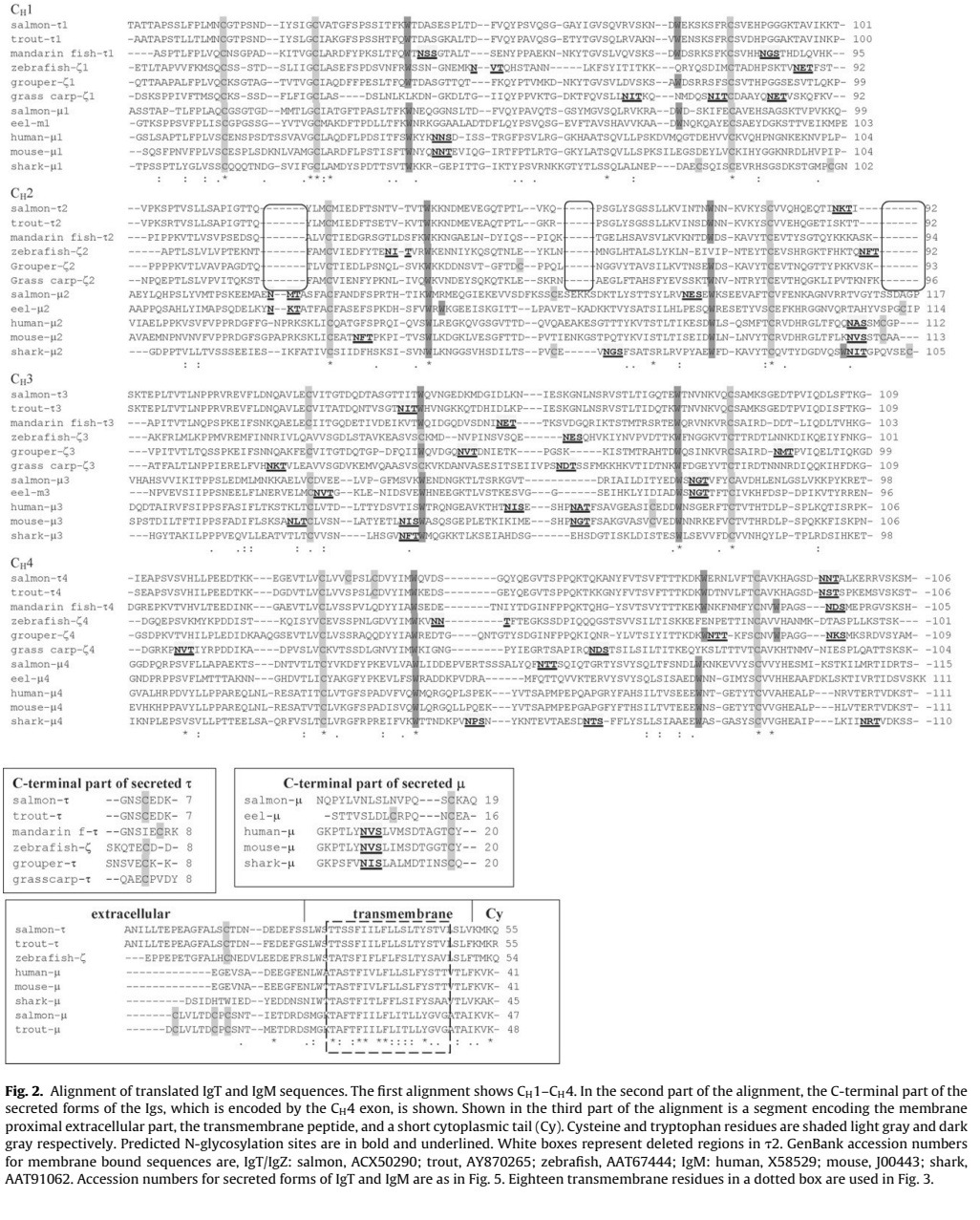

Fig. 2. Alignment of translated IgT and IgM sequences. The first alignment shows C_H1–C_H4. In the second part of the alignment, the C-terminal part of the secreted forms of the Igs, which is encoded by the C_H4 exon, is shown. Shown in the third part of the alignment is a segment encoding the membrane proximal extracellular part, the transmembrane peptide, and a short cytoplasmic tail (Cy). Cysteine and tryptophan residues are shaded light gray and dark gray respectively. Predicted N-glycosylation sites are in bold and underlined. White boxes represent deleted regions in τ2. GenBank accession numbers for membrane bound sequences are, IgT/IgZ: salmon, ACX50290; trout, AY870265; zebrafish, AAT67444; IgM: human, X58529; mouse, J00443; shark, AAT91062. Accession numbers for secreted forms of IgT and IgM are as in Fig. 5. Eighteen transmembrane residues in a dotted box are used in Fig. 3.

(Fig. 6 and supplementary data). Highest expression of IgM, IgT, and IgD was in head kidney and spleen ($P < 0.05$). Overall Ig transcript levels in the remaining tissues (gill, hind gut, thymus, liver, skin and brain) is not significantly different, however, based on mean values, the abundance of Ig transcripts was minimal in liver, muscle, skin, and brain (Fig. 6). The mean transcript levels of Ig genes in head kidney is higher than that of spleen, but not statisti-

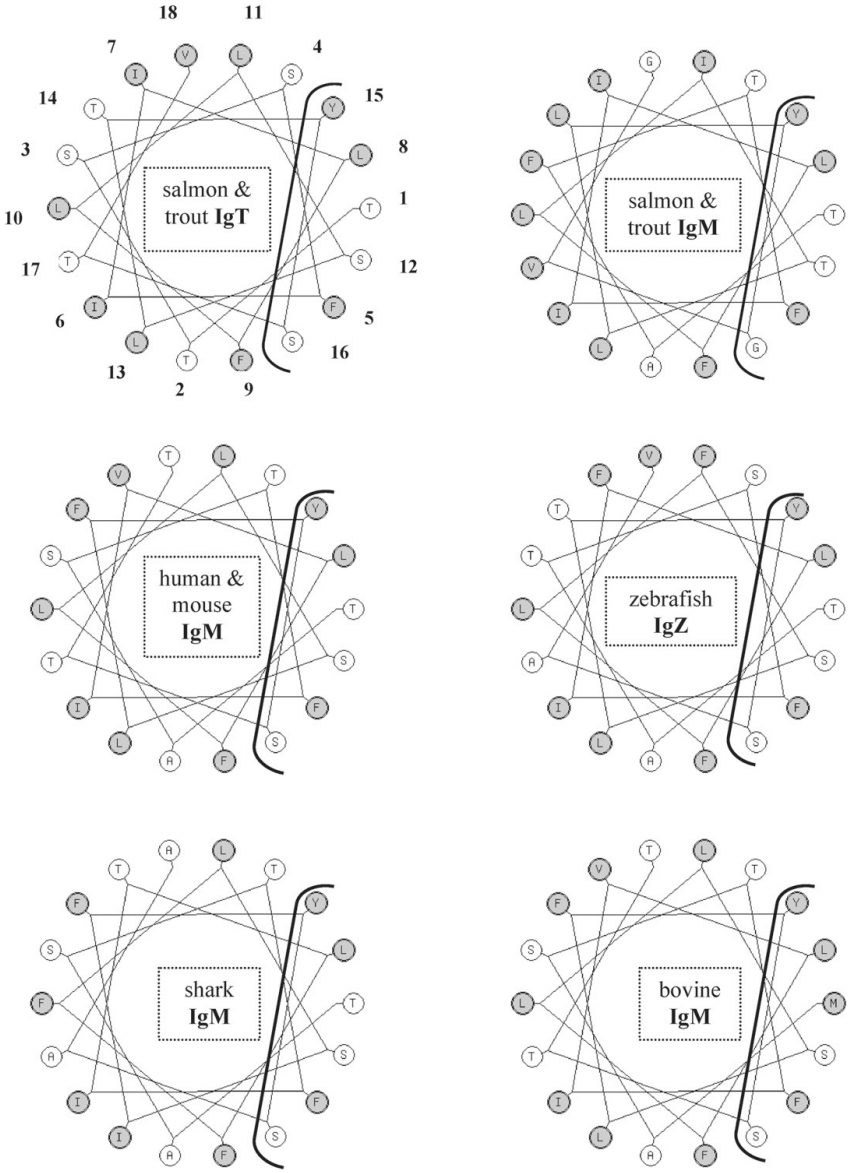

Fig. 3. Helical wheel (an axial projection of transmembrane alpha-helix regions) of salmon/trout IgT and IgM, zebrafish IgZ, human, mouse, bovine, and shark IgM. Conserved antigen receptor transmembrane (CART) motifs are represented by residues 15, 8, 1, 12, 5, and 16. The wheel is constructed using 18 transmembrane residues, marked from 1 to 18 (shown with a dotted box in the last section of Fig. 2), using the online helical wheel drawing program (http://kael.net/helical.htm). Accession number for bovine is AAB09545, and the rest are as in Fig. 2.

cally significant ($P = 0.4736$). A huge individual variation is observed for IgM especially in the head kidney and hind gut.

In a 2-step assay, the abundance of sIgM, mIgM, IgD, and spIgD were studied (Table 4). The analysis showed that transcripts for sIgM were most numerous as compared to mIgM and IgD ($P < 0.05$) especially in the spleen, hind gut, and the thymus. Overall Ig abundance is highest in head kidney and spleen, followed by hind gut and thy-

mus. The abundance of IgD transcripts was lower than that of transcripts encoding mIgM. An alternatively spliced IgD transcript (Hordvik et al., 1999) was verified to be present, but in very small amounts. This transcript was detected in head kidney and spleen at very minute levels. IgM-B transcripts were slightly more numerous than IgM-A transcripts in all the examined tissues of healthy fish (results not shown), as shown earlier (Hordvik et al., 2002), but there is no marked tissue specific difference between them.

4. Discussion

In the present study, it was revealed that IgM transcripts are most abundant followed by IgT, especially in head kidney and spleen, as compared to IgD, indicating that IgT is the dominant Ig next to IgM. The abundance of IgT transcripts in samples of mucosal tissues (skin and hind gut) is relatively weak. Nevertheless, preliminary RT-qPCR data shows an increase in transcript levels of IgT in skin following a challenge with salmon louse (*Lepeophtheirus salmonis*) copepods the first few days (3–15) post-infection (data not shown). This gives an indication of local production of τ transcripts following infection, and supports the hypothesis that IgT is involved in mucosal immunity (Sunyer et al., 2009). The fact that IgD is far less expressed than IgM and IgT in salmon is consistent with previous findings in most teleosts (Stenvik and Jorgensen, 2000; Stenvik et al., 2001; Hirono et al., 2003; Tian et al., 2009).

Highest expression of the different Igs was in head kidney followed by spleen, which is in accordance with previous studies (Stenvik and Jorgensen, 2000; Stenvik et al., 2001; Hirono et al., 2003; Hansen et al., 2005; Saha et al., 2005; Tian et al., 2009). The head kidney, having morphological and functional similarities to the mammalian bone marrow, is a major hematopoietic organ and site of production of antibodies and other immune cells in teleost fish (Rombout and Joosten, 1998; Press and Evensen, 1999). In the thymus, all Ig transcript levels were much lower than that in head kidney and spleen. Not unexpectedly, despite the clear trends in tissue distribution of the Ig genes, the analyses revealed considerable individual variation (except in the spleen), which has been the case in other studies as well, e.g. Bromage et al. (2006).

The expression and tissue distribution patterns of IgT exhibit considerable variation in different species. While IgT is expressed in various rainbow trout tissues, similar to the present finding, especially in spleen and head kidney (Hansen et al., 2005), expression of the zebrafish IgZ in adults is limited to primary lymphoid organs, including thymus (Danilova et al., 2005). A recently described membrane bound zebrafish IgZ-2 is expressed both in primary and secondary lymphoid organs (Hu et al., 2010). In fugu and mandarin fish, a gene similar to IgT is expressed in head kidney, spleen, and gill (Savan et al., 2005a; Tian et al., 2009).

Multiple alignments of single Ig (τ, μ, δ) domains from several species showed that τ1 and μ1 cluster together (NJ tree not shown, but can be inferred from Table 3). Likely, this is a result of co-evolution with the light chains, restricting the variability in this part of the molecule. Accordingly, μ1 is incorporated into the IgD heavy chain in teleosts,

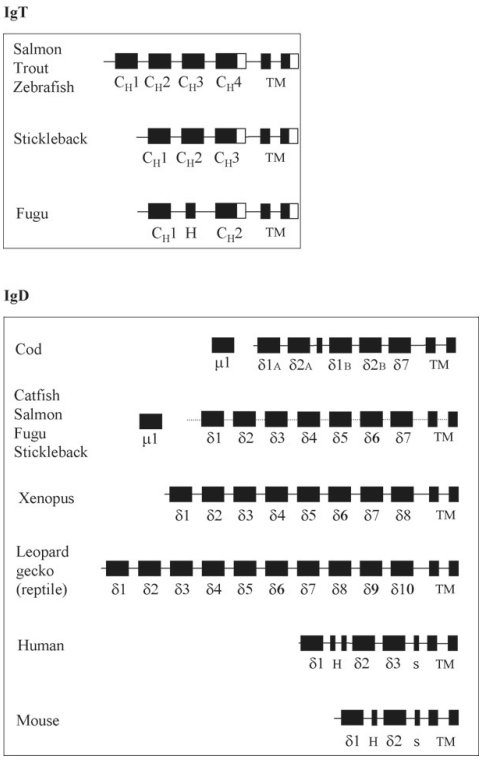

Fig. 4. IgT and IgD gene structures in different species. Exons encoding constant Ig domains, hinge regions and transmembrane/cytoplasmatic peptides are indicated, illustrating the diversity of overall structures in different animals. A number of teleost fish have IgD molecules with seven distinct δ domains. However, internal duplications are common, implicating that the molecules are considerably longer.

between the variable Ig domain and the δ chain. In cows and sheep, the first constant domain of IgD (δ1) shares almost the same sequence as their IgM μ1 domain (Zhao et al., 2002). In pigs, the δ1 domain is highly similar to μ1, and transcripts which includes μ1 instead of δ1 are also observed (Zhao et al., 2003). It is difficult to draw firm conclusions about the phylogenetic relationships of IgT, IgM and IgD, partly because non-random processes obviously are involved in the evolution of these genes. Furthermore, as illustrated in Fig. 4, the overall structure of IgD occurs in many different forms, varying from a gene comprising eight or nine constant Ig domains in non-mammalian vertebrates, to three (or two in mouse) C_H domains, and a hinge segment, in eutherian mammals (Wilson et al., 1997; Zhao et al., 2002; Ohta and Flajnik, 2006; Rogers et al., 2006; Zhao et al., 2006). In a previous study we suggested that the IgD heavy chain, comprising seven distinct Ig δ domains in salmon, arose by internal duplications from a smaller primordial molecule (Hordvik et al., 1999). Clustering of δ2 and δ5, δ3 and δ6, and δ4 and δ7 indicated that a fragment

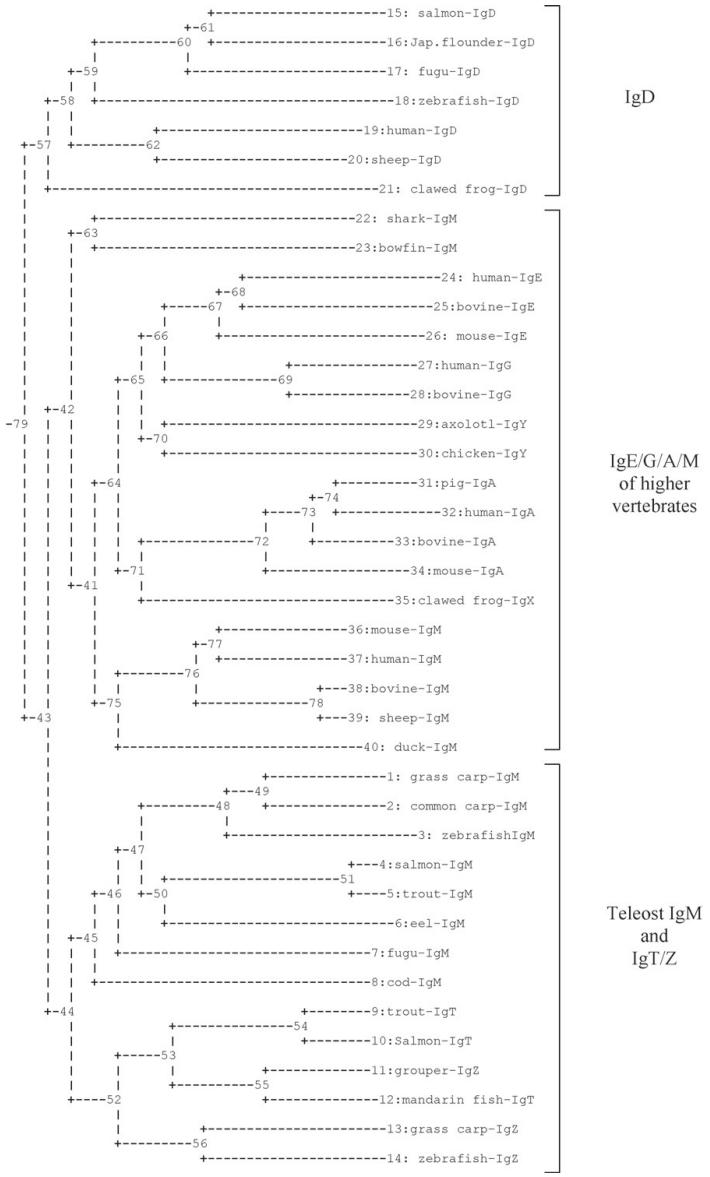

Fig. 5. Phylogenetic tree constructed from a series of Ig isotypes from different organisms using the ClustalW Multiple Alignment tool. IgT/Z sequences: salmon, ACX50291; trout, AY870268; mandarin fish, DQ016660; zebrafish, AAT67444; grouper, GU182366; grass carp, DQ478943. IgM: salmon, AAB24064; trout, AAB27359; eel, ACD76833; grass carp, ABD76396; common carp, BAA34718; cod, CAA41680; fugu, BAD26619, zebrafish AAT67445; human, CAC20458; mouse, AAB59650; bovine, AAC71048; duck, CAC43061; sheep, AAA51379; Shark, AAU04511; bowfin, AAC59687. IgD: salmon, AF141607; fugu, BAD34542.1; Japanese flounder, BAB41204; zebrafish, CAI11477; clawed frog, ABC75541; human, AAA52771.1; sheep, AAN03671.1. IgX: clawed frog, CAA32027. IgA: human, AAH65733; pig, AAA65943; mouse, CAA24161; bovine, AAC98391. IgG: Human, CAA27268; bovine, AAB37380. IgE: human, AAB59395; bovine, AAB09546.2; mouse, AAZ05128. IgY: axolotl, CAA49247; chicken, CAA30161. All IgT/IgM sequences used here are secreted forms, unless otherwise stated. Midpoint-rooted NJ tree was constructed with multiple alignment gap penalties of 10.0 (gap open penalty), and 0.05 (gap extension penalty), gonnet series weight matrix, and 1000 bootstrap replications.

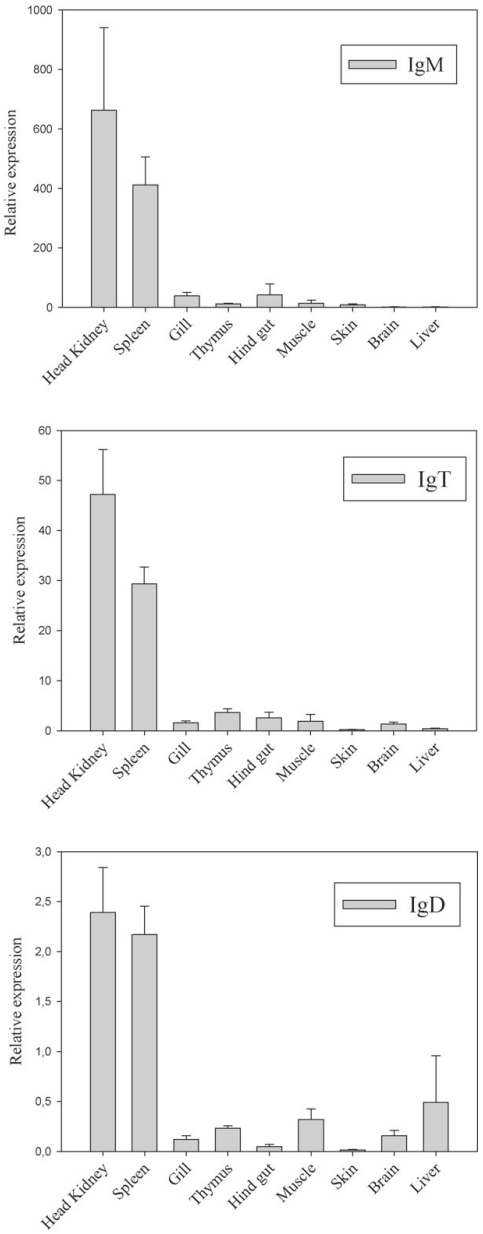

Fig. 6. Relative expression analysis of IgT, IgM, and IgD in various tissues using 1-step real time quantitative PCR, based on assays that measure the overall expression of the respective Ig genes. Data represents mean values (±SEM) of $n = 7$ (head kidney and skin), $n = 6$ (spleen), $n = 4$ (hind gut and gill), and $n = 3$ in all other tissues. The results are given in percentage relative to elongation factor 1A-A (100%).

comprising δ2, δ3 and δ4 was duplicated to generate the long IgD molecule. This hypothesis was recently supported by a larger body of data (Deza et al., 2009).

IgT overall structure varies to some degree (Fig. 4), but typically, transcripts of both membrane-bound and secreted forms are generated from these genes. Since IgT appears to be specific for teleosts; having four constant Ig domains in several species and an mRNA splicing pattern resembling that in shark and higher vertebrate IgM genes, it is tempting to speculate upon whether the IgT gene could have arisen during the diploidization process, e.g. by an unequal crossing-over event, after the whole genome duplication that occurred early in the evolution of bony fish (Jaillon et al., 2004; Dehal and Boore, 2005; Brunet et al., 2006). Interestingly, teleost IgT and IgM cluster together when constructing trees of a series of Ig isotypes from different animals (Fig. 5).

5. Concluding remarks

In the present study, IgT sequence information was collected and a survey of all types of Ig heavy chain gene transcripts was conducted in order to develop reliable qPCR assays and quantify the relative abundance of different Ig isotypes in representative tissues. Immediately before submission of the present paper BAC clones spanning the complete A and B heavy chain loci were submitted to GenBank (GU129139.1, GU129140.1), showing that there are several IgT genes and pseudogenes in salmon. However, all relevant τ, μ and δ sequences were included in the present study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetimm.2010.07.024.

References

Bao, Y., Wang, T., Guo, Y., Zhao, Z., Li, N., Zhao, Y., 2010. The immunoglobulin gene loci in the teleost Gasterosteus aculeatus. Fish Shellfish Immunol. 28, 40–48.

Bengten, E., Quiniou, S.M.A., Stuge, T.B., Katagiri, T., Miller, N.W., Clem, L.W., Warr, G.W., Wilson, M., 2002. The IgH locus of the channel catfish, Ictalurus punctatus, contains multiple constant region gene sequences: Different genes encode heavy chains of membrane and secreted IgD. J. Immunol. 169, 2488–2497.

Bromage, E.S., Ye, J., Kaattari, S.L., 2006. Antibody structural variation in rainbow trout fluids. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 143, 61–69.

Brunet, F.G., Crollius, H.R., Paris, M., Aury, J.M., Gibert, P., Jaillon, O., Laudet, V., Robinson-Rechavi, M., 2006. Gene loss and evolutionary rates following whole-genome duplication in teleost fishes. Mol. Biol. Evol. 23, 1808–1816.

Danilova, N., Bussmann, J., Jekosch, K., Steiner, L.A., 2005. The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z Nat. Immunol. 6, 295–302.

Dehal, P., Boore, J.L., 2005. Two rounds of whole genome duplication in the ancestral vertebrate. PLoS Biol. 3, e314.

