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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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


Paper II


Paper III

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
F\alpha\mu R  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)
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>m</td>
<td>Membrane</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosa-associated lymphoid tissue</td>
</tr>
<tr>
<td>MDIR</td>
<td>Modular domain immune type receptor</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>n</td>
<td>Number in study or group</td>
</tr>
<tr>
<td>NA</td>
<td>Not available</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer (cell)</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pIgR</td>
<td>Polymeric immunoglobulin receptor</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>RhoG</td>
<td>RAS homologue member G</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination-activating gene</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>s</td>
<td>Secreted</td>
</tr>
<tr>
<td>S</td>
<td>Secretory</td>
</tr>
<tr>
<td>SC</td>
<td>Secretory component</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SYK</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>V</td>
<td>Variable (region, gene segment)</td>
</tr>
<tr>
<td>V\text{H}, VH</td>
<td>Variable region, heavy chain</td>
</tr>
<tr>
<td>V\text{L}, VL</td>
<td>Variable region, light chain</td>
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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, 2012). 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.

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\(^1\) 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 salaris* (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).

![Image](image.png)

**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**


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**


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**


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 \(-\Delta Ct\) or \(-\Delta\Delta Ct\) values \([\Delta Ct = Ct \text{ of target gene (gene of interest)} - Ct \text{ of reference gene (internal control)}, \text{while } \Delta\Delta Ct = \Delta Ct \text{ of sample} - \Delta Ct \text{ 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 \(\Delta\Delta Ct\) 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 \(-\Delta\Delta Ct\) (or \(-\Delta Ct\) 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 $\tau$, $\mu$, and $\delta$ 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: $-\Delta \Delta Ct = -(\Delta Ct_{\text{Test}} - \Delta Ct_{\text{Control}})$, where $\Delta Ct = (Ct_{\text{target}} - Ct_{\text{EF1A}})$. 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-C8] (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-C8 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).](image)

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, \( \tau_1-\tau_4 \), like the \( \mu \) chains of other vertebrates. However, the membrane form of IgM includes only three constant Ig domains, \( \mu_1-\mu_3 \). A typical feature of IgD of most teleosts is a chimeric C\( \mu_1 \)-C\( \delta \) structure, where \( \mu_1 \) is incorporated between the variable Ig domain and the \( \delta \) chain. Internal duplications of the C\( \delta \) 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 \( \delta_7 \) 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, \( \tau 1-\tau 4 \), like the \( \mu \) chains of other vertebrates. However, the membrane form of IgM includes only three constant Ig domains, \( \mu 1-\mu 3 \). Internal duplications of the C\( \delta \) exons generates a longer IgD molecule. \( \mu 1 \) is incorporated into the IgD heavy chain, between the variable Ig domain and the \( \delta \) 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 \( \mu \), \( \tau \), and \( \delta \).

4.4. Expression of Ig isotypes in different tissues

The relative abundance of \( \tau \), \( \mu \), and \( \delta \) 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 at least by three DNA sequence elements: the enhancer, the promoter, and undefined intragenic sequences (Grosschedl and Baltimore, 1985).

4.5. plgR-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 plgR-like molecules of Atlantic salmon (Salsal plgR and Salsal plgRL) 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 plgR 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 plgR (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 plgR correspond to [D1] and [D5] of the mammalian counterparts. Teleost plgR 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 plgR, in addition to being shorter, share very low amino acid sequence identity to plgR of other vertebrates. Moreover, Paper II identified structurally and phylogenetically similar protein products in Atlantic salmon. Teleost plgR 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 plgR and Salsal plgRL also results in CD300A hits on top. There is also some similarity in the connecting region (e.g. O-glycosylation). However, BLAST search clearly indicated that the best hits are plgRs. 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, plgR do not possess activating or inhibitory motifs which is a typical feature of CD300 molecules (Clark et al., 2009). However, teleost plgR V domains have characteristic disulphide bridge topology (Cx7C motif) that makes them resemble the mammalian plgR [D1], and V domains of salmon CLM (partly), human CD300A, FcαμR, and NKp44 (Fig. 4).