Deza, F.G., Espinel, C.S., Mompo, S.M., 2009. Presence of a unique IgT on the IGH locus in Gasterosteus aculeatus and the very recent generation of a repertoire of VH genes. Dev. Comp. Immunol. 34, 114–122.
Hansen, J.D., Landis, E.D., Phillips, R.B., 2005. Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. Proc. Natl. Acad. Sci. U. S. A. 102, 6919–6924.
Harstad, H., Lukacs, M.F., Bakke, H.G., Grimholt, U., 2008. Multiple expressed MHC class II loci in salmonids; details of one non-classical region in Atlantic salmon (Salmo salar). BMC Genomics 9, 193.
Hirono, I., Nam, B.H., Enomoto, J., Uchino, K., Aoki, T., 2003. Cloning and characterisation of a cDNA encoding Japanese flounder Paralichthys olivaceus IgD. Fish Shellfish Immunol. 15, 63–70.
Hordvik, I., 1998. The impact of ancestral tetraploidy on antibody heterogeneity in salmonid fishes. Immunol. Rev. 166, 153–157.
Hordvik, I., 2002. Identification of a novel immunoglobulin delta transcript and comparative analysis of the genes encoding IgD in Atlantic salmon and Atlantic halibut. Mol. Immunol. 39, 85–91.
Hordvik, I., Berven, F.S., Solem, S.T., Hatten, F., Endresen, C., 2002. Analysis of two IgM isotypes in Atlantic salmon and brown trout. Mol. Immunol. 39, 313–321.
Hordvik, I., Thevarajan, J., Samdal, I., Bastani, N., Krossoy, B., 1999. Molecular cloning and phylogenetic analysis of the Atlantic salmon immunoglobulin D gene. Scand. J. Immunol. 50, 202–210.
Hordvik, I., Voie, A.M., Glette, J., Male, R., Endresen, C., 1992. Cloning and sequence analysis of two isotypic IgM heavy chain genes from Atlantic salmon, Salmo salar L. Eur. J. Immunol. 22, 2957–2962.
Hu, Y.L., Xiang, L.X., Shao, J.Z., 2010. Identification and characterization of a novel immunoglobulin Z isotype in zebrafish: implications for a distinct B cell receptor in lower vertebrates. Mol. Immunol. 47, 738–746.
Jaillon, O., Aury, J.M., Brunet, F., Petit, J.L., Stange-Thomann, N., Mauceli, E., Bouneau, L., Fischer, C., Ozouf-Costaz, C., Bernot, A., Nicaud, S., Jaffe, D., Fisher, S., Lutfalla, G., Dossat, C., Segurens, B., Dasilva, C., Salanoubat, M., Levy, M., Boudet, N., Castellano, S., Anthouard, V., Jubin, C., Castelli, V., Katinka, M., Vacherie, B., Biemont, C., Skalli, Z., Cattolico, L., Poulain, J., De Berardinis, V., Cruaud, C., Duprat, S., Brottier, P., Coutanceau, J.P., Gouzy, J., Parra, G., Lardier, G., Chapple, C., McKernan, K.J., McEwan, P., Bosak, S., Kellis, M., Volff, J.N., Guigo, R., Zody, M.C., Mesirov, J., Lindblad-Toh, K., Birren, B., Nusbaum, C., Kahn, D., Robinson-Rechavi, M., Laudet, V., Schachter, V., Quetier, F., Saurin, W., Scarpelli, C., Wincker, P., Lander, E.S., Weissenbach, J., Roest Crollius, H., 2004. Genome duplication in the teleost fish Tetraodon nigroviridis reveals the early vertebrate proto-karyotype. Nature 431, 946–957.
Kokubu, F., Hinds, K., Litman, R., Shamblott, M.J., Litman, G.W., 1988. Complete Structure and Organization of Immunoglobulin Heavy-Chain Constant Region Genes in a Phylogenetically Primitive Vertebrate. EMBO J. 7, 1979–1988.
Moore, L.J., Somamoto, T., Lie, K.K., Dijkstra, J.M., Hordvik, I., 2005. Characterisation of salmon and trout CD8 alpha and CD8 beta. Mol. Immunol. 42, 1225–1234.
Ohta, Y., Flajnik, M., 2006. IgD, like IgM, is a primordial immunoglobulin class perpetuated in most jawed vertebrates. Proc. Natl. Acad. Sci. U.S.A. 103, 10723–10728.
Olsvik, P.A., Lie, K.K., Jordal, A.E., Nilsen, T.O., Hordvik, I., 2005. Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. BMC Mol Biol 6, 21.
Press, C.M., Evensen, Ø., 1999. The morphology of the immune system in teleost fishes. Fish Shellfish Immunol. 9, 309–318.
Rogers, K.A., Richardson, J.P., Scinicariello, F., Attanasio, R., 2006. Molecular characterization of immunoglobulin D in mammals: immunoglobulin heavy constant delta genes in dogs, chimpanzees and four old world monkey species. Immunology 118, 88–100.
Rombout, J.H.W.M., Joosten, E.P.E.M., 1998. Immunology of fishes. In: Pastoret, P.P., Griebel, P., Bazin, H., Govaerts, A. (Eds.), Handbook of Vertebrate Immunology. Academic Press, London, pp. 1–43.
Saha, N.R., Suetake, H., Kikuchi, K., Suzuki, Y., 2004. Fugu immunoglobulin D: a highly unusual gene with unprecedented duplications in its constant region. Immunogenetics 56, 438–447.
Saha, N.R., Suetake, H., Suzuki, Y., 2005. Analysis and characterization of the expression of the secretory and membrane forms of IgM heavy chains in the pufferfish, Takifugu rubripes. Mol. Immunol. 42, 113–124.
Savan, R., Aman, A., Sato, K., Yamaguchi, R., Sakai, M., 2005a. Discovery of a new class of immunoglobulin heavy chain from fugu. Eur. J. Immunol. 35, 3320–3331.
Savan, R., Aman, A., Nakao, M., Watanuki, H., Sakai, M., 2005b. Discovery of a novel immunoglobulin heavy chain gene chimera from common carp (Cyprinus carpio L.). Immunogenetics 57, 458–463.
Stenvik, J., Jorgensen, T.O., 2000. Immunoglobulin D (IgD) of Atlantic cod has a unique structure. Immunogenetics 51, 452–461.
Stenvik, J., Schroder, M.B., Olsen, K., Zapata, A., Jorgensen, T.O., 2001. Expression of immunoglobulin heavy chain transcripts (VH-families, IgM, and IgD) in head kidney and spleen of the Atlantic cod (Gadus morhua L.). Dev. Comp. Immunol. 25, 291–302.
Sunyer, O., Zhang, Y.-A., Li, J., Parra, D., LaPatra, S.E., 2009. Is IgT the evolutionary equivalent of IgA? Insights into its structure and function. J. Immunol., 182.
Tian, J.Y., Sun, B.J., Luo, Y.P., Zhang, Y.A., Nie, P., 2009. Distribution of IgM, IgD and IgZ in mandarin fish, Siniperca chuatsi lymphoid tissues and their transcriptional changes after Flavobacterium columnare stimulation. Aquaculture 288, 14–21.
Wilson, M., Bengten, E., Miller, N.W., Clem, L.W., Du Pasquier, L., Warr, G.W., 1997. A novel chimeric Ig heavy chain from a teleost fish shares similarities to IgD. Proc. Natl. Acad. Sci. U.S.A. 94, 4593–4597.
Wilson, M.R., Ross, D.A., Miller, N.W., Clem, L.W., Middleton, D.L., Warr, G.W., 1995. Alternate pre-mRNA processing pathways in the production of membrane IgM heavy chains in holostean fish. Dev. Comp. Immunol. 19, 165–177.
Zhao, Y., Kacskovics, I., Pan, Q., Liberles, D.A., Geli, J., Davis, S.K., Rabbani, H., Hammarstrom, L., 2002. Artiodactyl IgD: the missing link. J. Immunol. 169, 4408–4416.
Zhao, Y., Pan-Hammarstrom, Q., Kacskovics, I., Hammarstrom, L., 2003. The porcine Ig delta gene: unique chimeric splicing of the first constant region domain in its heavy chain transcripts. J. Immunol. 171, 1312–1318.
Zhao, Y., Pan-Hammarstrom, Q., Yu, S., Wertz, N., Zhang, X., Li, N., Butler, J.E., Hammarstrom, L., 2006. Identification of IgF, a hinge-region-containing Ig class, and IgD in Xenopus tropicalis. Proc. Natl. Acad. Sci. U.S.A. 103, 12087–12092.

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Analysis of polymeric immunoglobulin receptor- and CD300-like molecules from Atlantic salmon

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Teleost

ABSTRACT

The polymeric immunoglobulin receptor (pIgR) plays a pivotal role in mucosal immune protection by transporting secretory immunoglobulins to mucosal epithelia, and protecting them from proteolytic degradation. It has been reported that a homolog of the pIgR has a similar role in teleost fish. Considering the role pIgR has in mucosal defenses, this study was initiated to characterize a possible pIgR homolog in Atlantic salmon (*Salmo salar*) and its relatedness to pIgR of other vertebrates and similar molecules. Two pIgR-like cDNAs and genes of Atlantic salmon (Salsal pIgR and Salsal pIgRL) were cloned and analyzed. In addition, we gathered sequence information of CMRF35-like molecules (CLM) 1, 7, and 8 (designated as CD300 in humans) and made a comparative evaluation to that of the Salsal pIgR and Salsal pIgRL polypeptides. Salsal pIgR and Salsal pIgRL, like pIgR in other teleosts, are composed of two IG V domains, a connecting, a transmembrane, and a cytoplasmic region. The same holds true for Atlantic salmon CLM1 and CLM7, except that they possess putative immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic tails. The abundance of Salsal pIgR transcript is significantly higher than Salsal pIgRL and CLM in the skin, while Salsal pIgRL transcripts were abundant in the gills, depicting their possible tissue-specific role in mucosal immunity. To further highlight the roles of these molecules in cutaneous mucosal defence, we compared their transcriptional changes in salmon skin and spleen infected with the ectoparasite *Lepeophtheirus salmonis* which targets skin and mucus of salmonid fish (sampled 3, 14 and 28 days post infection (dpi)). Salsal pIgR and Salsal pIgRL transcripts significantly increased after 14 dpi in skin and spleen. CLM1 was up-regulated in skin and down-regulated in spleen, possibly indicating that CLM1 expressing cells had migrated to the target site. Homology modeling using human pIgR domain 1 (PDB 1xed) identified structurally equivalent residues on both Salsal pIgR and Salsal pIgRL, and the same domain disulphide bridge topology. Cysteines 42 and 50 (IMGT numbering) are 7 residues apart in all V domains of Salsal pIgR, Salsal pIgRL, and mammalian [D1].

1. Introduction

Both mucosal and systemic immune compartments are essential for effective maintenance of immune homeostasis. It has been proposed that the specialization of IG isotypes into these compartments is a common feature of all jawed vertebrate immune systems (Flajnik, 2010; Zhang et al., 2010). The mucosal immune system protects the body from first-encounter of pathogens, playing a major defence role against invaders (Brandtzaeg, 1995; Kaetzel, 2005; Woof and Mestecky, 2005). In higher vertebrates, a key player of mucosal immunity is secretory immunoglobulin A (sIgA), which is produced by epithelial plasma cells and transported into apical surfaces by the polymeric immunoglobulin receptor (pIgR) (Brandtzaeg, 1995; Kaetzel, 2005). pIgR is a transmembrane glycoprotein that delivers multimeric immunoglobulins (IgA, and to a lesser extent IgM) into mucosal secretions (Kaetzel, 2005). A fragment of the pIgR which is bound to the IG (called the secretory component, SC) shields the molecule complex from proteolytic degradation (Kaetzel, 2005; Phalipon and Corthesy, 2003). Free SC and sIgA contribute to both innate and adaptive immune defence at mucosal surfaces in mammals (Kaetzel, 2005; Phalipon and Corthesy, 2003).

Mucosal immunity also exists in teleosts. Zhang et al. (2010) recently showed that rainbow trout (*Oncorhynchus mykiss*) IgT is dedicated to mucosal defences. Prior to this, possible involvement of teleost pIgR in the transport of polymeric IgM to the mucosal epithelia was reported in fugu (*Takifugu rubripes*), carp (*Cyprinus carpio*) and orange-spotted grouper (*Epinephelus coioides*) (Feng et al., 2009; Hamuro et al., 2007; Rombout et al., 2008).

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Mucosal surfaces of the fish are the main pathogen entry points, as the fish resides in aquatic environment, rich in pathogens and other stressors. The fish mucosa associated lymphoid tissues, including the gut, skin, and gill, represent initial barriers to pathogens (Rombout et al., 2010; Rombout and Joosten, 1998). Insight into the immune effector molecules on these tissues is crucial as it can aid in developing prophylactic measures against infection at these surfaces.

The pIgR contains several immunoglobulin superfamily (IgSF) variable (V) domains (V-LIKE-DOMAIN in IgSF other than immunoglobulin (IG) and T cell receptor (TR)). In contrast to the mammalian and avian pIgR containing five and four IgSF V domains respectively (Kaetzel, 2005; Norderhaug et al., 1999; Wieland et al., 2004), the extracellular portion of the teleost pIgR contains only two V domains (Feng et al., 2009; Hamuro et al., 2007; Rombout et al., 2008; Zhang et al., 2010). Although pIgR of non-mammalian vertebrates do not contain the conserved mammalian IG binding loops (BC, C′C″, and FG), pIgR associated with pIgA was detected in chicken (Wieland et al., 2004) and *Xenopus* (Braathen et al., 2007). In rabbits, a splice variant having three V domains (lacking [D2] and [D3]) was also able to bind pIgA (Kuhn and Kraehenbuhl, 1981). There is evidence that a two-domain containing teleost pIgR may function similarly (Feng et al., 2009; Hamuro et al., 2007; Rombout et al., 2008; Zhang et al., 2010). A delicate aspect of putative teleost pIgRs is that in addition to being shorter, they share very low amino acid sequence identity to pIgR of higher vertebrates. Besides, the presence of structurally and phylogenetically similar protein products in teleosts necessitates the need for analysis of related molecules.

When searching for teleost pIgR-like sequences in GenBank, top scores for pIgR are followed by CMRF35-like molecules (CLM1 and CLM7). These molecules, designated as CD300 in humans, are also members of the IgSF with V domains similar to those of pIgR (Green et al., 1998). They modulate a number of cell processes via their paired activating and inhibitory receptor functions (Clark et al., 2009a,b; Ju et al., 2008). While there is considerable information about similar molecules in higher vertebrates, CLM have not been described in teleost fish. Other similar molecules include novel immunoglobulin-like transcripts (NILT) and novel immune-type receptors (NITR) which are exclusive to teleosts, reported in rainbow trout, carp, and Atlantic salmon (Kock and Fischer, 2008; Ostergaard et al., 2009; Stet et al., 2005). Like in teleost pIgR, they often contain 1–2 V domains, followed by a connecting (CO), a transmembrane (TM), and a cytoplasmic (CY) region. Despite their similarities to the pIgR, a feature of NILT, NITR, CD300, and CLM is that many of them contain immunoreceptor tyrosine-based inhibitory motifs (ITIM) and/or immunoreceptor tyrosine-based activating motifs (ITAM) in their cytoplasmic regions.

Considering the pivotal role pIgR has in mucosal defenses, this study was initiated to clone and characterize a possible pIgR homolog in Atlantic salmon. Two pIgR-like cDNAs and genes of Atlantic salmon (Salsal pIgR and Salsal pIgRL) were identified, revealing their genomic organization, expression, similarity to known pIgR of other vertebrates, synteny, and 3D structural prediction. In addition, we gathered sequence information of CD300-like molecules (CLM1, CLM7 and CLM8), and made a comparative evaluation to that of Salsal pIgR and Salsal pIgRL. RT-qPCR was used to analyze the relative abundance of transcripts encoding for Salsal pIgR, Salsal pIgRL, and CLM proteins in skin, spleen, head kidney, hindgut, gill, and liver of healthy fish. To further highlight the role of Salsal pIgR, Salsal pIgRL, and CLM in cutaneous mucosal defense, we compared their transcriptional changes in skin and spleen from salmon infected with *Lepeophtheirus salmonis* (3, 14 and 28 days post infection (dpi)). This ectoparasitic copepod infects skin and mucus of salmonid fish. Since it has been demonstrated that domain 1 ([D1]) of the human pIgR is an appropriate model for structural prediction of other Ig-like domains (Hamburger et al., 2004), we used the same model as a template for 3D structural prediction of [D1] and [D2] of Salsal pIgR and Salsal pIgRL.

2. Materials and methods

2.1. Fish

Atlantic salmon in the size range of 100–200 g were obtained from the Aquatic and Industrial Laboratory at the High Technology Center in Bergen, Norway. Tissues of skin, gill, hindgut, spleen, head kidney and liver were collected from naïve fish. In addition, fish were challenged with *L. salmonis* (approximately 60 copepods per fish), as described in (Tadiso et al., 2011b). Sampling of skin and spleen tissues was done at 3, 14, and 28 days post infection (dpi).

2.2. RNA extraction and cDNA synthesis

RNA extraction was done by following the iPrep™ TRIzol® Plus kit (Invitrogen). The quantity and quality of the total RNA was assessed using a NanoDrop Spectrophotometer (NanoDrop Technologies). cDNA was synthesized by reverse transcription of RNA using the qScript™ cDNA Synthesis Kit (Quanta BioSciences). Total RNA and cDNA were stored at −80 and −20 °C respectively until use.

2.3. PCR and cloning of Salsal pIgR and Salsal pIgRL

Two putative full-length Atlantic salmon pIgR like cDNAs were cloned from spleen tissue by 3′ RACE using gene-specific primers from the conserved regions (Supplementary Table 1). For genomic DNA amplification, PCR was performed using appropriate gene specific primers (Supplementary Table 1) and AccuPrime™ *Taq* DNA Polymerase High Fidelity (Invitrogen). The PCR fragments obtained were TA cloned according to the manufacturer's protocol (TOPO TA cloning® kit for sequencing, Invitrogen). All PCR products were purified and ligated into the pCR®4-TOPO vector (Invitrogen) and positive clones were screened using the M13 forward and reverse primers included in the cloning kit. Plasmid DNA was isolated from positive clones (miniprep, Qiagen) and sequencing was performed using BigDye Version 3.0 fluorescent chemistry and run on an ABI Prism 7700 automated sequencing apparatus (Applied Biosystems). Sequences were assembled using NTI Vector 6.0 software and intron/exon patterns deduced from alignments of genomic and cDNA sequences. cDNA and genomic sequences were submitted to GenBank (Salsal pIgR: GQ892056, GQ892057; Salsal pIgRL: HM452378, HM452379).

Sequences were translated using Expasy translate tool (http://us.expasy.org/tools/dna.html) and aligned with ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Prediction of signal peptide and TM regions was confirmed by SignalP (http://cbs.dtu.dk/services/SignalP/) and SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/) softwares. Potential *O*-glycosylation sites were predicted by the online tool: http://www.cbs.dtu.dk/services/NetOGlyc/ (Julenius et al., 2005). Phylogenetic trees were constructed using MEGA 5.0 software (Tamura et al., 2007).

2.4. Real time quantitative PCR

For gene expression analysis, TaqMan® probe-based Real-Time PCR assays were designed using Primer express 3.0 software (Applied Biosystems). When possible, primer/probes were designed to span between two exons to avoid detection of genomic DNA (Table 1). Quantitative PCR was performed using 7500 Real-Time PCR System, and RNA was quantified in a two step

Table 1
RT-qPCR primers and probes.

Target	Forward primer 5′3′	Reverse primer 5′3′	Probe 5′3′
pIgR	CACAGCCTCCCTTCACATCA	TCCGCTCACCATGCTGTTC	TGTTATCCAAGGTATGTCTG
pIgRL	CAAAGTATCCGTGGACCTCACA	CCCCCCTCCTCACCAGATA	TCAGTGGTTGATGAGCTG
CLM1	TCCACAGGTACTGCAGGACTCTAT	CCAGCAGAAACCTCCAGAGTTAC	TGTCACTGTCAATTGTAAC
CLM7	CCTCATCTTCTACAGACTCTCAGCA	TCATAGCTACATGGGAAGGTGATG	TGTGAAGACAGGAGGCT
CLM8	TCCACATCTCCTGCCAGTATCA	CCCCTGCACCAGAATTTGA	CAGTTCAACAGGGACAAG
EF1A	CCCCTCCAGGACGTTTACAAA	CACACGGCCCACAGGTACA	ATCGGTGGTATTGGAAC

TaqMan® based assay (Applied Biosystems). The PCR reaction mix contained TaqMan® Universal Master mix (2×), 900 nM of each primer, 200 nM of MGB probe and 2 μl (50–100 ng) of cDNA in a final volume of 12.5 μl. Thermal cycling was carried out according to the manufacturers protocol (Applied Biosystems) as follows: an enzyme activation step of 95 °C for 10 min was followed by 40 cycles of 95 °C for 15 s (denaturation) and 60 °C for 60 s (annealing/extension). All samples were run in duplicate with non-reverse transcription and non-template controls on the same plate. Relative expression of mRNA was calculated using the $2^{-\Delta Ct}$ (healthy tissues) and $-\Delta\Delta Ct$ method (on infected tissues in relation to uninfected control) with elongation factor 1 alpha (EF1A) as internal reference gene (Olsvik et al., 2005).

2.5. Homology modeling and structural analysis of Salsal pIgR and Salsal pIgRL

Modeling and structure comparison of Salsal pIgR and Salsal pIgRL [D1] and [D2] was done using chain F of [D1] of human pIgR (PDB 1xed) with structural resolution of 1.9 angstrom (Hamburger et al., 2004). 1xed was found to be a good template (Identity ~ 25%, Similarity ~ 38% on average) for modeling using BLAST (www.ncbi.nih.nlm.gov) and SWISS-MODEL (http://swissmodel.expasy.org). This template satisfied the additional constraint of possible disulphide bond between the cysteine residues in the studied protein sequence. In the modeled structures, WHAT-IF analysis (http://swift.cmbi.ru.nl/servers/html/listcys.html) showed the possibility of cysteine bridges. RMSD calculations were done using DALI (http://ekhidna.biocenter.helsinki.fi/dali_lite/start). The Anolea and Gromos scores were in favorable negative range for most of the modeled residues with final energy lower than 2500 kJ/mol in average. The Modeller showed a mean DOPE score of ~8500 and GA341 ~0.6 for all the models on average. Visualisation and presentation of the models were done using RASMOL (http://openrasmol.org/) with command-line interaction.

2.6. Synteny/homology mapping

Genomic DNA sequences were subjected to BLAST search against the Ensembl database and the best hits were determined. Then, regions spanning +/−1 Mb around the best hits were analyzed. Hits which had an E-value <0.05, alignment-length >50 bp and percent identity >50% were considered. In addition, a region spanning 3 Mb in zebrafish chromosome 2 which harbors a cluster of pIgR-like sequences was aligned with the human genome database.

3. Results

3.1. pIgR cloning and sequence analysis

In this study two putative full-length Atlantic salmon pIgR-like genes were cloned and analyzed. The assumed true homolog of other teleost pIgRs was named Salsal pIgR, whereas a related molecule was named Salsal pIgRL.

The Salsal pIgR cDNA consists of 1361 bp, which includes 28 bp 5′UTR, an ORF of 1056 bp encoding 352 amino acids, and 277 bp 3′UTR (Supplementary Fig. 1A). The Salsal pIgRL cDNA contains 1406 bp, which includes 138 bp 5′UTR, an ORF of 1128 bp encoding 376 amino acids, and 137 bp 3′UTR (Supplementary Fig. 1B). Salsal pIgR sequences from the Norwegian Atlantic salmon, as compared to ESTs reported from Canada contain a tandem repeat of 4 codons (SVSP) in the connecting region. Compared to a recently reported rainbow trout pIgR sequence, Salsal pIgR contains tandem repeats of 12 additional codons $(SVSP)_3$ in the same region. Our reported Salsal pIgR gene, assembled from many overlapping fragments, is 5.1 kb, composed of eight exons and seven introns; while the Salsal pIgRL gene is 2670 bp long, with 6 exons and 5 introns (Fig. 1). The number of introns and the intron/exon pattern of the Salsal pIgR gene is similar to the one reported in fugu and grouper where [D2] is encoded by two exons (Feng et al., 2009; Hamuro et al., 2007). In the Salsal pIgRL gene each V domain is encoded by one exon. In human, each domain is encoded by one exon, except [D2] and [D3] which are encoded by the same exon (Krajci et al., 1992).

The translated Salsal pIgR and Salsal pIgRL sequences have an identity index of only 30%. Salsal pIgR is more similar to teleost pIgR, exhibiting amino acid sequence identity indices of 43–52% to fugu, zebrafish and grouper (Supplementary Table 2). Salsal pIgRL has 29–38% amino acid identity to the published teleost pIgR. Salsal pIgRL is very similar to the reported putative pIgR polypeptides (thus termed pIgR-like) in many aspects, i.e. it is composed of an extracellular region encoding two V domains, a connecting peptide, a transmembrane and a cytoplasmic region, it results in similar BLASTP hits, and it has typical conserved residues (e.g. Cx_7C motif). It also forms phylogenetic clusters together with teleost pIgR.

Like other teleost pIgR sequences, Salsal pIgR and Salsal pIgRL show low amino acid identity with pIgRs of higher vertebrates (Salsal pIgR has 15–18% identity whereas Salsal pIgRL has 17–21% identity). The same holds true for the transmembrane (TM) and cytoplasmic (CY) regions. However, the TM region of Salsal pIgRL and grouper pIgR exhibits ~31% identity to TM of human pIgR (Supplementary Table 2). The two V domains of Salsal pIgR and Salsal pIgRL show the highest amino acid identity to [D1] of human, which also holds true for most of the other teleosts (Supplementary Table 2). While KxWC (positions 39–42, according to the IMGT unique numbering (Lefranc et al., 2003)) motifs are highly conserved in the two V domains of teleost and [D1] and [D5] of mammalian pIgR, DxGWYWC motifs (positions 98–104) are highly conserved in [D1] and [D2] of teleost, and [D5] of higher vertebrates pIgR (Fig. 2). In human pIgR and FcαμR, and partly in teleost pIgR, there is a conserved region (G. . .CxY. . .KYW (positions 16–41)) predicted to be the binding site for IgA/IgM (Fig. 2). Within the teleost pIgR, this region is highly conserved in [D1] (71–80% identity) (Supplementary Table 2). A phylogenetic tree constructed from as many V domains as possible (including unpublished teleost pIgR sequences translated from ESTs) showed that teleost [D2] and [D1] cluster with higher vertebrates [D1] and [D5], respectively (Fig. 3).

Among the 5 characteristic amino acids of V domains (Lefranc et al., 2003; Williams and Barclay, 1988) which are conserved in human pIgR (Hamburger et al., 2004), 4 of them are conserved in

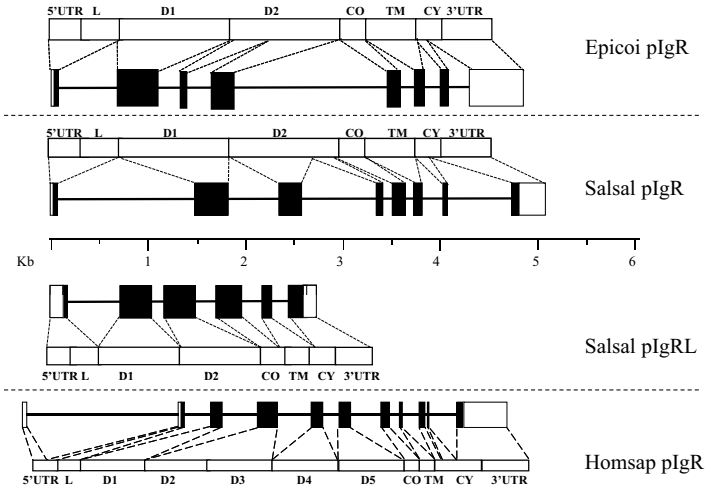

Fig. 1. Genomic organization of the Salsal pIgR and Salsal pIgRL genes. The exon–intron structures are displayed aligned to the cDNA. In both genes, the Leader (L) is encoded by exon 1, while exon 2 and 3 respectively encode for [D1] and [D2]. In Salsal pIgR, part of [D2] is encoded by exon 4, whereas exons 5, 6, and 7 respectively encode for the CO, TM, and CY regions, while part of the CY and 3′UTR are encoded by the last exon. In Salsal pIgRL, CO is encoded by exon 4 and part of exon 5, and the TM is encoded by exon 5. Part of exon 5 and exon 6 encode the CY. L, leader; CO, connecting region; TM, transmembrane region; CY, cytoplasmic region. Human (Homsap) and Grouper (Epicoi) pIgR are displayed for comparison (not to scale).

teleost pIgR (Fig. 2). In Salsal pIgR and Salsal pIgRL, the same conserved amino acids are found. They include Cys C23 (1st-CYS) and Cys C104 (2nd-CYS) (which are linked structurally by a disulphide bridge), Trp W41 (CONSERVED-TRP) (involved in the formation and stabilisation of the tertiary structure of the protein), and Asp D98. Arg R75 (which is involved in the formation of salt bridge with Asp D98) is only found conserved in mammalian [D1]. In [D5] of higher vertebrates, there is an additional cysteine residue responsible for covalent IgA binding (Fig. 2). The membrane proximal domain ([D2] in teleosts and [D5] in mammals) shows conservation of typical residues in pIgR and salmon CLM.

A prediction for functional sites on the protein sequences of Salsal pIgR and Salsal pIgRL was done using the Eukaryotic Linear Motif resource, ELM (http://elm.eu.org). The analysis indicated a number of instability motifs on both sequences in the connecting region, possibly serving as cleavage sites to generate the secretory

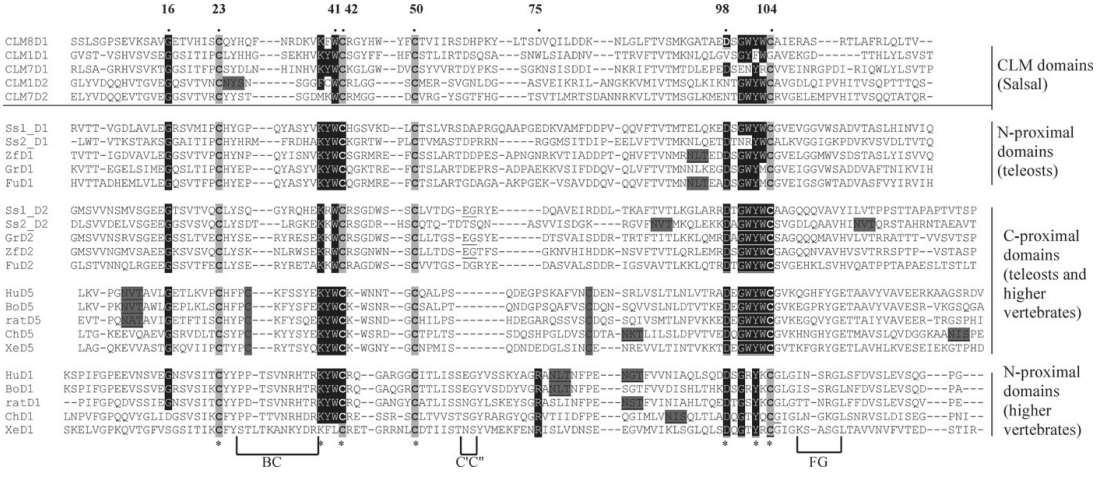

Fig. 2. Alignment of amino acid sequences of V domains of pIgR and related proteins from mammals, birds and teleost fish. The numbers of the key amino acids according to the IMGT unique numbering for V domain are indicated on top. Positions of fully conserved residues in all pIgR domains and species are indicated by (*). The positions of fully conserved cysteine residues are in bold and shaded light gray. The cysteine residues within [D5] of mammalian pIgR responsible for covalent IgA binding are shaded dark gray. All other important conserved amino acids are shown by white text in black background. DxGWYWC motif (positions 98–104), highly conserved motifs [D1] and [D2] of teleost pIgR, and [D5] of other vertebrates, are in bold and underlined. CDR-like loops (BC, C′C″ and FG) are shown below the sequence. Accession numbers for pIgR are: Gr, *Epinephelus coioides* (ACV91878.1); Fu, *Takifugu rubripes* (AB176853); Zf, *Danio rerio* (XP_001336190); Hu, *Homo sapiens* (NM_002644); rat, *Rattus norvegicus* (NP_036855); Bo, *Bos taurus* (NM_174143), Ch, *Gallus gallus* (AY233381); and Xe, *Xenopus laevis* (EF079076). Sequences of the Atlantic salmon CMRF35-like molecules (CLM), obtained from the nucleotide database, are CLM1 (NM_001140948), CLM7 (NM_001146562), and CLM8 (NP_001134362). Ss-1, Salsal pIgR; Ss-2, Salsal pIgRL; [D1], Domain 1; [D2], Domain 2; [D5], Domain 5.

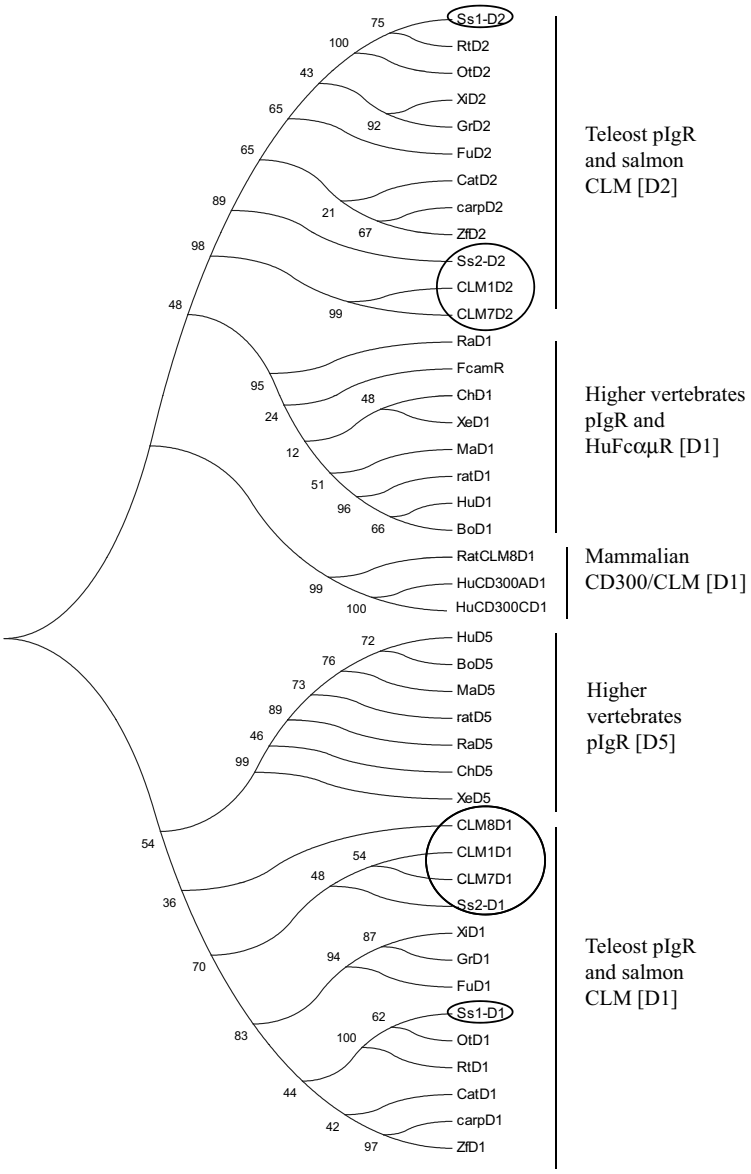

Fig. 3. Phylogenetic analyses involving 43 amino acid sequences of C and N terminal proximal pIgR V domains (i.e. [D1], [D2], [D5]) from several vertebrates, including salmon pIgRL and CLM, and human Fcαμ R and human/mouse CD300. NJ-tree was constructed using Mega 5.0 software (Tamura et al., 2007). Numbers at branch nodes indicate confidence levels obtained after 10,000 bootstrap replications. Accession numbers and species codes are as described in Fig. 2. Additional pIgR sequences included: Rt, *Oncorhynchus mykiss* (FJ940682), Ma, *Macropus eugenii* (AF317205); Ra, *Oryctolagus cuniculus* (X00412). Translated teleost pIgR ESTs: cat, *Ictalurus punctatus* (FD055077); Xi, *Xiphophorus* (FK036444); Ot, *Oncorhynchus tshawytscha* (EL562487). Others: human Fcαμ R (AAL51154.1); human CD300A (Q9UGN4.2); human CD300C (NP_006669.1); rat CLM8 (NP_001192277.1).

component during transcytosis. This region in Salsal pIgR spans from position 230–270 and include mainly phosphorylation, and glycosaminoglycan attachment sites. In Salsal pIgRL, this region is longer (245–315) (Fig. 4) and includes a number of phosphorylation sites, phosphothreonine motifs, and PDZ domains binding motifs, among others. These sites are confined to the connecting region and the prediction shows no such motifs in the V domains of Salsal pIgR and Salsal pIgRL. This is in contrast to salmon CLM where this instability motif is predicted to be distributed all over the sequence. In human pIgR this region spans residues 559–593 (Asano et al., 2004), and contains a number of cleavage and phosphorylation sites.

The connecting region is predicted to be highly *O*-glycosylated in Salsal pIgR and Salsal pIgRL (Fig. 5), allowing an extended stalk structure like for example in salmon CD8 (Moore et al., 2005). This

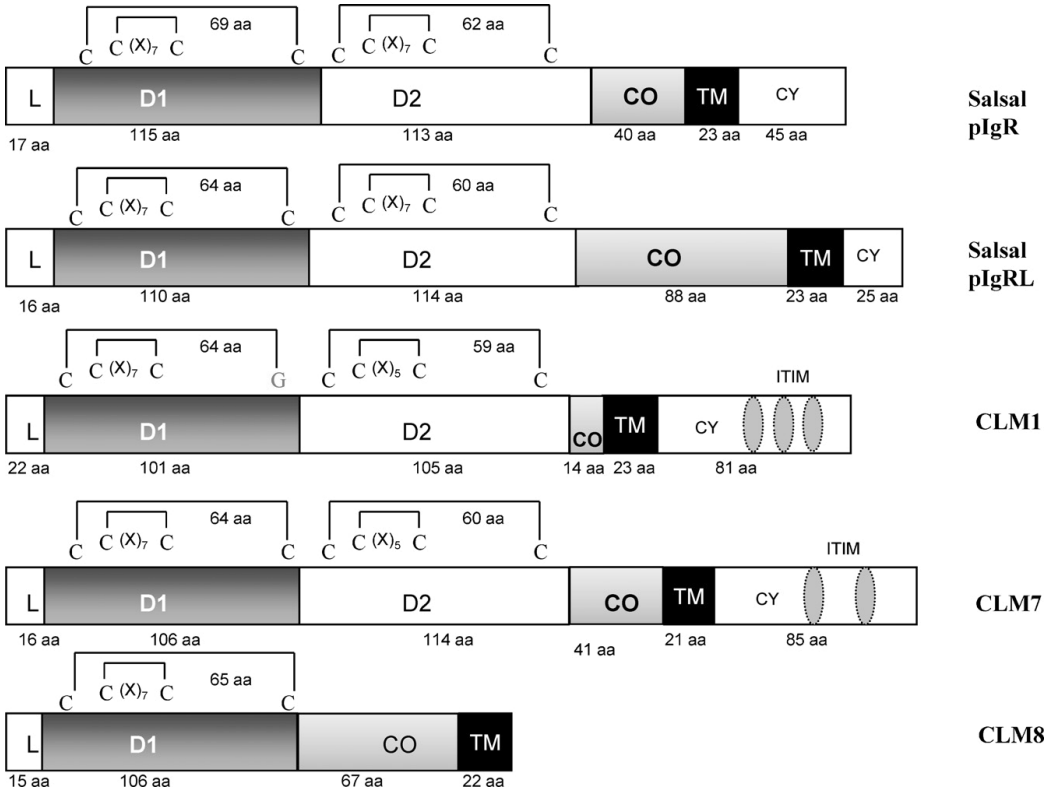

Fig. 4. The domain structure of *Salmo salar* pIgR, pIgRL, and CLM. L, leader; CO, connecting region; TM, transmembrane region; CY, cytoplasmic region. Despite a similar domain structure, CLM1 and CLM7 contain putative ITIM in their cytoplasmic regions.

is a typical feature of CMRF molecules in human (Jackson et al., 1992). However, very few *O*-glycosylation sites were found in human pIgR (Fig. 5).

3.2. Structural analysis

3D structural prediction for Salsal pIgR and Salsal pIgRL was done using [D1] of human pIgR as a template (PDB 1xed). Conserved cysteines and amino acids forming β-sheets are present in both molecules. Human pIgR exhibit the same domain disulphide bridge topology. Like in mammalian pIgR [D1], the two characteristic cysteines C23 and C104 of Salsal pIgR are separated by 69 residues. Furthermore, in all V domains of teleost pIgR and mammalian [D1], additional cysteines C42 and C50 are 7 residues apart (giving a Cx_7C motif) (Fig. 4). RASMOL as well as WHAT-IF predictions indicated that all Salsal pIgR domains form two disulphide bridges, which resulted in a similar stereochemistry (Fig. 6). Like in human pIgR, each domain is predicted to contain two disulphide bond linked β sheets composed of anti parallel β strands, B, E, D (A) and C′, C, F, G, A′ (Fig. 6A). It seems that C″ strands of Salsal pIgR and Salsal pIgRL are in a loop in the predicted structure, indicating loss of a few residues when modeling (Fig. 6B). The analysis showed that, like in human pIgR, Salsal pIgR and Salsal pIgRL domains have a single helical turn. In Salsal pIgR, Lys K96, Glu E97, and Asp D98 form a helix, whereas the equivalent residues in Salsal pIgRL are Glu E96, Thr T97, and Asp D98. The position of the α helix is shifted in this model. The FG loop is tilted towards the second sheet (C′ C F G A′), away from the BC loop (Fig. 6A). This is a typical conformation of the loops in pIgR that makes a distinction from IG V domains (Hamburger et al., 2004).

3.3. Search for syntenic regions

Mining into the Ensemble genome database (http://www.ensembl.org/index.html) using Salsal pIgR and Salsal pIgRL protein and gene sequences as a query, results in best hits to chromosome 17 of humans, chimpanzees, and orangutans, where a cluster of CD300 genes is located. This region is weakly similar with chromosome group IX of stickleback and chromosome 1 of zebrafish which harbors uncharacterized proteins; while pIgR related genes are clustered on chromosome 2 in zebrafish (51.8–52 Mb). In humans and mouse, the pIgR gene is located in close proximity to the Fc receptor for IgA and IgM (Fcα/μR) and interleukin genes on chromosome 1. No traces of such genes were found near this region in zebrafish chromosome 2. When a region spanning 50–53 Mb in zebrafish chromosome 2 was searched for orthologous regions in human, the best hits were distributed on different chromosomes. Thus, with the available sequence information it was not possible to detect synteny between teleost and human. This is also the case for the IgSF proteins such as NILT and SITR (Ribeiro et al., 2011; Stet et al., 2005).

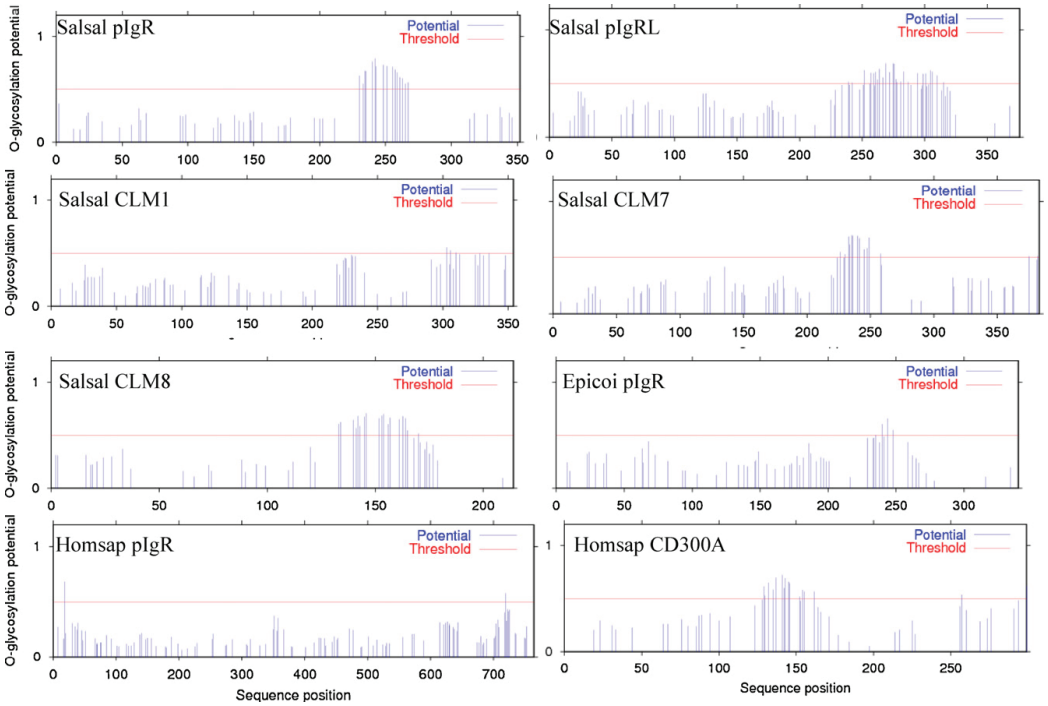

Fig. 5. Predicted *O*-glycosylation sites in the amino acid sequences of Salsal pIgR, Salsal pIgRL and Atlantic salmon CLM, compared to human pIgR and CD300A. Predicted by the NetOGlyc 3.1 Server: http://www.cbs.dtu.dk/services/NetOGlyc/ (Julenius et al., 2005).

3.4. Analysis for closely related genes

BLASTP search using Salsal pIgR and Salsal pIgRL as queries revealed similarity to CMRF35-like molecules (CLM7 and CLM1 respectively) next to a series of pIgR (E-value: 7E−27 to 1E−37) (Supplementary Tables 3 and 4). In fact, they are among the top 10 hits when zebrafish sequences, which take the lion's share of the BLAST hits, are excluded (Table 2). CLM (also known as CD300 in humans) are members of the IgSF with similar V domains to that of pIgR (Green et al., 1998). They modulate a diverse array of cell processes via their paired triggering and inhibitory receptor functions (Clark et al., 2009a,b; Ju et al., 2008). In Atlantic salmon, except being named CMRF35-like molecules (Leong et al., 2010), these proteins have not been described. Salmon CLM are structurally similar to the teleost pIgR, having a Cx_7C motif in [D1], while [D2] of CLM1 and CLM7 have a Cx_5C motif. CLM8 contains only one V domain (Fig. 4), and is similar to MDIR of *Raja eglanteria* and CLM8 of rats. In CLM1 [D1], the cysteine C104 (2nd-CYS) is replaced by glycine. The Cx_7C motif makes these molecules more similar to pIgR than NILT where $x = 3$, in most cases (Ostergaard et al., 2010; Stet et al., 2005). Moreover, in database searches for CLM related sequences in teleosts, pIgR are in the top 10 BLASTP hits (Supplementary Table 5). Salmon CLM1 and CLM7 show amino acid sequence identity of 24–27% to teleost pIgR, and ~20% to NILT. Teleost pIgR and salmon CLM1 and CLM7 V domains also cluster together in phylogenetic analysis, whereas salmon CLM8 diverges to some extent (Fig. 3). A notable difference from that of pIgR is that CLM1 and CLM7 possess putative ITIM in their cytoplasmic regions, which also holds true for teleost NILT. In salmon CLM7 and CLM8, like in Salsal pIgR and Salsal pIgRL, the connecting region is predicted to be heavily *O*-glycosylated, and CLM1 contains none (Fig. 5).

3.5. Differential expression of pIgR and CLM

RT-qPCR analysis revealed that Salsal pIgR and Salsal pIgRL are expressed in skin, gills, hindgut, spleen, and head kidney (Fig. 7A). Salsal pIgR transcripts, even though somewhat lower than that in lymphoid tissues, are up to 10 fold higher than Salsal pIgRL and CLM in skin, while Salsal pIgRL transcripts are abundant in gills (Fig. 7A). Both Salsal pIgRL transcripts significantly increased after 14 dpi in skin and spleen whereas CLM1 transcripts significantly increased in skin, with simultaneous depletion in spleen (Fig. 7B).

4. Discussion

This study was initiated to identify and characterize a pIgR homolog in Atlantic salmon. Accordingly, we first cloned a salmon counterpart of other teleost pIgRs (Salsal pIgR). A BLAST search using this sequence as query resulted in ESTs in salmon encoding for another molecule similar to Salsal pIgR, with 29–38% amino acid identity to teleost pIgR, and ~20% identity to mammalian pIgR. We then cloned the full length cDNA and gene of this molecule (Salsal pIgRL). When both sequences were subjected to BLAST searches against the salmon database, the pIgR comes to the forefront, followed by CLM7 and CLM1 respectively (E-value: 7E−27 to 1E−37). CMRF35 molecules are members of the IgSF having similar V domains as pIgR (Green et al., 1998). Salsal pIgR and Salsal pIgRL were further analyzed, with particular emphasis on their relative expression, domain structure and genomic organization. Also included were the sequence and expression analysis of salmon CLM1, CLM7, and CLM8.