Fig. 4. Schematic representation comparing the main features of plgR, plgRL, and CLM V domains with other molecules. Mammalian plgR contains 5 extracellular domains, with [D1] and [D5] being the corresponding domains of teleost counterparts. Domains with Cx7C motifs are shown in light gray texture. Salmon plgR, plgRL, CLM (partly), human CD300, FcαμR, and NKp44 V domains have Cx7C 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.
Following the binding of IgA to plgR, most of the IgA involved in immunity, produced locally at submucosal sites, is transported into external secretions as a plgR-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


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


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


Appendix
Appendix 1. Interval plot of IgM, IgT, and IgD expression in different tissues. 95% confidence interval for the mean (based on \( -\Delta Ct \) values normalized against EF1A) is shown. Tissues with highest expression (spleen and head kidney) are boxed. Note also individual variation.
**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.
<table>
<thead>
<tr>
<th>ATG</th>
<th>GTT</th>
<th>CTCTTTATCTTCTCATCCTTGTTCTTTTGCTCATGGA</th>
<th>MVSLYLIILVFVGHA</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATC</td>
<td>AGT</td>
<td>GAGGCACACACTGATGGCAACAAAGGGTACTGCAAAAGAGTGCCGGAGCC</td>
<td>PSGCNLSWTVKSTAKSGGA</td>
<td>34</td>
</tr>
</tbody>
</table>

**Appendix 2B. Nucleotide and deduced amino acid sequences of Salsal plgRL (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.
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.
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.
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.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 10</th>
<th>Day 15</th>
</tr>
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<tbody>
<tr>
<td>trypsin [Oncorhynchus masou]</td>
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<td>1.12</td>
<td>0.64</td>
<td>-2.30</td>
<td>-2.08</td>
</tr>
<tr>
<td>alpha amylase [Salmo salar]</td>
<td>1.39</td>
<td>1.57</td>
<td>-0.01</td>
<td>-2.53</td>
<td>-2.93</td>
</tr>
<tr>
<td>anionic trypsin [Oncorhynchus keta]</td>
<td>1.01</td>
<td>1.21</td>
<td>0.61</td>
<td>-2.36</td>
<td>-1.95</td>
</tr>
<tr>
<td>carboxylic ester hydrolase</td>
<td>1.12</td>
<td>1.55</td>
<td>1.65</td>
<td>-2.07</td>
<td>-2.06</td>
</tr>
<tr>
<td>Carboxypeptidase A1 precursor [Salmo salar]</td>
<td>1.13</td>
<td>0.91</td>
<td>0.80</td>
<td>-4.81</td>
<td>-2.96</td>
</tr>
<tr>
<td>Carboxypeptidase A2 precursor [Salmo salar]</td>
<td>0.35</td>
<td>1.56</td>
<td>0.58</td>
<td>-2.84</td>
<td>-2.77</td>
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<tr>
<td>Carboxypeptidase B precursor [Salmo salar]</td>
<td>0.64</td>
<td>0.98</td>
<td>0.22</td>
<td>-2.42</td>
<td>-2.11</td>
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<tr>
<td>Chymotrypsin B [Salmo salar]</td>
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<td>1.65</td>
<td>0.80</td>
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<td>-2.79</td>
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<td>Chymotrypsin B [Salmo salar]</td>
<td>1.06</td>
<td>1.48</td>
<td>0.26</td>
<td>-2.16</td>
<td>-2.04</td>
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<td>Chymotrypsin-like protease CTRL-1 precursor [Salmo salar]</td>
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<td>1.56</td>
<td>0.97</td>
<td>-3.38</td>
<td>-2.66</td>
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<tr>
<td>Elastase-1</td>
<td>1.40</td>
<td>1.30</td>
<td>0.71</td>
<td>-4.32</td>
<td>-2.80</td>
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<tr>
<td>Endoplasmic reticulum resident protein ERp27 [Salmo salar]</td>
<td>1.05</td>
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<td>1.27</td>
<td>-3.09</td>
<td>-2.91</td>
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<td>Guanidinoacetate N-methyltransferase [Salmo salar]</td>
<td>1.20</td>
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<td>0.63</td>
<td>-3.04</td>
<td>-2.59</td>
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<tr>
<td>Guanidinoacetate N-methyltransferase [Salmo salar]</td>
<td>1.51</td>
<td>0.27</td>
<td>0.81</td>
<td>-3.10</td>
<td>-2.43</td>
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<tr>
<td>High choriolytic enzyme 1 precursor [Salmo salar]</td>
<td>1.25</td>
<td>0.73</td>
<td>0.87</td>
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<td>-2.42</td>
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<tr>
<td>Proproteinase E precursor [Salmo salar]</td>
<td>0.10</td>
<td>1.19</td>
<td>0.74</td>
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<td>-3.56</td>
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<td>Proproteinase E precursor [Salmo salar]</td>
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<td>Trypsin II</td>
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<tr>
<td>Trypsin II</td>
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<td>1.19</td>
<td>0.44</td>
<td>-2.22</td>
<td>-1.68</td>
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<tr>
<td>Trypsin III</td>
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<td>1.25</td>
<td>0.96</td>
<td>-3.15</td>
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<tr>
<td>Trypsin precursor [Salmo salar]</td>
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<td>0.99</td>
<td>0.81</td>
<td>-1.18</td>
<td>-1.77</td>
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<tr>
<td>Trypsin-1 precursor [Salmo salar]</td>
<td>1.14</td>
<td>1.36</td>
<td>0.89</td>
<td>-1.67</td>
<td>-1.49</td>
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<tr>
<td>Trypsin-1 precursor [Salmo salar]</td>
<td>0.33</td>
<td>1.44</td>
<td>1.04</td>
<td>-2.42</td>
<td>-1.92</td>
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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
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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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; Hiroto 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 tetravalent 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 (μ1–μ4). As in mammal IgM, four 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
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 \( \mu \)-3-TM1 and \( \mu \)-4-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 Ig gene encodes only two Ig domains, corresponding to zebras (I \( \xi \) and C \( \xi \)) (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 which most likely occurred in the ancestor of salmonids; 4-TM1 splice variants are present in some primitive bony fish (Wilson et al., 1995). 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 IgT, IgM, and IgD.