Salsal pIgR and Salsal pIgRL are expressed in mucosal and lymphoid organs of Atlantic salmon, and also positively respond to

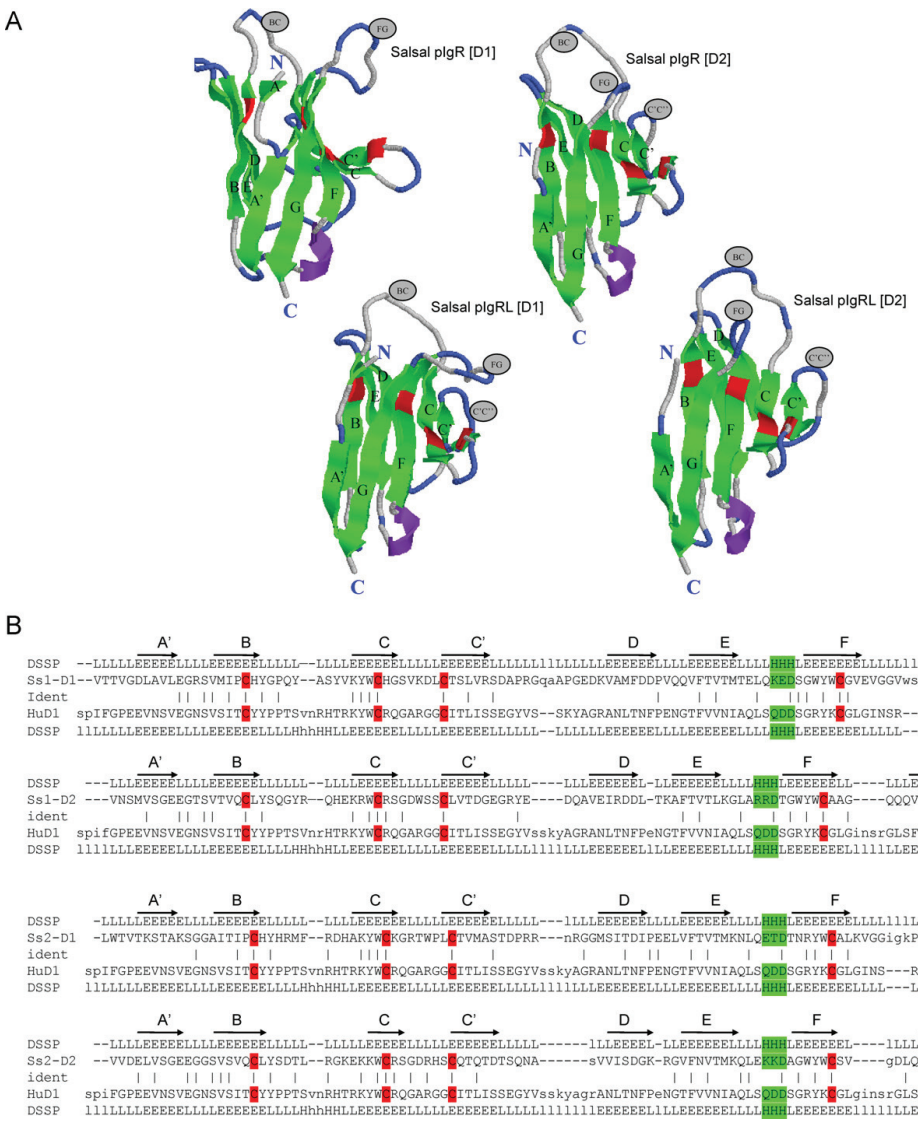

Fig. 6. (A) 3D structure model of [D1] and [D2] of Salsal pIgR and Salsal pIgRL using the human pIgR[D1] (PDB 1xed) as a template. Alpha helices, beta sheets and cysteines are shown in purple, green, and red respectively. (B) Pairwise structural alignments of human pIgR [D1], Salsal pIgR and Salsal pIgRL domains. Residues forming alpha helices are in green, and conserved cysteines are shaded red. Three-state secondary structure definitions by DSSP (reduced to H = helix, E = sheet, L = coil) are shown above the amino acid sequence. Structurally equivalent residues are in uppercase while structurally non-equivalent residues (e.g. in loops) are in lowercase. Amino acid identities are marked by vertical bars. Structural identity for Salsal pIgR [D1] and [D2] is 25%; and for Salsal pIgRL [D1] and [D2] are 25.0 and 34, respectively.

infection in skin by *L. salmonis*. It is worth noting that pIgR expression in salmon and other teleosts is not exclusive to mucosal tissues. pIgR expression levels in mucosal tissue homogenates of skin and gut are often low compared to transcript levels in head kidney and spleen, and the high pIgR transcripts possibly originate from lymphocytes which are the predominant cells in these lymphoid organs. In carp, it has been reported that IEL (Intraepithelial Lymphocytes) and lymphoid cells in the lamina propria and the thymus express the pIgR (Rombout et al., 2008). In the skin and gut, as there are relatively few cells expressing pIgR, pIgR expression is dependent on the tissue section sampled. While most of the epithelial cells in fugu skin expressed pIgR, other cells such as melanophores did not (Hamuro et al., 2007). In carp, *in situ* hybridization data showed high staining around the nuclei of skin epithelium, in contrast to the basal cells, which appeared to be less positive (Rombout et al., 2008). In accordance with pIgR expression, tetrameric IgM was present in the skin mucus and intestine of fugu, and sIgM was locally produced in the skin and intestine. IgM producing cells were distributed along the basal membrane of the skin, and lamina propria of the intestine (Hamuro et al., 2007).

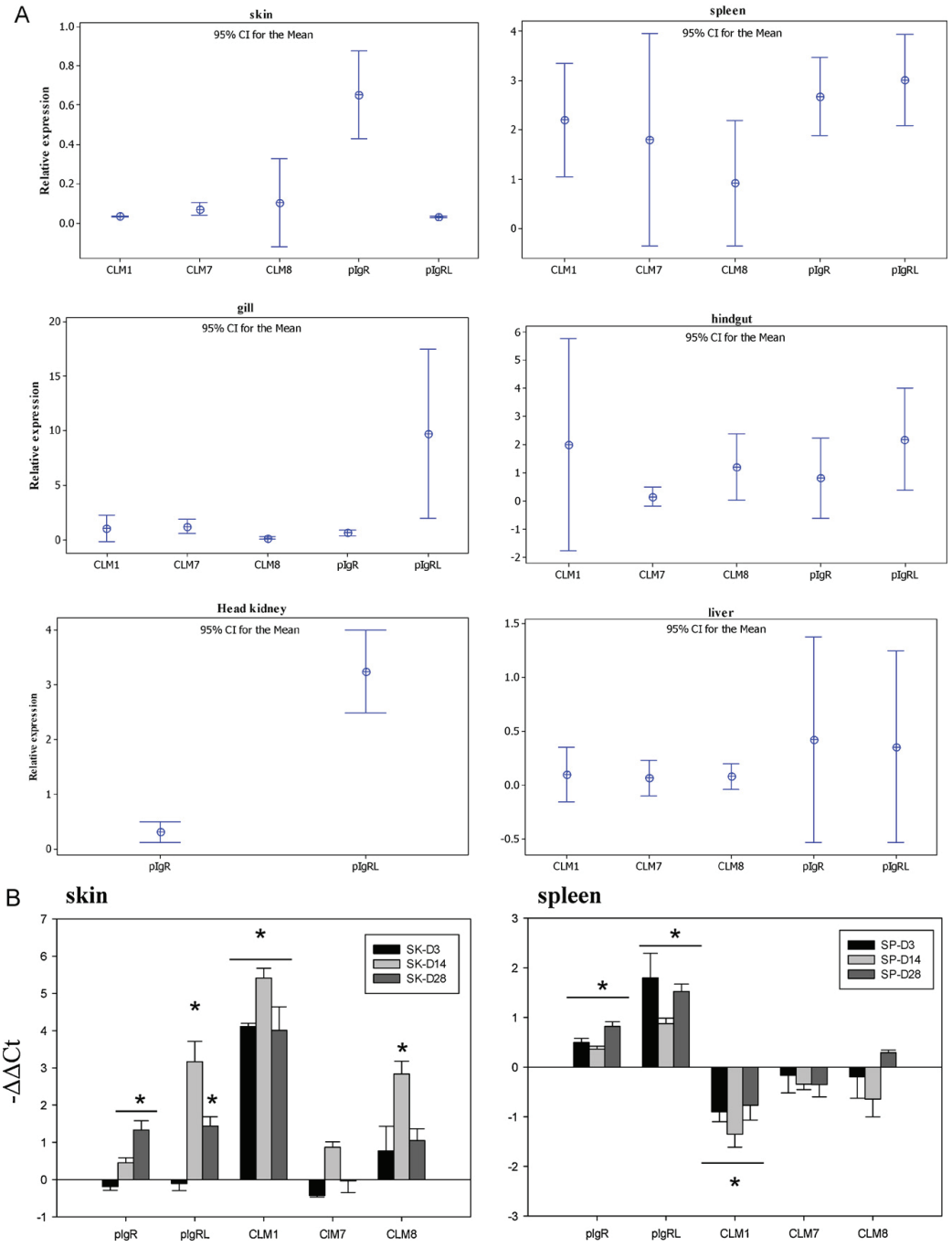

Fig. 7. Relative expression analysis of Salsal pIgR, Salsal pIgRL and CLM transcripts using TaqMan probe based real time quantitative PCR (RT-qPCR). (A) Relative expression in tissues of skin, spleen, head kidney, hindgut, gill and liver. Data represents 95% confidence interval for the mean ($n = 3$) of relative expression ratios normalized against elongation factor 1a (EF1A). (B) Relative expression of mRNA calculated using the $-\Delta\Delta$Ct method with EF1A as internal reference gene, and in relation to uninfected control. Data are mean$-\Delta\Delta$Ct $\pm$ SE. Significant differences from control ($n = 3$, $P < 0.05$) are indicated with *.

Table 2
BLASTP hits of Salsal pIgR and Salsal pIgRL (excluding zebrafish sequences).[b]

SN[a]	Accession	Description	Organism	Query coverage	E-value
Salsal pIgR					
0	ACX44838.1	pIgR	*Salmo salar*	100%	0.0
1	ADB81776.1	pIgR	*Oncorhynchus mykiss*	100%	0.0
2	ACV91878.1	pIgR	*Epinephelus coioides*	100%	1E−117
3	ADK91435.1	pIgR	*Paralichthys olivaceus*	98%	3E−109
5	ADB97624.1	pIgR	*Cyprinus carpio*	89%	6E−99
6	BAF56575.1	pIgR	*Takifugu rubripes*	92%	1E−91
7	ABQ10652.1	pIgR	*Danio rerio*	80%	1E−88
9	CAF97731.1	Unnamed protein	*Tetraodon nigroviridis*	61%	2E−53
10	ADM18014.1	pIgRL protein	*Salmo salar*	68%	8E−52
33	NP_001140034.1	CLM7	*Salmo salar*	98%	1E−34
48	ACM09186.1	CLM1 precursor	*Salmo salar*	57%	5E−27
49	NP_001134420.1	CLM1	*Salmo salar*	57%	7E−27
Salsal pIgRL					
0	ADM18014.1	pIgRL protein	*Salmo salar*	100%	0.0
5	ADB97624.1	pIgR	*Cyprinus carpio*	56%	5E−55
7	ACV91878.1	pIgR	*Epinephelus coioides*	78%	3E−53
13	ADK91435.1	pIgR	*Paralichthys olivaceus*	70%	2E−49
15	ADB81776.1	pIgR	*Oncorhynchus mykiss*	58%	4E−49
16	ACX44838.1	pIgR	*Salmo salar*	60%	4E−49
19	ABQ10652.1	pIgR	*Danio rerio*	57%	3E−48
29	BAF56575.1	pIgR	*Takifugu rubripes*	59%	2E−44
40	NP_001140034.1	CLM7	*Salmo salar*	56%	1E−37
45	ACM09186.1	CLM1 precursor	*Salmo salar*	58%	3E−36
46	NP_001134420.1	CLM1	*Salmo salar*	58%	4E−36

[a] BLASTP hits are listed according to the order they appear on the original list and full listing of the top 100 hits are available in Supplementary Tables 3 and 4.
[b] As *Danio rerio* sequences comprise ∼50% of the hits (mainly predicted pIgRL, and a number of unnamed proteins), they have been excluded from this list for simplicity.

Similarly, despite being the predominant Ig in mucosal tissues, there are only few IgT expressing cells in gut mucosa of rainbow trout (Zhang et al., 2010), and its transcript levels (e.g. in skin) are 10 times lower than that for IgM in salmon (Tadiso et al., 2011a). However, IgT can be up-regulated up to 700 fold in the gut of rainbow trout that survived infection with the parasite *Ceratomyxa shasta* (Zhang et al., 2010), and up to 10 fold in skin of salmon in response against infection with *L. salmonis* (Tadiso et al., 2011b).

The domain structures of Salsal pIgR and Salsal pIgRL are similar to teleost pIgR studied so far by having two V domains, while mammalian and avian pIgR contain five and four V domains respectively (Hamburger et al., 2004; Kulseth et al., 1995; Norderhaug et al., 1999; Wieland et al., 2004). As in humans, the first domain is encoded by one exon (Fig. 1). Structural prediction revealed that the residues encoding Salsal pIgR and Salsal pIgRL domains are structurally equivalent to [D1] of human pIgR (Fig. 6). [D1] of human pIgR has been described as sufficient for binding of polymeric IG (Bakos et al., 1994). As antibodies and their cognate antigens interact through the complementarity determining region (CDR) of IG, [D1] of mammalian pIgR non-covalently interacts with polymeric IG through its exposed CDR-like loops (BC, C′C″ and FG). In teleosts, these loops are not conserved. However, 3D structural prediction of Salsal pIgR and Salsal pIgRL showed that these loops are exposed (Fig. 6), suggesting that they may have IG binding roles.

The ability of pIgR to bind polymeric IG and mediate their transcytosis depends on the domain structure of pIgR and the nature of IG (Kaetzel et al., 1994; Norderhaug et al., 1999). Generally, ligand binding requires IG polymerization. Immune complexes containing only monomeric IG are not transported (Kaetzel et al., 1994). Whereas most mammalian IG isotypes occur solely in a monomeric (IgG, IgD, and IgE) or polymeric form (IgM), IgA is found in both forms (Woof and Mestecky, 2005). In teleosts, IgM predominantly occurs as a tetramer, while structural studies on IgT are at an infant stage. Very recently, it has been shown that rainbow trout IgT is present as a monomer in serum, and as a polymer in gut (Zhang et al., 2010), which is also the case for mammalian IgA. This structural plasticity makes IgT a suitable mucosal antibody. It has been demonstrated that ligand polymerization is a requirement for effective binding of IG with IG receptors (Ghumra et al., 2009). In this regard, a joining chain (J chain) plays a key role in IG polymerization and provision of a binding site for mammalian pIgR (Braathen et al., 2007; Brandtzaeg and Prydz, 1984). In teleosts, J chain containing antibodies have not been reported. In the human pIgR system, polymeric IgA and IgM have different binding requirements. Studies show that in terms of binding affinity, IgM is favored while IgA has a greater advantage of passive diffusion and epithelial uptake, having 6–12 fold greater external transfer compared to IgM (Natvig et al., 1997). In teleosts, however, this can be an immunological challenge. Passive immunization experiments of channel catfish (*Ictalurus punctatus*) against the parasite *Ichthyophthirius multifiliis* with murine monoclonal antibodies (mAbs) showed the failure of an IgM-class antibody to confer passive protection compared to other mAbs of IgG class (Lin et al., 1996). The amount of IgM in cutaneous mucus is extremely low; estimated to be below15 μg/ml in healthy fish (Hatten et al., 2001). The amounts of IgA in mammalian secretions exceeds the total daily production of IgG (Brandtzaeg, 1995). The values for IgT in gut mucus are even much lower than that for IgM (Zhang et al., 2010).

The predicted IG binding region of pIgR on human IgM overlaps with that of Fcα/μR (Ghumra et al., 2009). Fcα/μR, like the CD300 molecules, is a single domain containing protein with a very long connecting region, a transmembrane, and a cytoplasmic region. In catfish, a homolog of an Ig-binding receptor (FcRI) with three V domains but without transmembrane and cytoplasmic regions has been described (Stafford et al., 2006). Nevertheless, BLASTP search using FcRI and Fcα μR indicates that these molecules are distantly related to Salsal pIgR or CLM. Despite having low amino acid sequence identity to the pIgR, the Fcα μR has a conserved IgA/IgM binding motif (Shibuya et al., 2000), which is also partly conserved in salmon and other pIgRs. Within the teleost pIgR, this region is highly conserved in [D1] (Supplementary Table 2). This region is also conserved in CD300 molecules. However, the CD300 gene family has not been shown to bind IG (Clark et al., 2009b), except in human and mouse CD300LG, which is shown to interact with IgA2 and IgM (Takatsu et al., 2006). A recent study reported that IgM can bind to Fcα/μR without the need for the J chain (Yoo et al., 2011). It is tempting to speculate that J chain lacking teleost antibodies may also possibly follow this fashion. In amphibians, interaction of pIgR

to mucosal IG is possible without the need for a J chain (Mussmann et al., 1996).

In humans and mouse, the pIgR gene is located in close proximity to Fcα/μR and interleukin genes on chromosome 1(Kaetzel, 2005; Shibuya et al., 2000). However, mining into the genome database using Salsal pIgR and Salsal pIgRL as queries resulted in best hits to chromosome 17 of humans, chimpanzees, and orangutans, where a cluster of CD300 genes is located.

The CD300 proteins comprising seven members (A–G) have the ability to direct or terminate a protective immune response (Clark et al., 2009b). Among these, CD300A and CD300F (CLM8 and CLM1 in mouse) are inhibitory members with long cytoplasmic regions containing ITIM. Putative CLM in salmon (CLM1 and CLM7) fall into this category. CD300F are highly expressed in the spleen (Clark et al., 2009b). In this study, CLM1 is expressed mainly in the spleen, while it positively responds to infection by an ectoparasite in the skin, with simultaneous down-regulation in the spleen. A striking feature of the expression profiles of CLM in salmon is that their up-regulation in the skin is directly related to a decrease in their transcript levels in spleen, indicating migration to skin and depletion in spleen of these transcripts. It is worth noting that *L. salmonis* are known to modulate the immune response of Atlantic salmon (Fast et al., 2007). Thus, they may possibly stimulate secretion of these inhibitory motif containing proteins for their own good. Importantly, CLM1 in mouse has the ability to block myeloid specific responses (Chung et al., 2003). Salsal pIgR expression pattern is different from that of CLM and local proliferation of Salsal pIgR and Salsal pIgRL following infection is possible in skin. Their expression profiles imply that Salsal pIgR and CLM have different functions. Mammalian pIgR has important innate immune functions in addition to IG transport, by attaching to host and pathogenic factors (Phalipon and Corthesy, 2003).

Of particular importance is the fact that the V domains of Salsal pIgR, Salsal pIgRL and teleost pIgR are better related to the human CD300A V domain (25–30%). Furthermore, in contrast to human pIgR, human CD300A, salmon CLM7 and CLM8 (including other teleost pIgR), contain a region predicted to be heavily *O*-glycosylated. This however does not necessarily mean that teleost pIgRs are not true homologues of pIgR, partly because they contain typical common motifs, and they are expressed on mucosal tissues. A distinguishing feature of human and mouse CD300/CLM is that they are expressed mainly in cells of the myeloid lineage, and not in B or T-lymphocytes (Chung et al., 2003; Clark et al., 2009b). Besides, they often contain ITIM/ITAM in their cytoplasmic region (Clark et al., 2009b).

Salmo salar pIgR, pIgRL, and CLM sequences also show similarity to carp soluble immune-type receptor (SITR), teleost NILT as well as modular domain immune-type receptor-3 (MDIR3) of *Raja eglanteria*. SITR in carp possesses two domains and lacks transmembrane and cytoplasmic regions (Ribeiro et al., 2011). SITR [D2] shares similarities with human CD300 and Salsal pIgR, Salsal pIgRL, and CLM V domains. Particularly, this domain is 49% identical to Salsal pIgRL [D2], whereas the overall amino acid identity between SITR and Salsal pIgRL is 33% (data not shown). SITR is expressed mainly in myeloid cells (Ribeiro et al., 2011), while carp NILT are expressed in lymphoid tissues (Stet et al., 2005). NILT generally exhibits structural similarity to human natural cytotoxicity receptor (NKp44) (Stet et al., 2005). NKp44 have a Cx_7C motif and are related to salmon CLM8 (which are top in BLAST searches) and pIgR.

A series of unique IgSF proteins exist in mammals and teleosts. The various immunoglobulin isotypes, CLM in salmon, SITR in carp, NILT in carp, trout and salmon, FcRI in catfish, and the human and mouse Fcα/μR can be mentioned, among others (references are listed above). While the extracellular portions of CD300, CLM, Fc receptors, and NKp44 in mammals are encoded by one V domain, homologous molecules in teleosts (including putative pIgRs) mostly contain two V domains. Due to this, domain wise comparisons give better results while full length protein identities are very low (e.g. Salsal pIgRL vs human Fcα/μR). It is thus possible that two domain containing teleost pIgRs and CLM originate from the same ancestral gene having one domain. This hypothesis is supported by the fact that the second domains are relatively more similar between these molecules, indicating their recent emergence. This, however, requires a detailed phylogenetic analysis. Another possible mechanism contributing to this is termed exon shuffling by which an exon is duplicated or a new exon/domain is inserted into a gene/gene product (Keren et al., 2010).

5. Concluding remarks

In this study, salmon pIgR-like transcripts (Salsal pIgR and Salsal pIgRL) were isolated and a comparative assessment was done to published pIgR sequences and closely related molecules reported to GenBank. Although different features of the two Atlantic salmon pIgR-like protein products are similar, based on their similarity to the reported teleost pIgRs, Salsal pIgR is most likely to be a true homologue of the teleost pIgR. Salsal pIgR transcript levels are 10 times higher than that of Salsal pIgRL in skin. Conversely, other mucosal organs such as the gill contained a higher fraction of Salsal pIgRL transcripts, while both molecules responded clearly against cutaneous mucosal infection. However, it is not possible to rule out the possibilities of other roles. CLM in salmon are close relatives of teleost pIgRs. Their expression profiles imply that these groups of molecules have different functions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2011.09.013.

References

Asano, M., Takenouchi-Ohkubo, N., Matsumoto, N., Ogura, Y., Nomura, H., Suguro, H., Moro, I., 2004. Multiple cleavage sites for polymeric immunoglobulin receptor. Immunology 112, 583–589.

Bakos, M.A., Widen, S.G., Goldblum, R.M., 1994. Expression and purification of biologically active domain I of the human polymeric immunoglobulin receptor. Mol. Immunol. 31, 165–168.

Braathen, R., Hohman, V.S., Brandtzaeg, P., Johansen, F.E., 2007. Secretory antibody formation: conserved binding interactions between J chain and polymeric Ig receptor from humans and amphibians. J. Immunol. 178, 1589–1597.

Brandtzaeg, P., 1995. Basic mechanisms of mucosal immunity—a major adaptive defense system. The Immunologist 3, 89–96.

Brandtzaeg, P., Prydz, H., 1984. Direct evidence for an integrated function of J chain and secretory component in epithelial transport of immunoglobulins. Nature 311, 71–73.

Chung, D.H., Humphrey, M.B., Nakamura, M.C., Ginzinger, D.G., Seaman, W.E., Daws, M.R., 2003. CMRF-35-like molecule-1, a novel mouse myeloid receptor, can inhibit osteoclast formation. J. Immunol. 171, 6541–6548.

Clark, G.J., Ju, X., Azlan, M., Tate, C., Ding, Y., Hart, D.N., 2009a. The CD300 molecules regulate monocyte and dendritic cell functions. Immunobiology 214, 730–736.

Clark, G.J., Ju, X., Tate, C., Hart, D.N., 2009b. The CD300 family of molecules are evolutionarily significant regulators of leukocyte functions. Trends Immunol. 30, 209–217.

Fast, M.D., Johnson, S.C., Eddy, T.D., Pinto, D., Ross, N.W., 2007. *Lepeophtheirus salmonis* secretory/excretory products and their effects on Atlantic salmon immune gene regulation. Parasite Immunol. 29, 179–189.

Feng, L.N., Lu, D.Q., Bei, J.X., Chen, J.L., Liu, Y., Zhang, Y., Liu, X.C., Meng, Z.N., Wang, L., Lin, H.R., 2009. Molecular cloning and functional analysis of polymeric immunoglobulin receptor gene in orange-spotted grouper (*Epinephelus coioides*). Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 154, 282–289.

Flajnik, M.F., 2010. All GOD's creatures got dedicated mucosal immunity. Nat. Immunol. 11, 777–779.
Ghumra, A., Shi, J., McIntosh, R.S., Rasmussen, I.B., Braathen, R., Johansen, F.E., Sandlie, I., Mongini, P.K., Areschoug, T., Lindahl, G., Lewis, M.J., Woof, J.M., Pleass, R.J., 2009. Structural requirements for the interaction of human IgM and IgA with the human Fcalpha/mu receptor. Eur. J. Immunol. 39, 1147–1156.
Green, B.J., Clark, G.J., Hart, D.N.J., 1998. The CMRF-35 mAb recognizes a second leukocyte membrane molecule with a domain similar to the poly Ig receptor. Int. Immunol. 10, 891–899.
Hamburger, A.E., West Jr., A.P., Bjorkman, P.J., 2004. Crystal structure of a polymeric immunoglobulin binding fragment of the human polymeric immunoglobulin receptor. Structure 12, 1925–1935.
Hamuro, K., Suetake, H., Saha, N.R., Kikuchi, K., Suzuki, Y., 2007. A teleost polymeric Ig receptor exhibiting two Ig-like domains transports tetrameric IgM into the skin. J. Immunol. 178, 5682–5689.
Hatten, F., Fredriksen, A., Hordvik, I., Endresen, C., 2001. Presence of IgM in cutaneous mucus, but not in gut mucus of Atlantic salmon, *Salmo salar*. Serum IgM is rapidly degraded when added to gut mucus. Fish Shellfish Immunol. 11, 257–268.
Jackson, D.G., Hart, D.N., Starling, G., Bell, J.I., 1992. Molecular cloning of a novel member of the immunoglobulin gene superfamily homologous to the polymeric immunoglobulin receptor. Eur. J. Immunol. 22, 1157–1163.
Ju, X., Zenke, M., Hart, D.N., Clark, G.J., 2008. CD300a/c regulate type I interferon and TNF-alpha secretion by human plasmacytoid dendritic cells stimulated with TLR7 and TLR9 ligands. Blood 112, 1184–1194.
Julenius, K., Molgaard, A., Gupta, R., Brunak, S., 2005. Prediction, conservation analysis, and structural characterization of mammalian mucin-type O-glycosylation sites. Glycobiology 15, 153–164.
Kaetzel, C.S., 2005. The polymeric immunoglobulin receptor: bridging innate and adaptive immune responses at mucosal surfaces. Immunol. Rev. 206, 83–99.
Kaetzel, C.S., Robinson, J.K., Lamm, M.E., 1994. Epithelial transcytosis of monomeric IgA and IgG cross-linked through antigen to polymeric IgA. A role for monomeric antibodies in the mucosal immune system. J. Immunol. 152, 72–76.
Keren, H., Lev-Maor, G., Ast, G., 2010. Alternative splicing and evolution: diversification, exon definition and function. Nat. Rev. Genet. 11, 345–355.
Kock, H., Fischer, U., 2008. A novel immunoglobulin-like transcript from rainbow trout with two Ig-like domains and two isoforms. Mol. Immunol. 45, 1612–1622.
Krajci, P., Kvale, D., Tasken, K., Brandtzaeg, P., 1992. Molecular-cloning and exon–intron mapping of the gene encoding human transmembrane secretory component (the poly-Ig receptor). Eur. J. Immunol. 22, 2309–2315.
Kuhn, L.C., Kraehenbuhl, J.P., 1981. The membrane-receptor for polymeric immunoglobulin is structurally related to secretory component—isolation and characterization of membrane secretory component from rabbit liver and mammary-gland. J. Biol. Chem. 256, 2490–2495.
Kulseth, M.A., Krajci, P., Myklebost, O., Rogne, S., 1995. Cloning and characterization of two forms of bovine polymeric immunoglobulin receptor cDNA. DNA Cell Biol. 14, 251–256.
Lefranc, M.P., Pommie, C., Ruiz, M., Giudicelli, V., Foulquier, E., Truong, L., Thouvenin-Contet, V., Lefranc, G., 2003. IMGT unique numbering for immunoglobulin and T cell receptor variable domains and Ig superfamily V-like domains. Dev. Comp. Immunol. 27, 55–77.
Leong, J.S., Jantzen, S.G., von Schalburg, K.R., Cooper, G.A., Messmer, A.M., Liao, N.Y., Munro, S., Moore, R., Holt, R.A., Jones, S.J., Davidson, W.S., Koop, B.F., 2010. *Salmo salar* and *Esox lucius* full-length cDNA sequences reveal changes in evolutionary pressures on a post-tetraploidization genome. BMC Genomics 11, 279.
Lin, T.L., Clark, T.G., Dickerson, H., 1996. Passive immunization of channel catfish (*Ictalurus punctatus*) against the ciliated protozoan parasite *Ichthyophthirius multifiliis* by use of murine monoclonal antibodies. Infect. Immun. 64, 4085–4090.
Moore, L.J., Somamoto, T., Lie, K.K., Dijkstra, J.M., Hordvik, I., 2005. Characterisation of salmon and trout CD8 alpha and CD8 beta. Mol. Immunol. 42, 1225–1234.
Mussmann, R., Du Pasquier, L., Hsu, E., 1996. Is *Xenopus* IgX an analog of IgA? Eur. J. Immunol. 26, 2823–2830.
Natvig, I.B., Johansen, F.E., Nordeng, T.W., Haraldsen, G., Brandtzaeg, P., 1997. Mechanism for enhanced external transfer of dimeric IgA over pentameric IgM: studies of diffusion, binding to the human polymeric Ig receptor, and epithelial transcytosis. J. Immunol. 159, 4330–4340.
Norderhaug, I.N., Johansen, F.E., Krajci, P., Brandtzaeg, P., 1999. Domain deletions in the human polymeric Ig receptor disclose differences between its dimeric IgA and pentameric IgM interaction. Eur. J. Immunol. 29, 3401–3409.
Olsvik, P.A., Lie, K.K., Jordal, A.E., Nilsen, T.O., Hordvik, I., 2005. Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. BMC Mol. Biol. 6, 21.
Ostergaard, A.E., Lubieniecki, K.P., Martin, S.A., Stet, R.J., Davidson, W.S., Secombes, C.J., 2010. Genomic organisation analysis of novel immunoglobulin-like transcripts in Atlantic salmon (*Salmo salar*) reveals a tightly clustered and multigene family. BMC Genomics 11, 697.
Ostergaard, A.E., Martin, S.A., Wang, T., Stet, R.J., Secombes, C.J., 2009. Rainbow trout (*Oncorhynchus mykiss*) possess multiple novel immunoglobulin-like transcripts containing either an ITAM or ITIMs. Dev. Comp. Immunol. 33, 525–532.
Phalipon, A., Corthesy, B., 2003. Novel functions of the polymeric Ig receptor: well beyond transport of immunoglobulins. Trends Immunol. 24, 55–58.
Ribeiro, C.M., Bird, S., Raes, G., Ghassabeh, G.H., Schijns, V.E., Pontes, M.J., Savelkoul, H.F., Wiegertjes, G.F., 2011. A novel soluble immune-type receptor (SITR) in teleost fish: carp SITR is involved in the nitric oxide-mediated response to a protozoan parasite. PLoS One 6, e15986.
Rombout, J.H., Abelli, L., Picchietti, S., Scapigliati, G., Kiron, V., 2010. Teleost intestinal immunology. Fish Shellfish Immunol. 31, 616–626.
Rombout, J.H., van der Tuin, S.J., Yang, G., Schopman, N., Mroczek, A., Hermsen, T., Taverne-Thiele, J.J., 2008. Expression of the polymeric Immunoglobulin Receptor (pIgR) in mucosal tissues of common carp (*Cyprinus carpio* L.). Fish Shellfish Immunol. 24, 620–628.
Rombout, J.H.W.M., Joosten, E.P.E.M., 1998. Immunology of fishes. In: Pastoret, P.P., Griebel, P., Bazin, H., Govaerts, A. (Eds.), Handbook of Vertebrate Immunology. Academic Press, London, pp. 1–43.
Shibuya, A., Sakamoto, N., Shimizu, Y., Shibuya, K., Osawa, M., Hiroyama, T., Eyre, H.J., Sutherland, G.R., Endo, Y., Fujita, T., Miyabayashi, T., Sakano, S., Tsuji, T., Nakayama, E., Phillips, J.H., Lanier, L.L., Nakauchi, H., 2000. Fc alpha/mu receptor mediates endocytosis of IgM-coated microbes. Nat. Immunol. 1, 441–446.
Stafford, J.L., Wilson, M., Nayak, D., Quiniou, S.M., Clem, L.W., Miller, N.W., Bengten, E., 2006. Identification and characterization of a FcR homolog in an ectothermic vertebrate, the channel catfish (*Ictalurus punctatus*). J. Immunol. 177, 2505–2517.
Stet, R.J., Hermsen, T., Westphal, A.H., Jukes, J., Engelsma, M., Lidy Verburg-van Kemenade, B.M., Dortmans, J., Aveiro, J., Savelkoul, H.F., 2005. Novel immunoglobulin-like transcripts in teleost fish encode polymorphic receptors with cytoplasmic ITAM or ITIM and a new structural Ig domain similar to the natural cytotoxicity receptor NKp44. Immunogenetics 57, 77–89.
Tadiso, T.M., Krasnov, A., Skugor, S., Afanasyev, S., Hordvik, I., Nilsen, F., 2011b. Gene expression analyses of immune responses in Atlantic salmon during early stages of infection by salmon louse (*Lepeophtheirus salmonis*) revealed bi-phasic responses coinciding with the copepod-chalimus transition. BMC Genomics 12, 141.
Tadiso, T.M., Lie, K.K., Hordvik, I., 2011a. Molecular cloning of IgT from Atlantic salmon, and analysis of the relative expression of tau, mu, and delta in different tissues. Vet. Immunol. Immunopathol. 139, 17–26.
Takatsu, H., Hase, K., Ohmae, M., Ohshima, S., Hashimoto, K., Taniura, N., Yamamoto, A., Ohno, H., 2006. CD300 antigen like family member G: a novel Ig receptor like protein exclusively expressed on capillary endothelium. Biochem. Biophys. Res. Commun. 348, 183–191.
Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.
Wieland, W.H., Orzaez, D., Lammers, A., Parmentier, H.K., Verstegen, M.W., Schots, A., 2004. A functional polymeric immunoglobulin receptor in chicken (*Gallus gallus*) indicates ancient role of secretory IgA in mucosal immunity. Biochem. J. 380, 669–676.
Williams, A.F., Barclay, A.N., 1988. The immunoglobulin superfamily—domains for cell surface recognition. Annu. Rev. Immunol. 6, 381–405.
Woof, J.M., Mestecky, J., 2005. Mucosal immunoglobulins. Immunol. Rev. 206, 64–82.
Yoo, E.M., Trinh, K.R., Lim, H., Wims, L.A., Morrison, S.L., 2011. Characterization of IgA and IgM binding and internalization by surface-expressed human Fcalpha/mu receptor. Mol. Immunol. 48, 1818–1826.
Zhang, Y.A., Salinas, I., Li, J., Parra, D., Bjork, S., Xu, Z., LaPatra, S.E., Bartholomew, J., Sunyer, J.O., 2010. IgT, a primitive immunoglobulin class specialized in mucosal immunity. Nat. Immunol. 11, 827–835.

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Gene expression analyses of immune responses in Atlantic salmon during early stages of infection by salmon louse (*Lepeophtheirus salmonis*) revealed bi-phasic responses coinciding with the copepod-chalimus transition

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Abstract

Background: The salmon louse (*Lepeophtheirus salmonis* Krøyer), an ectoparasitic copepod with a complex life cycle causes significant losses in salmon aquaculture. Pesticide treatments against the parasite raise environmental concerns and their efficacy is gradually decreasing. Improvement of fish resistance to lice, through biological control methods, needs better understanding of the protective mechanisms. We used a 21 k oligonucleotide microarray and RT-qPCR to examine the time-course of immune gene expression changes in salmon skin, spleen, and head kidney during the first 15 days after challenge, which encompassed the copepod and chalimus stages of lice development.

Results: Large scale and highly complex transcriptome responses were found already one day after infection (dpi). Many genes showed bi-phasic expression profiles with abrupt changes between 5 and 10 dpi (the copepod-chalimus transitions); the greatest fluctuations (up- and down-regulation) were seen in a large group of secretory splenic proteases with unknown roles. Rapid sensing was witnessed with induction of genes involved in innate immunity including lectins and enzymes of eicosanoid metabolism in skin and acute phase proteins in spleen. Transient (1-5 dpi) increase of T-cell receptor alpha, CD4-1, and possible regulators of lymphocyte differentiation suggested recruitment of T-cells of unidentified lineage to the skin. After 5 dpi the magnitude of transcriptomic responses decreased markedly in skin. Up-regulation of matrix metalloproteinases in all studied organs suggested establishment of a chronic inflammatory status. Up-regulation of putative lymphocyte G0/G1 switch proteins in spleen at 5 dpi, immunoglobulins at 15 dpi; and increase of IgM and IgT transcripts in skin indicated an onset of adaptive humoral immune responses, whereas MHCI appeared to be down-regulated.

Conclusions: Atlantic salmon develops rapid local and systemic reactions to *L. salmonis*, which, however, do not result in substantial level of protection. The dramatic changes observed after 5 dpi can be associated with metamorphosis of copepod, immune modulation by the parasite, or transition from innate to adaptive immune responses.

Background

The salmon louse (*Lepeophtheirus salmonis* Krøyer) is a widespread disease-causing marine ectoparasitic copepod infecting wild and farmed salmonids. The development of *L. salmonis* encompasses ten stages: two nauplii, a copepodid, four chalimus, two pre-adult, and an adult stage [1]. The nauplii hatch directly from egg-strings attached to the female lice. The two nauplii stages and the copepodid are free-living larvae that utilize yolk and other components provided maternally. The copepodid is the infectious stage of *L. salmonis*; its ability to settle and to recognize a relevant host is of critical importance for the parasite. We have observed that *L. salmonis* copepodids use 7-11 days (at 9.3°C) before they all have completed the molt to chalimus I. The four chalimus stages are physically attached to the host

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by a frontal filament. Even though an increase in virulence by *L. salmonis* has been observed as the parasite reaches the pre-adult stages [2], the chalimus stage can also account for smolt mortalities (e.g. in small pink salmon [3]). Lice damage fish by feeding on their mucus, skin, and blood and the wounds increase the risk of secondary infections. At present, *L. salmonis* is recognized as one of the major problems in salmon aquaculture in Norway, UK, USA, and Canada; whereas in Chile, a *Caligus* species (*C. rogercresseyi*) gives similar problems. The annual global loss due to sea lice in salmonid aquaculture is estimated to be more than 300 million USD [4]. Moreover, lice originating from farmed salmon may cause infections and mortality on wild salmonids [4,5].

L. salmonis is controlled mainly by pesticides and at present only a few types are available, emamectin benzoate being the most commonly used [6]. However, increasing concerns about development of pesticide resistance, occurrence of treatment failures, and undesirable environmental impacts raise questions about the future of this strategy. The need for new methods of parasite control is fully recognized by the industry, authorities and society. At this time multiple studies assess improvement of salmon resistance to lice with an aid of selective breeding, special feeds and immune stimulants. The possibility of immunization and vaccination against *L. salmonis* infection is discussed [7,8]. However, protective antibody responses following repeated challenge are weak. Better understanding of acquired immune responses is essential for vaccine development. However, data on factors related to adaptive immunity are lacking in this host-parasite system [7,9]. Development of biological methods of protection needs better understanding of mechanisms underlying resistance to lice. The ability to suppress and reject parasites shortly after infection can be associated with innate immunity. Early innate responses are especially important since they greatly influence the subsequent responses that develop in the immune cascade. Such responses are believed to explain considerable differences between the salmonid species in susceptibility to lice [10]. Limited epithelial hyperplasia and inflammation after infection with the parasite were reported in Atlantic salmon (*Salmo salar L.*) and this was in contrast to highly resistant coho salmon (*Oncorhynchus kisutsch*) and chinook (*O. nerka*) salmon [11]. This can be related to inherent constraints of the immune system or its modulation by the parasite. In addition, Atlantic salmon possesses thin epidermal layer, sparsely distributed mucus cells, and exhibits low mucus lysozyme and protease activity as compared to other salmonids [12].

Knowledge of salmon immune responses to lice and their roles in protection against parasite is still limited. Until present, studies have addressed a relatively small number of immune parameters. Development of high-throughput analytical methods makes it possible to expand the search and to monitor large number of immune pathways in parallel at the gene expression level. In a previous study, we used a 1.8 k cDNA microarray (SFA2 or immunochip) to examine the local and systemic responses of Atlantic salmon to lice within the whole infection period [13]. This platform included a relatively small number of genes and the early responses were represented with only one time-point - 3 days post infection (dpi.). In this paper we report immune related responses during the first 15 dpi, divided in five time-points. This enabled us to see how the host is responding during the early infection period. In this study, we used the Atlantic salmon oligonucleotide platform discussed in detail in [14]. Gene expression profiling was done in skin and spleen and real-time RT-qPCR analyses were performed in these tissues, and also in the head kidney.

Results

Lice count and a summary of gene expression changes

The number of lice was determined at 15 dpi (the last day of experiment), and high counts (58.4 ± 9.48 lice per fish, all at chalimus I to III stage) from 100 copepodids per fish of initial infection confirmed the lack of Atlantic salmon's ability to clear the parasite. However, the microarray analyses suggested rapid and sizeable transcriptomic responses to lice. The total number of differentially expressed features was 2438 in skin and 922 in spleen (Figure 1A and Additional file 1). Given low redundancy of the platform, these numbers are close to numbers of differentially expressed genes (DEG). While the magnitude of responses remained relatively stable within the whole study period in the spleen, the number of genes with expression changes in skin decreased markedly after 5 dpi. For validation of microarray results, genes that covered the whole range of expression ratios were chosen, and RT-qPCR analyses were performed in the same individuals (Figure 1B). The results of two independent methods were in good concordance: coefficients of linear regression and correlation (Pearson r) were equal to 0.84 and 0.80 respectively (complete RT-qPCR results are in Additional file 2).

Hierarchical clustering suggested high consistency of the gene expression changes (Figure 1C, D). The samples (biological replicates) were grouped by the time-points with exclusion of one outlier (D15-1), which deviated from the common trend in both analyzed tissues. The samples from spleen and skin were divided in two large clusters (days 1-5 and days 10-15), which were sharply separated, especially in the skin. This suggested a bi-phasic response to lice and the K-mean clustering confirmed abrupt expression changes in a major part of

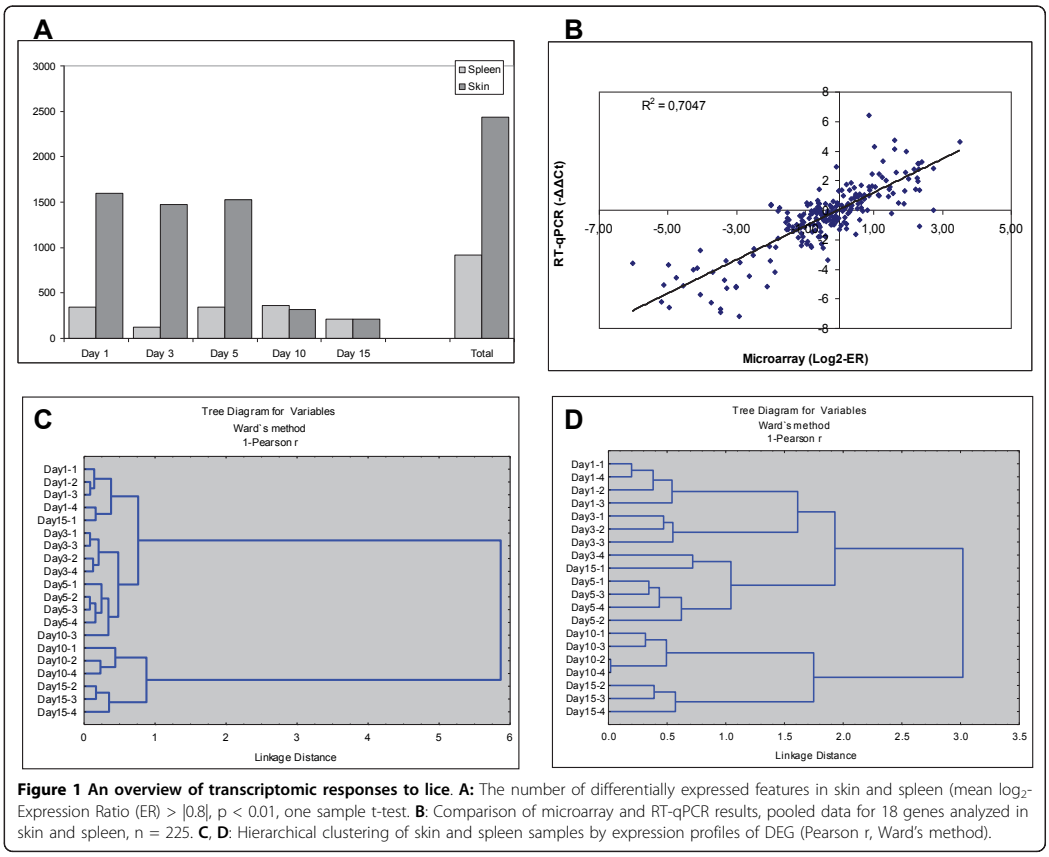

Figure 1 An overview of transcriptomic responses to lice. **A:** The number of differentially expressed features in skin and spleen (mean $\log_2$-Expression Ratio (ER) > |0.8|, $p < 0.01$, one sample t-test. **B**: Comparison of microarray and RT-qPCR results, pooled data for 18 genes analyzed in skin and spleen, n = 225. **C**, **D**: Hierarchical clustering of skin and spleen samples by expression profiles of DEG (Pearson r, Ward's method).

genes between days 5 and 10 (data not shown). A notable example of bi-phasic regulation is a group of splenic proteases (trypsins and chemotrypsins, carbopeptidases and carboxylic ester hydrolases, elastase, proteinase E and choriolytic enzyme) and proteins involved in regulation of exocytosis (syncollin and endoplasmic reticulum protein ERp27). The microarray results were confirmed with RT-qPCR (Figure 2 and Additional file 3).

Search for the enriched functional classes and pathways in the present microarray data illustrate the thematic associations of gene expression changes. By functions of DEG, responses to lice were much more diverse and complex in skin, which was the target site for the parasite (Table 1). The changes were associated with cell maintenance (metabolism of amino acids and sugars, mitochondrion and cytoskeleton (including motor proteins), protein biosynthesis, modification and transport, regulation of redox status, DNA replication and repair), cell communication and reparation of tissues. By result of statistical analysis, enrichment was greatest in classes related to basic metabolic functions (mitochondrion, glycolysis and ribosomes). The immune functional groups comprised a relatively small fraction of changes in the skin (only two KEGG pathways) but were predominant in the spleen (five of ten terms included in Table 1); inflammatory response and complement and coagulation cascades were the most enriched terms. The study focused on the immune responses and therefore in presentation of results preference is given to genes with known immune roles.

Humoral immunity and inflammation

Rapid responses to the parasite and transmission of signal from the damaged sites to the internal organs were confirmed with up-regulation of pro-inflammatory genes in both skin and spleen. The complement system is part of both innate and adaptive immune system, and plays a major role in recognition and elimination of pathogens.

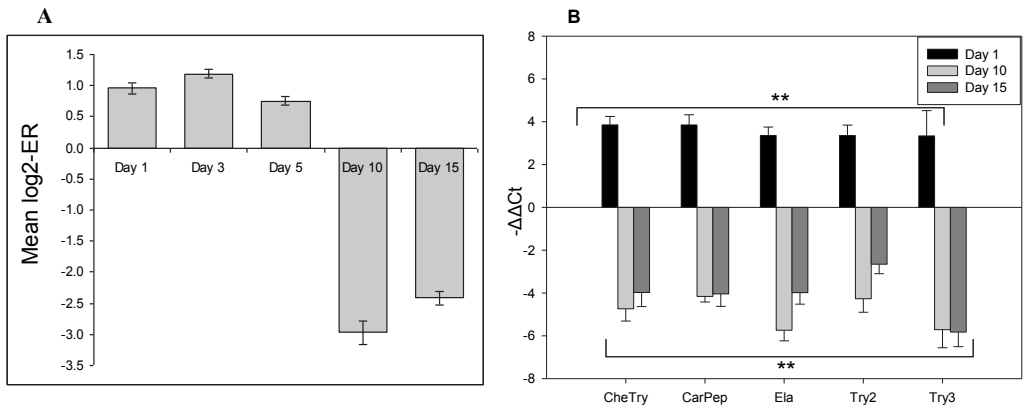

Figure 2 Expression changes of proteases in spleen. **A**: Microarray data are mean log_2-ER ± SE for 24 genes with highly coordinated expression profiles. The lists of genes and accession numbers are in Additional file 3. **B:** RT-qPCR analyses performed with SYBR Green assays. Data are mean -ΔΔCt ± SE. Significant differences from control (n = 4, P < 0.01) are indicated with**. Note significant differences from control of all genes at all time points. CheTry, Chemytrypsin; CarPep, Carboxypeptidase; Ela, Elastase; Try2, trypsin-2; Try3, trypsin-3.