Table 1

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product A</th>
<th>Product B</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgT: ( -3 \rightarrow 4 )</td>
<td>GCCAAAGCGGAGACGGTGGAG</td>
<td>GGTGCTGACGAGGCTTGCTGGA</td>
<td>629 bp</td>
<td>826 bp</td>
</tr>
<tr>
<td>IgM: ( \mu )-4-( \mu )-TM1</td>
<td>GCCAGGGCTGGGCAAAAGCCTAA</td>
<td>GACAGATCGACTCAAGCAGA</td>
<td>1058 bp</td>
<td>951 bp</td>
</tr>
<tr>
<td>IgD: 8TM1–8TM2</td>
<td>GAAATGGACGAGGGCTTGCTGGA</td>
<td>CGAATGTCTGAAACGAGCAGC</td>
<td>564 bp</td>
<td>611 bp</td>
</tr>
</tbody>
</table>

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 \(-80^\circ\text{C}\) 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 \(-20^\circ\text{C}\), 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 \( ^{32}\text{P}-\text{CTP} \) 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

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward primer 5’</th>
<th>Reverse primer 5’</th>
<th>Exon</th>
<th>Probe 5’</th>
<th>Exon</th>
</tr>
</thead>
<tbody>
<tr>
<td>3’</td>
<td>TGCTTCTTCTGAGGACAGCTCA</td>
<td>GTTCTTCTTCTGAGGACAGCTCA</td>
<td>3’</td>
<td>TCGTCTTCTTCTGAGGACAGCTCA</td>
<td>3’</td>
</tr>
</tbody>
</table>
| 4’   | GAGGACGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGA

The exons from which the primer/probes were designed are shown in the last column. Note that primers and probes between t2 and t3, \( \mu \)2 and \( \mu \)3 amplify both secreted and membrane forms of IgT/M.
showed that the inserts of these clones were restricted by an EcoRI 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 t2 and t3 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 QPCR 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).