Several lectins with early (1 dpi) induction in skin (Figure 3) have unknown roles but may be needed for detection of pathogen; the calcium dependent (C-type) lectin domain family 4 E is expressed in macrophages and other Ag presenting cells [15]. In theory, lectins can activate one of the complement pathways. In this respect, it is noteworthy to mention down-regulation of several genes for C1Q-like proteins that can trigger the classical pathway, which could mean preferential activation of the lectin pathway. Decreased expression was shown for two negative regulators of complement: CD59 and C4b-binding protein. Phospholipase A2 and prostaglandin E synthase 3 are involved in biosynthesis of inflammatory regulators and several more immune effectors showed rapid up-regulation. The RT-qPCR analyses of IL-1B, IL-12, TNF-α did not find significant expression changes in skin, spleen, and head kidney (see Additional file 2). The components of the NFKB pathway changed expression in both directions while a panel of IFN-dependent proteins were down-regulated; many of these have shown strong responses to viruses [14]. Given large distance between the spleen and the skin, we could anticipate preferential regulation of genes for proteins exported to plasma and body fluids including acute phase proteins (serum amyloids, lysozyme C and transferrin) (Figure 4). Several lesser known proteins have been attributed to this functional group; these are jeltraxin, which is similar to C-reactive P component and serum amyloid P component, differentially regulated trout protein 1 [16] and LPS neutralizing protein cathelicidin. In addition, rapid up-regulation was observed in a number of possible pro-inflammatory genes including several TNF-dependent genes (TNF decoy receptor, metalloreductase STEAP4 and TSG6). In parallel, a large group of genes for plasma proteins decreased expression: highly coordinated changes were seen in the components of complement and coagulation cascade (26 genes) and in a diverse group of 55 genes that among other included apolipoproteins and glycoproteins, macroglobulins and protease inhibitors, proteins binding copper, iron and heme, scavengers, chemokines and cytokines (Figure 5A).

The temporal patterns of inflammatory changes were different in the analyzed tissues. In skin, many genes had similar expression profiles during 1-5 dpi, while in spleen the acute phase proteins showed a short-term increase only at 1 dpi. However in both skin and spleen the character of innate immune responses changed dramatically after 5 dpi. The switch of transcriptomic program was marked with abrupt temporary down-regulation of splenic plasma proteins, which was similar to that observed at 1 dpi but with greater magnitude (Figure 5A). A hallmark of the second phase was up-regulation of several matrix metalloproteinases: MMP9 (gelatinase) and MMP13 (collagenase 3), which was observed in skin, spleen and head kidney - the latter was analyzed with RT-qPCR (Figure 5B-D). These inducible enzymes have a wide range of roles, from massive degradation of extracellular matrix and tissue remodeling to limited proteolysis and subtle regulation of immune processes [17,18]. Various pro-inflammatory genes including chemokines and effectors showed up-regulation after 5 dpi.

Cellular responses, acquired immunity

The gene expression profiles in skin (Figure 6A) indicated rapid alterations of the composition of immune

Table 1 Enrichment of GO classes and KEGG pathways in the lists of DEG

Functional group, pathway	Features[1]	p-value[2]	Vocabulary
Skin			
Mitochondrion	177 / 1104	0.000	GO
Glycolysis / Gluconeogenesis	33 / 117	0.000	KEGG
Pentose phosphate pathway	13 / 48	0.009	KEGG
Glutamate metabolism	16 / 48	0.000	KEGG
Glutathione metabolism	13 / 49	0.011	KEGG
Ribosome	47 / 172	0.000	GO
Protein folding	33 / 178	0.015	GO
Protein modification	44 / 246	0.009	GO
Protein transport	78 / 473	0.004	GO
Cytoskeleton	89 / 579	0.013	GO
Myosin complex	23 / 86	0.000	GO
Endoplasmic reticulum	140 / 992	0.027	GO
Cell redox homeostasis	11 / 43	0.027	GO
Double-strand break repair	7 / 24	0.050	GO
Anti-apoptosis	41 / 200	0.001	GO
Positive regulation of apoptosis	12 / 51	0.037	GO
Antigen processing and presentation[3]	17 / 69	0.007	KEGG
Leukocyte transendothelial migration	32 / 186	0.043	KEGG
Cell adhesion	83 / 545	0.020	GO
Tight junction	38 / 208	0.011	KEGG
Heparin binding	18 / 90	0.043	GO
Keratinization	8 / 21	0.006	GO
TGF-beta signaling pathway	24 / 113	0.008	KEGG
Spleen			
Inflammatory response	22 / 213	0.000	GO
Complement and coagulation cascades	29 / 85	0.000	KEGG
Peptidase activity	23 / 145	0.000	GO
Acute-phase response	8 / 20	0.000	GO
Chemotaxis	10 / 81	0.003	GO
Basement membrane	10 / 68	0.000	GO
Cell adhesion	36 / 545	0.020	GO
Extracellular space	47 / 375	0.000	GO
Heparin binding	13 / 90	0.000	GO
Neuroactive ligand-receptor interaction	14 / 173	0.036	KEGG

[1] Numbers of genes among DEG and on the microarray platform. [2]Yates' corrected chi-square. [3]Immune related groups and pathways are highlighted with bold.

cells in the target site. Stable up-regulation during 1-5 dpi was observed in a panel of signal transducers: LCK2, protein kinase D3, RAS homologue member G (RhoG), spleen tyrosine kinase (SYK), GRB2-related adaptor protein 2, G protein-coupled receptor kinase 5, RAS guanyl-releasing protein 2, which are known for their important roles in regulating immune cell movement [19,20]. Several of these genes have shown preferential expression in salmon peripheral blood leukocytes in previous microarray study [14]; however, their association with specific cell lineages remains unknown. Microarray analyses showed decreased abundance of transcripts for proteins that have a major part in transendothelial migration of leukocytes, including annexin 2, myosin 9 - a non-muscle motor protein, and integrin beta; CD9 and CD63 expressed on leukocyte membranes interact with integrins and proteins of extracellular matrix. Down-regulated CD53 mediates activation of leukocytes and MafB is the myeloid associated differentiation marker. We did not see induction of myeloid-specific genes while a number of events suggested recruitment and activation of lymphoid cells. Increase was observed in a panel of T-cell-specific genes including T-cell receptor alpha (TCRα), serine/threonine-protein phosphatase 2B, L-plastin, drebrin suggesting preponderance of T lymphocytes among immune cells that appeared in the target sites (Figure 6A). Up-regulation of TCRα and CD3ε in the head kidney at 1 dpi and decrease at 5 dpi

Gene	D1	D3	D5	D10	D15
Rhamnose-binding lectin WCL1	2.07	0.42	0.82	0.09	0.61
CD209 antigen-like protein D	1.07	0.94	0.63	-0.30	-0.19
C-type lectin domain family 4 E	1.60	4.28	1.60	-0.03	0.53
Mannose receptor C type 1	1.45	0.46	0.57	-0.39	0.32
Mannose receptor, C type 2	3.15	4.10	2.09	0.50	0.37
Mannose-binding protein C	-0.58	-3.09	-3.34	0.25	0.29
Polymeric immunoglobulin receptor	1.72	1.18	1.33	-0.47	0.53
Complement factor H1 protein	2.79	2.61	2.44	0.15	1.16
C4b-binding protein alpha chain	-0.78	-1.50	-1.06	0.40	-0.50
CD59 glycoprotein	-0.94	-1.67	-1.14	-0.43	-0.52
Complement C1q-like protein 4	-0.44	-1.79	-1.84	-0.18	0.06
Complement C1q subcomponent subunit B	-1.08	-1.92	-1.61	0.66	-0.24
C1q-like protein 4	-2.84	-0.24	0.56	5.24	3.78
Prostaglandin E synthase 3	1.42	0.62	0.49	0.01	0.29
Phospholipase A2, group VI isoform b	1.41	1.03	1.18	-0.29	0.09
C-C chemokine receptor type 3	1.77	0.93	0.90	-0.22	0.13
Amyloid beta A4 protein , isoform a	2.63	1.74	2.38	-0.01	1.14
SAPS domain family member 3	2.37	3.37	1.07	-0.21	0.14
Tax1	2.01	1.90	1.19	0.15	0.11
Nuclear factor NF-kappa-B p100 subunit	1.38	0.70	0.86	-0.08	0.09
Tax1	-1.91	-1.49	-1.58	0.04	-0.29
Transcription factor AP-1	-1.04	-0.87	-0.25	-0.63	-0.33
Nuclear factor NF-kappa-B p100 subunit	-0.69	-1.08	-0.80	-0.10	-0.23
Transcription factor jun-B	-0.01	-1.44	-1.23	0.61	0.18
Galectin-9	-0.42	-1.14	-1.26	0.64	0.42

Gene	D1	D3	D5	D10	D15
Interferon regulatory factor 1	-0.23	-1.16	-0.98	0.51	-0.08
Gamma-interferon-inducible thiol reductase	-0.58	-1.28	-1.06	0.30	0.29
Interferon regulatory factor 2-binding protein 1	-0.79	-1.43	-0.75	0.40	-0.21
Interferon-gamma receptor alpha chain	-0.75	-1.29	-1.14	0.31	0.05
Interferon-inducible protein Gig2-like	-0.92	-1.30	-1.13	0.01	0.06
Probable ATP-dependent RNA helicase DHX58	-1.36	-1.56	-1.38	-0.36	-0.08
Janus kinase 1	-1.62	-1.03	-0.64	-0.56	-0.03
VHSV-induced protein-10	-0.91	-1.51	-0.77	-0.61	-0.15
Sacsin	-1.37	-1.48	-1.38	-0.86	0.16
Interferon-induced protein 44	-1.05	-1.62	-0.74	-0.79	-0.55
Interferon-induced with tetratricopeptide repeats 5	-1.49	-1.06	-0.78	-1.16	0.04
Interferon regulatory factor 7	-1.19	-0.39	-1.39	-0.92	-0.50
Interferon-induced protein 44	-0.90	-1.35	-0.93	-1.07	-0.78
Inducible nitric oxide synthase	-2.01	-1.32	-0.69	-0.06	0.02
C-reactive protein	-1.31	-1.81	-1.80	0.41	-0.33
Lysozyme g	-1.35	-1.14	-1.04	-0.39	-0.45
Cytochrome b558 alpha-subunit	-2.70	-0.71	-1.33	-0.72	-0.29
Integrin beta-1	-0.08	-1.22	-1.55	1.38	0.49
Integrin beta-2	-1.00	-1.02	-1.01	1.28	0.33
Matrix metalloproteinase-9	-0.31	-0.95	-0.72	2.19	0.47
Collagenase 3	-0.44	-0.30	-0.11	2.52	-0.14
Matrix metalloproteinase-9	-1.51	-0.78	-1.52	1.67	0.74
Serum amyloid A-5 protein	-0.95	-3.33	-3.59	2.88	1.68
Cathelicidin antimicrobial peptide	-1.62	-2.16	-3.25	1.84	0.26
C3a anaphylatoxin chemotactic receptor	-0.58	-0.05	-0.04	1.42	0.57

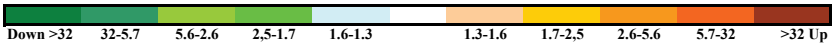

Figure 3 Examples of immune genes with differential expression in skin (microarray results). Data are mean log_2-ER (n = 4).

(Figure 6D) implied rapid recruitment of T-cells from this depot. The nature of these cells remains unknown. No expression changes of CD8 were detected though the microarray platform included probes to alpha and beta chains whose performance was confirmed in studies with viral diseases including cardiomyopathy syndrome (CMS), heart and skeletal muscle inflammation (HSMI), and the infectious salmon anemia (ISA) (unpublished results). The RT-qPCR analyses found a short-term up-regulation of CD4-1 in skin (Figure 6C). An interesting finding was expression changes of genes that control differentiation of lymphocytes. This was shown by an increase in several genes that regulate early lymphopoiesis, such as kin of IRRE like 3, myeloid/

Gene	D1	D3	D5	D10	D15
Acute phase serum amyloid A (SAA)	3.17	-0.35	0.53	-0.69	0.72
Cathelicidin antimicrobial peptide	1.88	-0.13	0.70	-0.54	0.83
Differentially regulated trout protein 1	2.54	0.89	0.40	-2.17	-0.70
Jeltraxin precursor	1.51	-0.06	0.90	-0.39	0.03
Lysozyme C II precursor	1.40	-0.11	0.20	-0.76	0.75
Metalloreductase STEAP4-1	1.59	0.92	-0.18	-0.11	0.20
Metalloreductase STEAP4-2	2.05	1.47	0.08		0.45
Serum amyloid A	2.42	-0.91	0.49	-1.70	0.25
Transferrin	1.81	0.54	-2.47	-0.82	0.58
C-X-C motif chemokine 10 precursor	1.04	-0.09	0.94	-1.39	0.24
C type lectin receptor A	1.29	0.91	0.23	-0.67	0.51
TNF decoy receptor	1.16	-0.42	0.14	-1.44	0.93
Tumor necrosis factor-inducible protein	1.55	0.52	0.63	-1.02	0.88
MAP kinase-activated protein kinase 3	0.61	0.52	0.98	2.16	-0.05
Natterin-like protein	-0.09	1.13	-0.14	1.63	0.02

Gene	D1	D3	D5	D10	D15
C-C motif chemokine 28-1	-0.04	0.11	0.80	1.85	-0.96
C-C motif chemokine 28-2	0.14	-0.07	0.77	1.84	-0.21
C-x-c chemokine receptor type 3A-1	0.17	0.59	0.45	1.23	0.51
Complement factor H1 protein	0.46	0.39	0.75	2.31	-0.11
Complement factor D precursor	0.35	0.30	0.17	1.56	0.96
IL-8 receptor	-0.44	1.24	0.66	1.87	1.27
Interleukin-2 receptor subunit beta	0.01	-0.04	0.85	1.90	-0.34
Neutrophil cytosolic factor 1	0.41	1.09	0.09	1.01	1.09
Myeloperoxidase precursor	-0.23	-0.31	0.05	1.27	0.93
Arachidonate 5-lipoxygenase-activating p	-0.11	0.63	0.13	1.16	0.15
Leukotriene A-4 hydrolase	-0.11	0.77	-0.05	1.02	0.79
Rhamnose-binding lectin WCL1	0.44	0.22	0.91	2.14	-0.25
Matrix metalloproteinase	0.27	1.62	0.70	1.59	1.31
Matrix metalloproteinase-9	-0.30	1.63	0.00	1.71	1.44
Collagenase 3 precursor	-0.27	1.64	0.21	1.70	1.72

Figure 4 Examples of immune genes with differential expression in spleen (microarray results). Data are mean log_2-ER (n = 4).

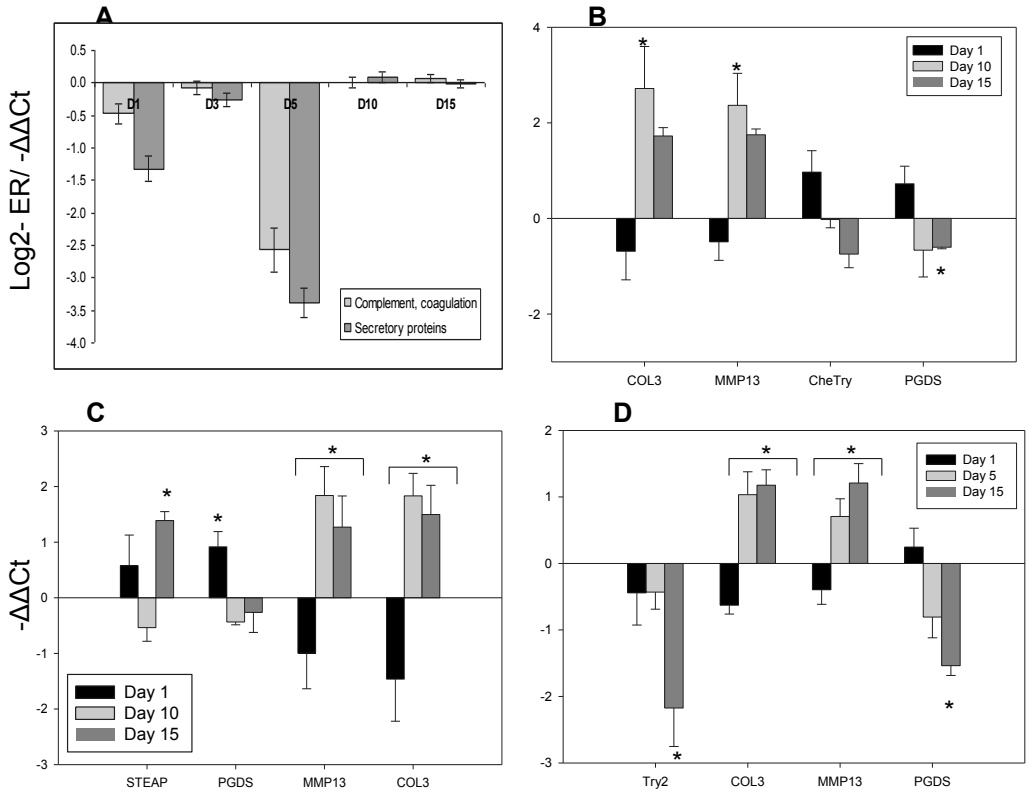

Figure 5 Temporal expression changes. **A**: plasma proteins in spleen (microarray results). Data are mean log_2-ER ± SE for 26 genes (complement and coagulation cascade) and 55 genes (secretory proteins) with similar expression profiles. The lists of genes and accession numbers are in Additional file 4. **B-D:** RT-qPCR analyses performed with SYBR Green based assays in skin **(B)**, spleen **(C)**, and head kidney **(D)**. Data are mean -ΔΔCt ± SE. Significant differences from control (n = 4, P < 0.05) are indicated with *. COL3, Collagenase 3 precursor; MMP13, Matrix metalloproteinase-13; CheTry, Chemytrypsin; PGDS, Prostaglandin D synthase; STEAP, Metalloreductase STEAP4; Try2, trypsin 2.

lymphoid or mixed-lineage leukemia, notch 1, Ikaros, growth factor independent (Figure 6A). Translin and translin-associated X interacting protein 1 are required for somatic recombination of genes encoding immunoglobulins (Ig) and T-cell receptors [21], while BTG3 and IRF4 stimulate their transcription. This may mean that terminal differentiation of T-cells takes place in the infected sites and we came to a similar conclusion in our studies of a viral disease CMS (unpublished data).

We did not find any indications of T-cell mediated immunity in subsequent responses. Down-regulation of MHCI, B2M in skin and head kidney during 1-5, and 15 dpi, and MHCII in skin 15 dpi (Figure 6), suggested absence of antigen presentation to T-cells. After 5 dpi the T-cell related genes showed no expression changes in skin. In contrast, there was evidence for the development of B cell mediated immunity. Despite an early regulation in skin of polymeric immunoglobulin receptor (pIgR), a key molecule in transcytosis of Igs (Figure 3), neither microarray nor RT-qPCR analyses showed early regulation of Ig genes in skin. Rapid (1 dpi) up-regulation of IgM and IgT in the head kidney followed with decrease at 5 dpi (additional file 2) suggested recruitment of B cells. However since no increase of B cell-specific transcripts were detected in skin at 1-3 dpi, they probably did not appear in the target site. However, RT-qPCR analyses revealed gradual increase of IgM and IgT transcripts from 10 to 15 dpi (Figure 7). Up-regulation of several isoforms of lymphocyte G0/G1 switch protein 2 at 5 dpi (data not shown) probably marked an onset of adaptive immune responses in the spleen. A large panel of Ig transcripts

A

Gene	D1	D3	D5	D10	D15
Tyrosine-protein kinase SYK	3.51	2.11	2.82	-0.48	1.16
Rho-related GTP-binding protein RhoG	1.78	1.19	1.66	0.10	0.91
Nuclear receptor coactivator 7, isoform 1	1.68	2.40	1.89	-0.12	0.55
LCK2	1.21	1.32	0.68	-0.16	0.13
protein kinase D3	1.15	0.83	1.23	-0.32	0.25
GRB2-related adaptor protein 2	0.75	2.02	1.03	0.28	0.37
G protein-coupled receptor kinase 5	0.69	1.71	0.89	-0.09	0.28
myeloid/lymphoid or mixed-lineage leukemia	1.89	1.71	1.25	-0.55	0.55
kin of IRRE like 3	1.63	0.77	1.49	-0.18	0.55
DNA-binding protein Ikaros	1.14	1.40	1.01	-0.90	0.22
notch 1	0.79	1.50	0.80	-0.53	-0.30
Myeloid-associated differentiation marker	-0.57	-1.27	-1.40	0.61	0.00
Hematopoietic lineage cell-specific protein	-0.89	-0.76	-1.28	0.61	0.12
Transcription factor SOX-4	-0.99	-1.84	-0.43	-0.56	-0.36
Transcription factor MafB	-1.28	-0.94	-0.73	0.52	-0.16
Interferon regulatory factor (IRF4)	3.06	4.66	1.85	-0.03	0.33
Translin	2.99	4.23	1.70	-0.57	0.25
Tanslin-associated factor X interacting protein 1	2.81	4.53	1.85	0.24	0.85
Serine/threonine-protein phosphatase 2B	3.62	3.00	3.35	0.62	1.73
Drebrin-like protein	3.25	3.94	1.54	-0.16	0.37
modified T cell receptor alpha	3.14	4.05	1.57	-0.19	0.19
T cell receptor alpha-1	2.92	3.95	1.40	-0.06	0.04
Growth factor independent 1	2.68	3.38	1.07	-0.41	0.30

Gene	D1	D3	D5	D10	D15
RAS guanyl-releasing protein 2	2.64	3.49	1.15	-0.33	-0.03
SRC-like-adapter	2.42	0.64	1.80	-0.25	0.73
kinase C-binding protein 1	1.87	1.62	1.37	0.00	0.46
lymphocyte cytosolic protein 1	1.68	4.19	1.76	0.10	0.54
T cell receptor alpha-2	1.68	4.89	2.32	0.34	0.51
T cell receptor alpha-3	1.56	4.54	1.83	0.14	0.47
T cell receptor alpha-4	1.08	4.52	2.19	0.06	0.33
BTG3 associated nuclear protein isoform a	1.25	1.29	1.40	-0.29	0.20
CD4 T-cell surface glycoprotein	-0.93	-1.07	-0.31	-0.66	0.14
CD3gammadelta-A	-1.18	-0.88	-0.24	-0.56	-0.32
Plastin-2 lymphocyte cytosolic protein	-1.38	-1.13	-1.20	0.56	0.08
CD63 antigen	-0.98	-1.69	-0.79	0.74	0.28
CD9 antigen	-1.03	-1.47	-0.68	-0.31	-0.48
Integrin beta-1	-1.23	-1.71	-1.81	0.03	0.36
Integrin beta-1	-0.08	-1.22	-1.55	1.22	0.65
Integrin beta-2	-1.00	-1.02	-1.01	1.14	0.47
Leukocyte surface antigen CD53	-1.25	-1.29	-0.49	-0.45	-0.16
Annexin A2-A	-1.64	-1.83	-1.63	0.51	0.28
Myosin-9	-1.71	-1.15	-1.68	0.43	0.34
nonclassical MHC class I antigen	1.13	0.86	0.67	-0.63	-0.17
Beta-2-microglobulin-1	-0.77	-1.77	-1.13	-1.34	-0.56
Beta-2-microglobulin-2	-1.39	-1.79	-1.39	-0.09	-0.64
H-2 class I histocompatibility antigen	-1.64	-2.41	-1.66	-0.50	-0.40

Down >32 32-5.7 5.6-2.6 2,5-1.7 1.6-1.3 1.3-1.6 1.7-2,5 2.6-5.6 5.7-32 >32 Up

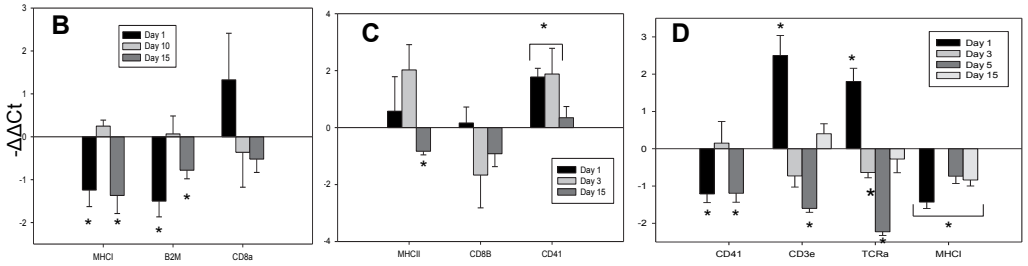

Figure 6 Examples of genes involved in cellular immunity with differential expression in skin and head kidney. **A**: microarrays in skin, data are mean log_2-ER (n = 4). The gene functions are explained in the text. **B-D**: RT-qPCR analyses of MHC and T-cell markers in skin (**B** &**C**), and in head kidney (**D**) performed with SYBR Green and TaqMan based assays. Data are mean -ΔΔCt ± SE. Significant differences from control (n = 4, P < 0.05) are indicated with *.

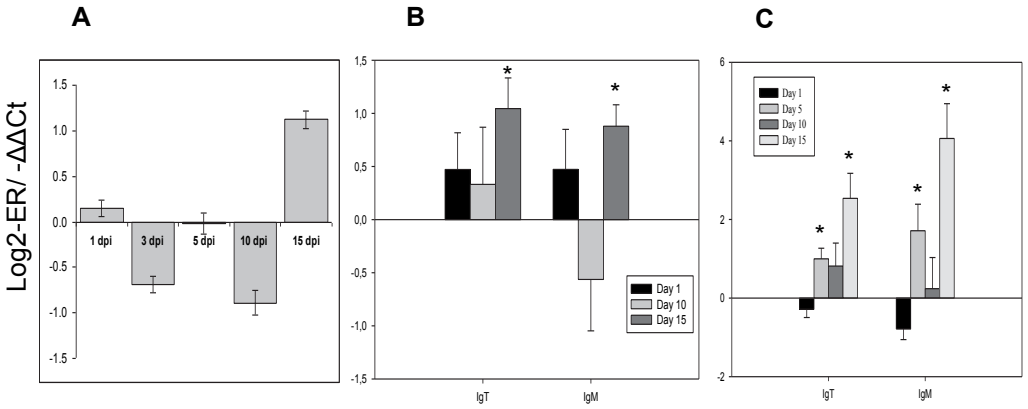

Figure 7 Expression of immunoglobulins. **A**: microarray analyses in spleen. Data are log_2-ER ± SE for 28 independent probes. **B, C**: RT-qPCR analyses in spleen (**B**), and skin **(C)** performed with SYBR Green and TaqMan based assays. Data are mean -ΔΔCt ± SE. Significant differences from control (n = 4, P < 0.05) are indicated with *.

showed decrease at 10 dpi followed with up regulation at 15 dpi.

Discussion

The Atlantic salmon is highly susceptible to *L. salmonis* and the present study was thus designed to identify host responses due to the early infectious stages from 1 to 15 dpi. After settlement, copepodids spend 7 to 11 days (at 9°C) on the host before all have completed the molt and are physically attached to the host by a frontal filament. We focused on the first 15 days after infection and the host responses were related to copepodids (day 1 to 5), mixed copepodid and chalimus (day 10) and chalimus (day 15) (Figure 8). We used advantages of multiple

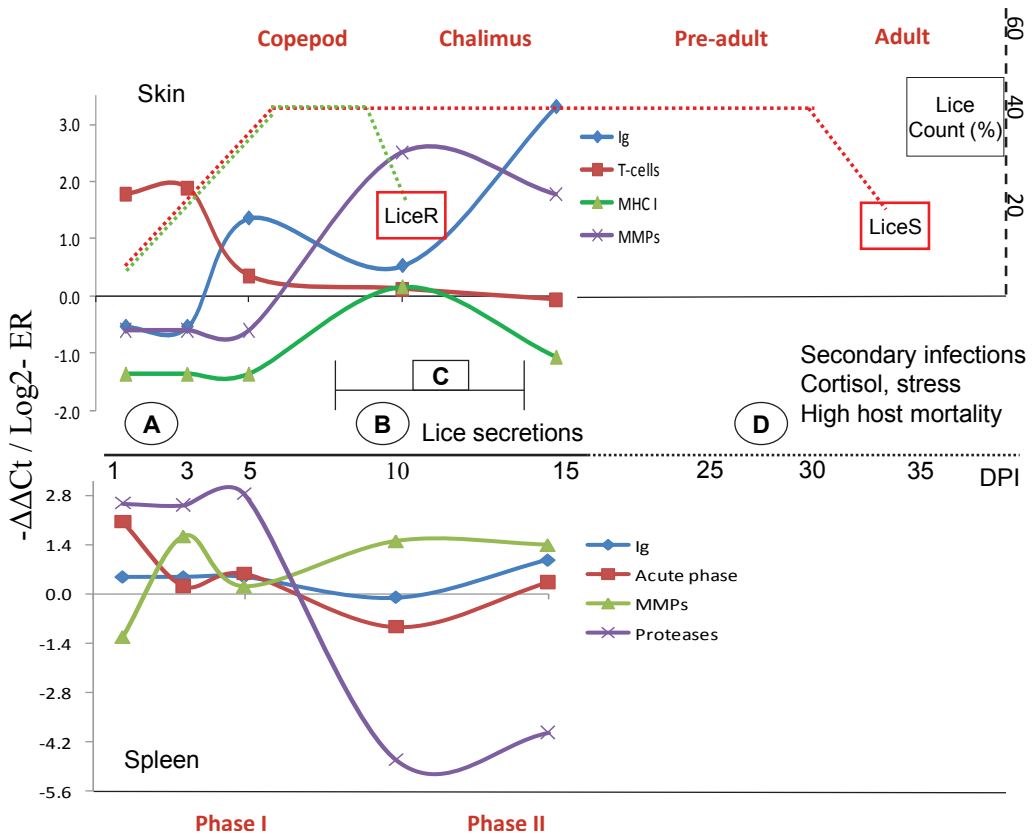

Figure 8 Summary of salmon responses to lice in skin and spleen. The present study dealt with responses until 15 dpi as shown in the left side of the figure. Host responses to mature lice stages from literature are shown by a dotted line (right side). The figure illustrates the bi-phasic responses to *L. salmonis* with abrupt changes in gene expression profiles taking place between 5 and 10 dpi, when lice molts from copepodids to chalimus. Ig genes show an initial decrease followed by gradual increase. MHC and related genes are down regulated. **A**: early sensing was witnessed both in skin and spleen. At point **B**, PGE_2 and other secreted components start to increase [31,34] and this may lead to immune modulation, and can partly explain the pronounced shift in immune responses. Resistant salmonids (e.g. coho salmon) reject lice at point **C**, 7-14 dpi [10,11] and the number of lice per fish (LiceR) starts to decrease (see the hypothetical curve on the right side). In susceptible species including Atlantic salmon, the lice number (LiceS) remains relatively stable until they reach the pre-adult/adult stages where lice falls off the fish possibly due to aging, competition, and other factors. In spleen, secretory proteases show dramatic fluctuation. Increased MMPs in all tissues suggest inflammation. When the louse enters the pre-adult stage (i. e, ~ after day 20), the effects on host increase and can cause high mortality. The risk of secondary infections increase, and cortisol levels rise significantly (point **D**), which indicates severe stress [9,26,34]. At this point, it is difficult to differentiate the direct and stress mediated effects of lice [26]. The figure summarizes responses observed after single pulse infection. However, under natural conditions, salmon can carry parasites at different developmental stages and responses can be modified with diverse environmental factors.

gene expression profiling with a 21 k oligonucleotide microarray, which covers the major fraction of protein-coding genes in Atlantic salmon. Transcriptional responses to salmon louse were analyzed in skin, the first entry point for the parasite, and in the spleen. The latter was selected due to the important role as a lymphoid organ [22] and furthermore, in our previous microarray study [13] we found greater gene expression changes in salmon spleen in comparison with the head kidney, another major immune organ of teleost fish. Our data revealed a strong host response one day post infection with a pronounced switch in gene expression pattern taking place between 5 and 10 dpi. This switch corresponds to the period where lice molt from copepodids to chalimus I ending up with a new transcription pattern at day 15 where all surviving parasites have developed into chalimus (Figure 8). In contrast to Pacific salmon, Atlantic salmon show limited tissue response to *L. salmonis* infections [10]. The reason for this is unknown but a recent study [23] indicates that the Pacific and Atlantic form of *L. salmonis* may represent two different species and this may account for some of the differences. In addition to immune parameters, differing resistance of salmonids to *L. salmonis* can be related to the structure of skin, composition of mucus, and environmental factors [5,9-12,24].

Little is known about how teleosts respond to parasites in general and to the salmon louse in particular. By using a microarray approach it is possible to screen a large number of markers and to identify both known and novel host responses to the pathogen of interest. Use of genomic tools allowed reconsidering of views based on the studies with limited sets of immune parameters. It was thought that louse do not cause significant effect in Atlantic salmon at early stages [25]. Weak inflammation at the site of attachment was regarded as a plausible explanation of higher susceptibility of Atlantic salmon in comparison with closely related species, such as sockeye and coho salmon (reviewed in [26]). However, transcriptomic analyses did not show low levels of immune responses to lice in Atlantic salmon. Dramatic gene expression changes were seen immediately after infection in the target site (skin) and in the spleen; both local and systemic sensing was rapid and large by scale. Given that most differentially expressed genes are not those that are commonly included in studies of salmon immunity, it would be difficult to detect these changes based on the candidate genes approach. An unexpected finding was involvement of splenic proteases. Dramatic expression changes of a group of genes encoding functionally related proteins imply their important role, which remains completely unknown. Thus, results of transcriptome analyses suggest that low resistance of Atlantic salmon to lice appears to be accounted for by the character of immune response rather than the scale of the response. The results elucidated immune processes that are activated but most likely do not confer substantial protection against the parasite.

High-throughput analyses revealed a bi-phasic response to lice. Modulation of responses by the parasite can be considered as one possible explanation. It is well documented that parasites have the ability to modulate host response to avoid rejection by the host and by this increase survival. For ectoparasites this can be conducted by releasing excreted products to the host surface or the site of feeding. Based on knowledge from other ectoparasites it is likely that *L. salmonis* releases a diversity of secretory/excretory products when it settles on a suitable host. Recently it was shown that horse fly (*Tanabus yao*) release a wide diversity of molecules when feeding and these molecules where shown to affect a range of biochemical and physiological processes in the host [27]. Salivary gland extracts from ticks suppress lymphocyte proliferation and cytokine response [28]. Parasites such as *Leishmania* utilize a number of immune avoidance strategies [29], some of which resembling that of tumor cells [30]. Immune suppression by lice has been reported in several publications. Salmon louse releases molecules that affect host response [31] and a few of these have been identified [31,32]. Lice produce PGE2, trypsin-like proteases, and other products that suppress the immune system of Atlantic salmon [33]. Significant reduction of oxidative and phagocytic activities of macrophages [25], and reduced transcription of IL-1β and COX-2 in lice infected salmon has been reported [31,34]. In the present study, a panel of pro-inflammatory cytokines analyzed with RT-qPCR (IL1-β, IL1R1, TNFα and IL-12) did not show significant response to lice. Furthermore, our findings indicated down-regulation of Ag presentation after infection with salmon lice, possibly affecting the conventional T-cell mediated adaptive immune response. Similar down-regulation of genes involved in Ag processing has been documented in Atlantic salmon infected by the protozoan ectoparasite that causes amoebic gill disease (AGD) [35]. This is interesting because *L. salmonis* is also implicated as a possible risk factor for AGD [36]. In similar host-parasite interaction studies, MHC II gene expression decreased in head kidney and skin after infection of carp with *Trypanoplasma borreli* [37], and rainbow trout with *Gyrodactylus derjavini* [38]. Besides, our microarray data shows down-regulation of lysosomal proteases (cathepsins), which process exogenous antigens for presentation by MHC II [39,40].

The character of inflammation changed during the copepodid-chalimus transition as well. Commonly acute and chronic inflammation is associated with cells of

myeloid origin and lymphocytes, respectively. However, an opposite trend was observed in our study. As shown in Figure 6A and 6C, gene expression changes provided evidence for a rapid recruitment of T-cells in the damaged sites, indicating a short term T-cell mediated response early during infection (1-5 dpi), which completely disappeared after 5 dpi. It is worth noting that Atlantic salmon possesses diversified numbers T-cells and receptors [41,42]. Their functional roles remain undetermined, as the true cytokine profile of CD4 response is dependent on interactions between the pathogen and antigen-presenting-cells [43]. In mammals, natural T- cells expressing a conserved TCRα-chain can exhibit both $CD4^+$ and $CD4^-/8^-$ double-negative phenotype [44]. It is possible that in the present study, at least some of the lymphocyte responses could be MHC-independent, possibly belonging to unidentified lineages that are not associated with acquired immunity or immune memory either, as Atlantic salmon used in this study was not immunized previously against lice. These cells could be similar to innate T-like cells which function as natural killer T-cells recognising antigens presented by non-classical MHC molecules [45]. Microarray data showed up-regulation of non-classical MHCI molecules in the skin (Figure 6A). Induction of genes that control early stages of lymphocyte differentiation suggests involvement of precursor cells, which either resided in skin or were delivered with blood. Concurrent down-regulation of several genes in skin that control transendothelial migration indicates the depletion of leukocytes.

A hallmark of transit from acute to chronic inflammation was the systemic increase of MMP9 (gelatinase) and MMP13 (collagenase), which did not show expression changes during the first phase. Earlier we found preferential expression of these genes in salmon leukocytes [14]. The changes of transcript abundance could be due to either MMPs induction in activated resident immune cells (macrophages) or influx of leukocytes. The latter possibility is supported with simultaneous up-regulation of integrins and C3a anaphylatoxin chemotactic receptor in skin and neutrophil cytosolic factor in spleen (Figs 3 & 4). The observed changes can be a consequence of chronic stress and increased production of cortisol. In this respect, it is noteworthy that Fast et al. [34] found no changes of plasma cortisol levels during the first 15 dpi in Atlantic salmon infected with *L. salmonis*, while its increase at 26-33 dpi was in parallel with the induction of pro-inflammatory mediators (IL-1β and TNFα). Overall, cortisol has an immune suppressive action. However, a remarkable feature of salmon MMPs is induction with both inflammatory stimuli [46] and stress [47]. Recently we observed up-regulation of MMPs in salmon with cortisol implants (manuscript under preparation). Previously we reported a sustained induction of MMPs as a characteristic feature of lice infection in Atlantic salmon [13]. MMP-9 in carp LPS stimulated leucocytes shows a bi-phasic profile: increase until 48 hours, decline, and another increase at 168 hours, indicating its role both in early inflammation and later stages of tissue remodelling [17].

Vaccines are discussed as a possible measure against salmon louse. Immunization of fish against *L. salmonis* may be facilitated by an improved understanding of the adaptive immune system and molecules involved therein, particularly how the host responds to parasites. One of the limitations with vaccine development could be the limited exposure of louse to blood and thereby serum antibodies (reviewed in [8]), and mucosal immunity might play a major role here as *L. salmonis* are colonizers of cutaneous mucosa of salmonids. Mucosal epithelial cells serve as an initial barrier and, in addition, they are involved in adaptive immunity by Ag presentation and production of Igs along with complement, lectins, CRP, lysozymes, proteolytic enzymes and other effectors [48-50]. Antibodies at the surface of skin mucus can block ectoparasites from infestation or reduce infestation success [50]. IgT/IgZ is a teleost specific antibody class first discovered in rainbow trout and zebrafish [51,52]. In salmon, there are three highly similar IgT sub-variants [53]. A recent study indicated that they might be differentially regulated [54]. IgT is associated with mucosal immunity, similarly to the mammalian IgA [55]. IgT transcription in rainbow trout gut was up-regulated more than 700 fold in fish that survived infection with the parasite *Ceratomyxa shasta* [55]. This immunoglobulin may coat gut luminal bacteria thus preventing their attachment and invasion of the gut epithelium [55]. While transcript levels of IgT and IgM in mucosal tissues of naïve Atlantic salmon are relatively low [53], we documented up to ten fold increase in skin after infection with *L. salmonis* (Figure 7C). An increase of IgT and IgM transcripts in skin and spleen may indicate an onset of adaptive immune responses at later stages of infection. It is worth mentioning here that microarray data has showed an early up-regulation of pIgR, a key molecule involved in the transport of Igs to mucosal surfaces [55-57]. However, we did not observe an early increase in Ig transcripts at the target site. In addition to transcytosis of Igs, pIgR has an important role in innate immune functions by attaching to host and pathogenic factors, as well as protecting Igs from proteolytic degradation [57,58].

Conclusions

In this paper we studied gene expression changes in Atlantic salmon skin, spleen and head kidney during the first 15 days post infection by *L. salmonis*, using microarray and RT-qPCR (results are summarised in

Figure 8). The findings clearly indicated early sensing at 1 dpi with induction of genes involved in innate immune reactions, including lectins and enzymes of eicosanoid metabolism in skin and acute phase proteins in spleen. This was followed by regulation of a diverse array of genes including MMPs and immunoglobulins. The responses are bi-phasic with large shift in transcript profiles of many genes during the time window corresponding to the copepod-chalimus transition. Gradual increase of Ig transcripts from 1-15 dpi in skin and spleen, possibly indicated mounting of adaptive immunity, which was supported by the up-regulation of putative lymphocyte G0/G1 switch proteins at 5 dpi in the spleen. The responses, however, did not result in appreciable level of protection, as revealed by the lice load on fish at the end of the study. Down-regulation of the antigen presenting MHCI and related molecules, and absence of T-cell induction at later stages suggested lack of T-cell dependent acquired immunity. Further biochemical and functional studies of immune mechanisms of IgT at mucosal sites in salmon, in the context of lice infection will greatly contribute to a better understanding of how adaptive immunity is orchestrated in salmon with regard to mucosal defences. Furthermore, the large group of secretory splenic proteases, which show the greatest transcriptional fluctuations (up- and down-regulation), deserve a closer attention.

Methods

Challenge experiment

Atlantic salmon in the size range of 100-200 g, which has not previously been in contact with *L. salmonis*, were placed in two different tanks containing full salinity water (one for control and another for *L. salmonis* infection), at the Institute of Marine Research (IMR) in Bergen. A hatchery and culturing system that enables laboratory maintenance of salmon louse throughout its life-cycle was developed recently [59]. Salmon louse of LsGulen strain [59] were reared in the hatchery and egg strings were collected and placed into an incubator until they reached the copepodid stage. Atlantic salmon were challenged with *L. salmonis* (approximately 100 copepodids per fish). Commonly, approximately one third of the copepodids added to a tank are found on fish during the pre-adult/adult stages [59]. Following infection, tissues of skin, spleen, and head kidney were sampled 1, 3, 5 dpi (corresponding to the copepod stage); 10 and 15 dpi (chalimus stage). Control fish were sampled in a similar manner (Figure 9). Immediately after sampling, tissues were stored in liquid nitrogen and then transferred to -80°C freezer. The fish were kept at a temperature of 9 ± 1°C during the entire experimental period, and fed with commercial diet once daily. Louse load on each fish was counted at the end of the experiment, 15 dpi.

RNA and cDNA preparation

RNA was isolated using the iPrep™ TRIzol® Plus kit (Invitrogen), and purified with RNeasy mini kit (QIAGEN). The quantity and quality of the total RNA was assessed using a NanoDrop Spectrophotometer (NanoDrop Technologies) and an Agilent 2100 Bioanalyzer (Agilent Technologies). Samples with RNA integrity number (RIN) of 8 or higher were accepted for microarray analyses. cDNA was synthesized by reverse transcription of RNA using the qScript™ cDNA Synthesis Kit (Quanta BioSciences). Total RNA and cDNA were stored at -80 and -20°C respectively until use.

Microarray analyses

RNA amplification and labelling were performed using Two-Colour Quick Amp Labelling Kit and Gene Expression Hybridization kit (for fragmentation of labeled cRNA) following the manufacturer's instructions for 4 × 44 k microarrays (Agilent Technologies). Nofima Marin's salmon oligonucleotide microarray SIQ3 (GEO GPL10706) was fabricated by Agilent Technologies and annotated with STARS bioinformatic package [14]. The features were assigned to the functional classes (GO) and pathways (KEGG). In addition, custom annotations were implemented based on literature and other public sources. Four biological replicates in each of 5 time-points per tissue (skin and spleen), which comprise a total of 40 microarrays (one array per sample), were included in the analyses (Figure 9). The input of total RNA used in each reaction was 500 ng. Pooled control samples were prepared by mixing equal RNA concentrations from each individual sample of control fish per each time point. Individual samples of infected fish were labeled with Cyanine 5 (Cy5), while control samples were labeled with Cy3. Following labeling, amplification, purification, and quantification, 825 ng of both the Cy5-labeled test cRNA samples and Cy3-labelled control samples were mixed and hybridized (competitively) to the arrays (Figure 9). Over night hybridization (17-hours, 65°C, and rotation speed of 10 rpm) was performed in hybridization oven (Agilent Technologies). After hybridization, arrays were washed with Gene Expression Wash Buffers 1 and 2 and scanned with a GenePix 4100A (Molecular Devices, Sunnyvale, CA, USA). GenePix Pro 6.0 was used for spot to grid alignment, feature extraction and quantification. Assessment of spot quality was done with aid of GenePix. After filtration of flagged low quality spots, Lowess normalization of $\log_2$-expression ratios (ER) was performed. The differentially expressed genes (DEG) were selected by criteria: $\log_2 ER > |0.8|$ and $p < 0.01$, (sample t-test, null hypothesis $\log_2 ER = 0$) in at least one time-point. The microarray data were submitted to GEO, GSE26981 (skin) and GSE26984 (spleen).

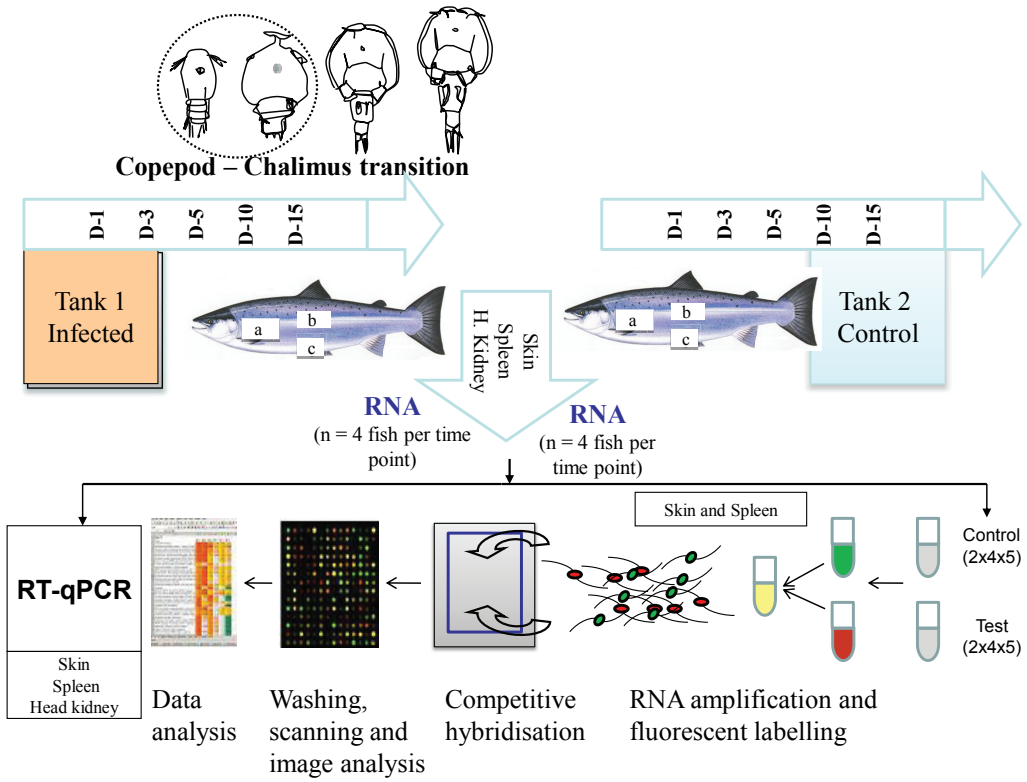

Figure 9 Set-up of the experiment. Atlantic salmon was challenged with *L. salmonis* at the copepod stage. Skin, spleen, and head kidney were sampled from challenged and control fish at 1, 3, 5 dpi (corresponding to the copepod stage); 10 and 15 dpi (chalimus stage). Boxes a, b, and c respectively indicate approximate positions on the fish where tissues (head kidney, skin, and spleen) were removed. A total of forty samples of spleen and skin form infected salmon (4 individuals from the 5 time points) were used for microarray analyses. Test samples were labeled with Cy5 and hybridized to pooled control samples labeled with Cy3 from the same time-points. Competitive hybridization to the arrays was followed by washing, scanning, image analysis, and data analysis. Selected genes were analyzed with RT-qPCR.

RT-qPCR

Eighteen differentially expressed genes from the microarray experiment, covering the entire dynamic range of expression, were selected for verification with RT-qPCR. Preference was given to immune genes and to genes with unknown roles that showed strong responses to the parasite. In addition, T-cell markers, immunoglobulins (Ig), and genes of antigen presentation, and pro-inflammatory cytokines were included. RT-qPCR analyses were carried out with skin, spleen and head kidney. TaqMan® probe, and SYBR Green based Real-Time PCR assays were designed using Primer express 3.0 (Applied Biosystems), and Beacon Designer 7.8 (PREMIER Biosoft, USA) respectively (Table 2). When possible, primers or probes were designed to span between two exons. Fast SYBR® Green Master Mix based PCR was performed using 7500 Fast Real-Time PCR System (Applied Biosystems). The PCR reaction mix contained 5 μl of Fast SYBR® Green Master Mix (2 ×), 500 nM of each primer, and 2 μl of cDNA in a final volume of 10 μl. Thermal cycling was carried out according to the manufacturers protocol (Applied Biosystems) as follows: enzyme activation at 95°C for 20 sec, followed by 40 cycles of denaturation (95°C for 3 sec), and annealing/extension (60°C for 30 sec). In addition, Taqman probe based quantitative PCR was performed on a few selected genes using the 7500 Fast Real-Time PCR System (Applied Biosystems), and quantification of mRNA was performed in a single step assay (both RT and PCR steps carried out in the same tube)

Table 2 Primer/ probe list

S. N	Sequence Definition	Code		Primer/ probe (5'-3')	Product length	GenBank Accession (GI)
1.	Apoptosis regulator Bcl-X	Apo	Fwd	TACTAAGTGTTGCCGTTGA	109	209734267
			Rev	TAATCCAATCTGTGTCATTCTG		
2.	Beta-2-microglobulin precursor	B2M	Fwd	CACAGACAGACACAGACA	102	221220497
			Rev	CAACGATTGACAGAATAGACTT		
3.	Carboxypeptidase A1 precursor	CarPep	Fwd	AGCATACCAAGGACAACAC	75	209732661
			Rev	TACAACAGTACAATGACACAGT		
4.	Chymotrypsin B	ChyTry	Fwd	CTGTCCATACTGTATATTGCTAT	111	209734305
			Rev	GCTATAATGCTTAGGTGTTGTA		
5.	Collagenase 3 precursor	COL3	Fwd	ATCTGTGCTTACTACTAATCAAC	81	209156091
			Rev	GGGCTTCATCTTCTTTACTG		
6.	Complement C1q subcomponent subunit B precursor	C1qB	Fwd	CTGTCTGTCGTTGTCTTC	89	223649475
			Rev	ATGGTCTGTTGGTCTGTA		
7.	Elastase-1	Ela	Fwd	ACCGTCAACAAAGTCTTCA	75	S31963336
		Rev		CAGCAGAGCGATGTCATA		
8.	Fish virus induced TRIM protein	TRIM	Fwd	GCATGGCACAATAATAACT	75	S35697379
			Rev	GTCCAGATACACTCCTAC		
9.	Trypsin-2	Try2	Fwd	CAGTTGTCGGTTGAGATG	81	S18845530
			Rev	CAAGATGTGCCAGATAGC		
10	Trypsin-3	Try3	Fwd	CATTATTCTTCTCGCTCTG	81	S31964271
			Rev	TCATACCCTCCAACAATC		
11	Tumor necrosis factor alpha	TNFα	Fwd	ACAAAGAGGGCCAGGGATTC	100	126507266
			Rev	GAGGCCTGGCTGTAGACGAA		
12	Interleukin-1 receptor-like protein	IL1R1	Fwd	AGCAGGATGTCCTCGGTCTA	202	185136290
			Rev	TGGGTAGCGGTGTAGTTTCC		
13	Interleukin-1 beta	IL1β	Fwd	ACCGAGTTCAAGGACAAGGA	196	186288127
			Rev	GCAGCTCCATAGCCTCACTC		
14	Gamma-interferon-inducible lysosomal thiol reductase	GIILTR	Fwd	CTATGTGCCTTGGATTGT	79	209732609
			Rev	CAGAGTGAAGAGTGAAGAC		
15	HSP70_ONCMY Heat shock cognate 70 kDa protein	HSP70	Fwd	TCACTAGAGTCCTATGCT	85	CL7Contig1
			Rev	TTGTCTTGTCCTCATCAC		
16	Lipopolysaccharide-induced TNF-a factor homolog	LPSi-TNFα	Fwd	CAATTCCTTCGACCTCAT	85	209734201
			Rev	GCTCTTCTCCATACTGTC		
17	Metalloreductase STEAP4	STEAP4	Fwd	CTCCAACTCTGAAGACTATT	103	S48396453
			Rev	GAGCACTGTCAATCAATG		
18	Myosin, heavy polypeptide 9, non-muscle	Myo	Fwd	GCAGTTGAGACTCTACAGTGGAA	75	CB498954
			Rev	ACAGCGTGTTGAGTGTGGTT		
19	Programmed cell death protein 2	PCDP	Fwd	GCATAGACAGCCACAATC	81	209735097
			Rev	GAGCGTAACAACCTGAAG		
20	Prostaglandin E synthase 3	PGES	Fwd	TCCAGCCAATGTCTTAGT	99	223672934
			Rev	AAGCACGGTATAACTGAAC		
21	P-selectin precursor	P-sel	Fwd	CTGGTGATTCTATTGATGAC	86	209154193
		Rev		TTGACGCTGTAGTTGTAT		
22	CD8α	CD8α	Fwd	CGTCTACAGCTGTGCATCAATCAA	118	185135177
			Rev	GGCTGTGGTCATTGGTGTAGTC		
23	Interleukin-12	IL-12	Fwd	TCTACCTACACGACATTGTCCAGCC	62	209736091
			Rev	ATCCATCACCTGGCACTTCATCC		
24	Prostaglandin D synthase	PGDS	Fwd	ATCCCAGGCCGCTTCAC	59	304376917
			Rev	ACACGCATGTCATTTTCATTGTT		

Table 2 Primer/ probe list *(Continued)*

			Probe	TTCACCAGCCAGCGTT		
25	Matrix metalloproteinase-13	MMP13	Fwd	GCCAGCGGAGCAGGAA	56	213514499
			Rev	AGTCACCTGGAGGCCAAAGA		
			Probe	TCAGCGAGATGCAAAG		
26	T-cell receptor alpha	TCRα	Fwd	GACAGCTACTACAGCCAGGTT		209736003
			Rev	CAGAATGGTCAGGGATAGGAAGTT		
			Probe	ACACAGATGCAAAGATC		
27	CD3ε	CD3ε	Fwd	TCAGGGCTCGGAAGAAGTCT	68	185135943
			Rev	GCCACGGCCTGCTGA		
			Probe	CCAAAAACCCACTTCCC		
28	CD4-like protein, variant 1	CD4-1	Fwd	GAATCTGCCGCTGCAAAGAC	75	185135736
			Rev	AGGGATTCCGGTCTGTATGATATCT		
			Probe	CCCAAACCAAAAGGATTC		
29	CD8β	CD8β	Fwd	GGAGGCCAGGAGTTCTTCTC	70	185135192
			Rev	GGCTTGGGCTTCGTGACA		
			Probe	ACCCGGAGAAACTC		
30	MHC class I antigen	MHC-I	Fwd	CAACGCCACAGGCAGTCA	64	25573077
			Rev	CGGTACTCATTCTGAGCTGTGTTAC		
			Probe	CACCAAACTCAAGTGGG		
31	MHC class II antigen	MHC-II	Fwd	CTCACTGAGCCCATGGTGTAT	117	223672978
			Rev	GAGTCCTGCCAAGGCTAAGATG		
			Probe	CTGGGACCCGTCCCTG		
32	Immunoglobulin mu	IgM	Fwd	TGAGGAGAACTGTGGGCTACACT	69	2182101
			Rev	TGTTAATGACCACTGAATGTGCAT		
			Probe	CATCAGATGCAGGTCC		
33	Immunoglobulin tau	IgT	Fwd	CAACACTGACTGGAACAACAAGGT	97	260766539
			Rev	CGTCAGCGGTTCTGTTTTGGA		
			Probe	AGTACAGCTGTGTGGTGCA		
34	Elongation factor 1A	EF1A	Fwd	CCCCTCCAGGACGTTTACAAA	57	224587629
			Rev	CACACGGCCCACAGGTACA		
			Probe	ATCGGTGGTATTGGAAC		

according to the Verso™ 1-step QRT-PCR low ROX kit (Thermo Scientific). PCR reaction mix containing 1-step qPCR low ROX mix (2 ×), enzyme mix, RT enhancer, 900 nM of each primer, 200 nM of TaqMan probe was mixed with 2 μl (50-100 ng) of RNA in a final volume of 12.5 μl. This is followed by thermal cycling steps of cDNA synthesis at 50°C for 15 min, an enzyme activation step of 95°C for 15 min, and 45 cycles of 95°C for 15 sec (denaturation) and 60°C for 60 sec (annealing/ extension). All samples were run in duplicates (or triplicates) with non-template controls and non-reverse transcription controls on the same plate. Amplification of genomic DNA was checked by a melting curve analysis, which resulted in a single peak, indicating single product amplification. Elongation factor 1 alpha (EF1A) was used as an internal reference gene, as it has been shown to be the best reference gene for RT-qPCR studies in Atlantic salmon tissues [60]. We also have recent experience with samples similar to those analysed in this study and EF1A has shown an appropriate stability [53]. This transcript has also been represented in the microarray and did not show response to lice. To calculate ΔCt, the respective reference Ct values were subtracted from target Ct values of the control and test samples; and -ΔΔCt was calculated as: $-\Delta\Delta Ct = -(\Delta Ct_{Test} - \Delta Ct_{Control})$. PCR efficiencies were in acceptable range (Additional file 2). Statistical analysis of the RT-qPCR data was done using Microsoft excel and SigmaPlot 11.0. The data were presented as mean -ΔΔCt ± SE. Differences between control and test at each sampling point were assessed with Student's t-test.

Additional material

Additional file 1: Differentially expressed genes, complete microarray results.

Additional file 2: Complete RT-qPCR results and PCR efficiencies.

Additional file 3: Proteases with differential expression in spleen, microarray results.

Additional file 4: Secretory proteins, components of complement and coagulation cascade differential expression in spleen, microarray results.

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Authors' contributions
TMT contributed to the overall experimental design, conducted the challenge test, the RT-qPCR analysis, performed the microarray experiment, involved in data analysis, drafted and wrote the manuscript. AK analysed the microarray data, drafted and wrote the manuscript. SS performed the microarray experiment and contributed to the overall manuscript. SA developed computer programs and database for management of microarray results and data mining. IH designed the study and contributed to the overall manuscript. FN designed the study, was involved in the challenge experiment, and contributed to the overall manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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References

1. Johnson SC, Albright LJ: **The developmental stages of *Lepeophtheirus salmonis* (Krøyer, 1837) (Copepoda: Caligidae).** *Can J Zool* 1991, **69**:929-950.
2. Grimnes A, Jakobsen PJ: **The physiological effects of salmon lice infection on post-smolt of Atlantic salmon.** *J Fish Biol* 1996, **48**:1179-1194.
3. Jones S, Kim E, Bennett W: **Early development of resistance to the salmon louse, *Lepeophtheirus salmonis* (Kroyer), in juvenile pink salmon, *Oncorhynchus gorbuscha* (Walbaum).** *J Fish Dis* 2008, **31**:591-600.
4. Costello MJ: **How sea lice from salmon farms may cause wild salmonid declines in Europe and North America and be a threat to fishes elsewhere.** *Proceedings of the Royal Society B-Biological Sciences* 2009, **276**:3385-3394.
5. Costello MJ: **Ecology of sea lice parasitic on farmed and wild fish.** *Trends Parasitol* 2006, **22**:475-483.
6. Gustafson L, Ellis S, Robinson T, Marenghi F, Endris R: **Efficacy of emamectin benzoate against sea lice infestations of Atlantic salmon, *Salmo salar L.*: evaluation in the absence of an untreated contemporary control.** *J Fish Dis* 2006, **29**:621-627.
7. Alvarez-Pellitero P: **Fish immunity and parasite infections: from innate immunity to immunoprophylactic prospects.** *Vet Immunol Immunopathol* 2008, **126**:171-198.
8. Raynard RS, Bricknell IR, Billingsley PF, Nisbet AJ, Vigneau A, Sommerville C: **Development of vaccines against sea lice.** *Pest Manag Sci* 2002, **58**:569-575.
9. Tully O, Nolan DT: **A review of the population biology and host-parasite interactions of the sea louse *Lepeophtheirus salmonis* (Copepoda: Caligidae).** *Parasitology* 2002, **124(Suppl)**:S165-182.
10. Fast MD, Ross NW, Mustafa ASims DE, Johnson SC, Conboy GA, Speare DJ, Johnson G, Burka JF: **Susceptibility of rainbow trout *Oncorhynchus mykiss*, Atlantic salmon *Salmo salar* and coho salmon *Oncorhynchus kisutch* to experimental infection with sea lice *Lepeophtheirus salmonis*.** *Dis Aquat Organ* 2002, **52**:57-68.
11. Johnson SC, Albright LJ: **Comparative Susceptibility and Histopathology of the Response of Naive Atlantic, Chinook and Coho Salmon to Experimental-Infection with *Lepeophtheirus-Salmonis* (Copepoda, Caligidae).** *Dis Aquat Organ* 1992, **14**:179-193.
12. Fast MD, Sims DE, Burka JF, Mustafa A, Ross NW: **Skin morphology and humoral non-specific defence parameters of mucus and plasma in rainbow trout, coho and Atlantic salmon.** *Comp Biochem Physiol A Mol Integr Physiol* 2002, **132**:645-657.
13. Skugor S, Glover KA, Nilsen F, Krasnov A: **Local and systemic gene expression responses of Atlantic salmon (*Salmo salar* L.) to infection with the salmon louse (*Lepeophtheirus salmonis*).** *BMC Genomics* 2008, **9**:498.
14. Krasnov A, Timmerhaus G, Afanasyev S, Jorgensen SM: **Development and assessment of oligonucleotide microarrays for Atlantic salmon (Salmo salar L.).** *Comp Biochem Physiol Part D Genomics Proteomics* 2011, **6**:31-38.
15. Arce I, Martinez-Munoz L, Roda-Navarro P, Fernandez-Ruiz E: **The human C-type lectin CLECSF8 is a novel monocyte/macrophage endocytic receptor.** *Eur J Immunol* 2004, **34**:210-220.
16. Bayne CJ, Gerwick L, Fujiki K, Nakao M, Yano T: **Immune-relevant (including acute phase) genes identified in the livers of rainbow trout, *Oncorhynchus mykiss*, by means of suppression subtractive hybridization.** *Dev Comp Immunol* 2001, **25**:205-217.
17. Chadzinska M, Baginski P, Kolaczkowska E, Savelkoul HF, Kemenade BM: **Expression profiles of matrix metalloproteinase 9 in teleost fish provide evidence for its active role in initiation and resolution of inflammation.** *Immunology* 2008, **125**:601-610.
18. Hasebe T, Kajita M, Fujimoto K, Yaoita Y, Ishizuya-Oka A: **Expression profiles of the duplicated matrix metalloproteinase-9 genes suggest their different roles in apoptosis of larval intestinal epithelial cells during *Xenopus laevis* metamorphosis.** *Dev Dyn* 2007, **236**:2338-2345.
19. Maghazachi AA: **Insights into seven and single transmembrane-spanning domain receptors and their signaling pathways in human natural killer cells.** *Pharmacol Rev* 2005, **57**:339-357.
20. Salmond RJ, Filby A, Qureshi I, Caserta S, Zamoyska R: **T-cell receptor proximal signaling via the Src-family kinases, Lck and Fyn, influences T-cell activation, differentiation, and tolerance.** *Immunol Rev* 2009, **228**:9-22.
21. Aoki K, Inazawa J, Takahashi T, Nakahara K, Kasai M: **Genomic structure and chromosomal localization of the gene encoding translin, a recombination hotspot binding protein.** *Genomics* 1997, **43**:237-241.
22. Whyte SK: **The innate immune response of finfish–a review of current knowledge.** *Fish Shellfish Immunol* 2007, **23**:1127-1151.
23. Yazawa R, Yasuike M, Leong J, von Schalburg KR, Cooper GA, Beetz-Sargent M, Robb A, Davidson WS, Jones SR, Koop BF: **EST and mitochondrial DNA sequences support a distinct Pacific form of salmon louse, *Lepeophtheirus salmonis*.** *Mar Biotechnol (NY)* 2008, **10**:741-749.
24. Bricknell IR, Dalesman SJ, O'Shea B, Pert CC, Luntz AJ: **Effect of environmental salinity on sea lice *Lepeophtheirus salmonis* settlement success.** *Dis Aquat Organ* 2006, **71**:201-212.
25. Mustafa A, MacWilliams C, Fernandez N, Matchett K, Conboy GA, Burka JF: **Effects of sea lice (*Lepeophtheirus salmonis* Kroyer, 1837) infestation on macrophage functions in Atlantic salmon (*Salmo salar* L.).** *Fish Shellfish Immunol* 2000, **10**:47-59.
26. Wagner GN, Fast MD, Johnson SC: **Physiology and immunology of *Lepeophtheirus salmonis* infections of salmonids.** *Trends Parasitol* 2008, **24**:176-183.
27. Xu X, Yang H, Ma D, Wu J, Wang Y, Song Y, Wang X, Lu Y, Yang J, Lai R: **Toward an understanding of the molecular mechanism for successful blood feeding by coupling proteomics analysis with pharmacological testing of horsefly salivary glands.** *Mol Cell Proteomics* 2008, **7**:582-590.
28. Ramachandra RN, Wikel SK: **Modulation of host-immune responses by ticks (Acari: Ixodidae): effect of salivary gland extracts on host macrophages and lymphocyte cytokine production.** *J Med Entomol* 1992, **29**:818-826.
29. Bogdan C, Rollinghoff M: **The immune response to Leishmania: mechanisms of parasite control and evasion.** *Int J Parasitol* 1998, **28**:121-134.
30. Ouaissi A, Ouaissi M: **Molecular basis of *Trypanosoma cruzi* and Leishmania interaction with their host(s): exploitation of immune and defense mechanisms by the parasite leading to persistence and chronicity, features reminiscent of immune system evasion strategies in cancer diseases.** *Arch Immunol Ther Exp (Warsz)* 2005, **53**:102-114.
31. Fast MD, Johnson SC, Eddy TD, Pinto D, Ross NW: ***Lepeophtheirus salmonis* secretory/excretory products and their effects on Atlantic salmon immune gene regulation.** *Parasite Immunol* 2007, **29**:179-189.

32. Fast MD, Burka JF, Johnson SC, Ross NW: **Enzymes released from *Lepeophtheirus salmonis* in response to mucus from different salmonids.** *J Parasitol* 2003, **89**:7-13.
33. Firth KJ, Johnson SC, Ross NW: **Characterization of proteases in the skin mucus of Atlantic salmon (*Salmo salar*) infected with the salmon louse (*Lepeophtheirus salmonis*) and in whole-body louse homogenate.** *J Parasitol* 2000, **86**:1199-1205.
34. Fast MD, Muise DM, Easy RE, Ross NW, Johnson SC: **The effects of *Lepeophtheirus salmonis* infections on the stress response and immunological status of Atlantic salmon (*Salmo salar*).** *Fish Shellfish Immunol* 2006, **21**:228-241.
35. Young ND, Cooper GA, Nowak BF, Koop BF, Morrison RN: **Coordinated down-regulation of the antigen processing machinery in the gills of amoebic gill disease-affected Atlantic salmon (*Salmo salar* L.).** *Mol Immunol* 2008, **45**:2581-2597.
36. Nowak BF, Bryan J, Jones SRM: **Do salmon lice, *Lepeophtheirus salmonis*, have a role in the epidemiology of amoebic gill disease caused by *Neoparamoeba perurans*?** *J Fish Dis* 2010, **33**:683-687.
37. Saeij JP, de Vries BJ, Wiegertjes GF: **The immune response of carp to *Trypanoplasma borreli*: kinetics of immune gene expression and polyclonal lymphocyte activation.** *Dev Comp Immunol* 2003, **27**:859-874.
38. Lindenstrom T, Secombes CJ, Buchmann K: **Expression of immune response genes in rainbow trout skin induced by Gyrodactylus derjavini infections.** *Vet Immunol Immunopathol* 2004, **97**:137-148.
39. Riese RJ, Chapman HA: **Cathepsins and compartmentalization in antigen presentation.** *Curr Opin Immunol* 2000, **12**:107-113.
40. Lennon-Duménil A-M, Bakker AH, Wolf-Bryant P, Ploegh HL, Lagaudrière-Gesbert C: **A closer look at proteolysis and MHC-class-II-restricted antigen presentation.** *Curr Opin Immunol* 2002, **14**:15-21.
41. Collet B, Collins C: **Comparative gene expression profile in two Atlantic salmon cell lines TO and SHK-1.** *Vet Immunol Immunopathol* 2009, **130**:92-95.
42. Yazawa R, Cooper GA, Hunt P, Beetz-Sargent M, Robb A, Conrad M, McKinnel L, So S, Jantzen S, Phillips RB, *et al*: **Striking antigen recognition diversity in the Atlantic salmon T-cell receptor alpha/delta locus.** *Dev Comp Immunol* 2008, **32**:204-212.
43. Jankovic D, Sher A, Yap G: **Th1/Th2 effector choice in parasitic infection: decision making by committee.** *Curr Opin Immunol* 2001, **13**:403-409.
44. Joyce S: **Natural T cells: cranking up the immune system by prompt cytokine secretion.** *Proc Natl Acad Sci USA* 2000, **97**:6933-6935.
45. Medzhitov R: **Recognition of microorganisms and activation of the immune response.** *Nature* 2007, **449**:819-826.
46. MacKenzie S, Iliev D, Liarte C, Koskinen H, Planas JV, Goetz FW, Molsa H, Krasnov A, Tort L: **Transcriptional analysis of LPS-stimulated activation of trout (*Oncorhynchus mykiss*) monocyte/macrophage cells in primary culture treated with cortisol.** *Mol Immunol* 2006, **43**:1340-1348.
47. Krasnov A, Koskinen H, Pehkonen P, Rexroad CE, Afanasyev S, Molsa H: **Gene expression in the brain and kidney of rainbow trout in response to handling stress.** *BMC Genomics* 2005, **6**:3.
48. Jones SR: **The occurrence and mechanisms of innate immunity against parasites in fish.** *Dev Comp Immunol* 2001, **25**:841-852.
49. Palaksha KJ, Shin GW, Kim YR, Jung TS: **Evaluation of non-specific immune components from the skin mucus of olive flounder (*Paralichthys olivaceus*).** *Fish Shellfish Immunol* 2008, **24**:479-488.
50. Maki JL, Dickerson HW: **Systemic and cutaneous mucus antibody responses of channel catfish immunized against the protozoan parasite Ichthyophthirius multifiliis.** *Clin Diagn Lab Immunol* 2003, **10**:876-881.
51. Hansen JD, Landis ED, Phillips RB: **Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish.** *Proc Natl Acad Sci USA* 2005, **102**:6919-6924.
52. Danilova N, Bussmann J, Jekosch K, Steiner LA: **The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z.** *Nat Immunol* 2005, **6**:295-302.
53. Tadiso TM, Lie KK, Hordvik I: **Molecular cloning of IgT from Atlantic salmon, and analysis of the relative expression of tau, mu, and delta in different tissues.** *Vet Immunol Immunopathol* 2011, **139**:17-26.
54. Yasuike M, de Boer J, von Schalburg KR, Cooper GA, McKinnel L, Messmer A, So S, Davidson WS, Koop BF: **Evolution of duplicated IgH loci in Atlantic salmon, *Salmo salar*.** *BMC Genomics* 2010, **11**:486.
55. Zhang YA, Salinas I, Li J, Parra D, Bjork S, Xu Z, LaPatra SE, Bartholomew J, Sunyer JO: **IgT, a primitive immunoglobulin class specialized in mucosal immunity.** *Nat Immunol* 2010, **11**:827-835.
56. Hamuro K, Suetake H, Saha NR, Kikuchi K, Suzuki Y: **A teleost polymeric Ig receptor exhibiting two Ig-like domains transports tetrameric IgM into the skin.** *J Immunol* 2007, **178**:5682-5689.
57. Kaetzel CS: **The polymeric immunoglobulin receptor: bridging innate and adaptive immune responses at mucosal surfaces.** *Immunol Rev* 2005, **206**:83-99.
58. Phalipon A, Corthesy B: **Novel functions of the polymeric Ig receptor: well beyond transport of immunoglobulins.** *Trends Immunol* 2003, **24**:55-58.
59. Hamre LA, Glover KA, Nilsen F: **Establishment and characterisation of salmon louse (*Lepeophtheirus salmonis* (Kroyer 1837)) laboratory strains.** *Parasitol Int* 2009, **58**:451-460.
60. Olsvik PA, Lie KK, Jordal AE, Nilsen TO, Hordvik I: **Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon.** *BMC Mol Biol* 2005, **6**:21.

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