2.5. Statistical analysis

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

<table>
<thead>
<tr>
<th>Salmon IgT</th>
<th>Grouper IgZ</th>
<th>Mandarin fish IgT</th>
<th>Zebrasfish IgZ</th>
<th>Grass carp IgZ</th>
<th>Salmon IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>τ1</td>
<td>τ2</td>
<td>τ3</td>
<td>τ4</td>
<td>μ1</td>
<td>μ2</td>
</tr>
<tr>
<td>Salmon</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>τ1</td>
<td>45</td>
<td>20</td>
<td>12</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>τ2</td>
<td>17</td>
<td>41</td>
<td>7</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>τ3</td>
<td>10</td>
<td>17</td>
<td>47</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>τ4</td>
<td>21</td>
<td>24</td>
<td>11</td>
<td>43</td>
<td>21</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Tissue</th>
<th>N</th>
<th>slgM Mean ± SEM</th>
<th>mlgM Mean ± SEM</th>
<th>mlgD Mean ± SEM</th>
<th>splgD Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head kidney</td>
<td>12</td>
<td>874.36 ± 553</td>
<td>51.9 ± 26.20</td>
<td>17.80 ± 12.30</td>
<td>0.003</td>
</tr>
<tr>
<td>Spleen</td>
<td>12</td>
<td>774.80 ± 197</td>
<td>26.12 ± 7.12</td>
<td>10.42 ± 3.10</td>
<td>0.001</td>
</tr>
<tr>
<td>Hind gut</td>
<td>12</td>
<td>253.97 ± 95.3</td>
<td>1.592 ± 0.57</td>
<td>0.2658 ± 0.08</td>
<td>Undet.</td>
</tr>
<tr>
<td>Thymus</td>
<td>12</td>
<td>39.45 ± 16.4</td>
<td>1.517 ± 0.63</td>
<td>0.599 ± 0.36</td>
<td>Undet.</td>
</tr>
<tr>
<td>Gill</td>
<td>7</td>
<td>4.66 ± 2.6</td>
<td>0.816 ± 0.21</td>
<td>0.2550 ± 0.04</td>
<td>Undet.</td>
</tr>
<tr>
<td>Skin from side</td>
<td>7</td>
<td>1.99 ± 0.783</td>
<td>0.284 ± 0.23</td>
<td>0.0584 ± 0.03</td>
<td>Undet.</td>
</tr>
<tr>
<td>Skin from head</td>
<td>6</td>
<td>0.40 ± 0.362</td>
<td>0.0425 ± 0.01</td>
<td>0.0212 ± 0.01</td>
<td>Undet.</td>
</tr>
<tr>
<td>Brain</td>
<td>6</td>
<td>8.01 ± 4.34</td>
<td>0.901 ± 0.25</td>
<td>0.1493 ± 0.03</td>
<td>Undet.</td>
</tr>
<tr>
<td>Liver</td>
<td>7</td>
<td>1.31 ± 0.86</td>
<td>0.0659 ± 0.02</td>
<td>0.0261 ± 0.00</td>
<td>Undet.</td>
</tr>
</tbody>
</table>

* N = 11 for mlgD.
Fig. 2. Alignment of translated IgT and IgM sequences. The first alignment shows CH1–CH4. In the second part of the alignment, the C-terminal part of the secreted forms of the Igs, which is encoded by the CH4 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 /H9270 2. GenBank accession numbers for membrane bound sequences are, IgT/IgZ: salmon, ACX50290; trout, AY870265; zebrafish, AAT67444; IgM: human, X58529; mouse, J00443; shark, AA191062. 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 splgD 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 IgT 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., 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 \((\gamma, \mu, \delta)\) domains from several species showed that \(\gamma 1\) and \(\mu 1\) 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, \(\mu 1\) is incorporated into the IgG heavy chain in teleosts, between the variable Ig domain and the \(\delta\) chain. In cows and sheep, the first constant domain of IgG \((\delta 1)\) shares almost the same sequence as their IgM \(\mu 1\) domain (Zhao et al., 2002). In pigs, the \(\delta 1\) domain is highly similar to \(\mu 1\), and transcripts which includes \(\mu 1\) instead of \(\delta 1\) 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) \(C 1\) 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 \(\delta\) domains in salmon, arose by internal duplications from a smaller primordial molecule (Hordvik et al., 1999). Clustering of \(\delta 2\) and \(\delta 5\), \(\delta 3\) and \(\delta 6\), and \(\delta 4\) and \(\delta 7\) 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, AAB27339; eel, ACD76833; grass carp, AB76396; common carp, BAA34718; cod, CA44680; fugu, BAD6619; zebrafish, AAT67445; human, CAC20458; mouse, AAB59650; bovine, AAB54751; sheep, AA51379; shark, AAB94511; bowfin, AAC59687. IgD: salmon, AF141607; fugu, BAD34542; Japanese flounder, BAB412044; zebrafish, CA41477; clawed frog, AABC57541; human, AA52771.1; IgX: clawed frog, CA42027. IgA: human, AAY5733; pig, AAT5943; mouse, CA42461; bovine, AAT98391. IgG: human, AAT76445; mouse, AAT5943; bovine, AAT98391. IgE: human, AAT76445; mouse, AAT5943; bovine, AAT98391. IgY: axolotl, AAT76445; chicken, AAT76445. 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%).

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 sequences were included in the present study.

Acknowledgements

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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


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

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\textbf{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 (\textit{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 transcripts is significantly higher than Salsal PIgRL and CLM in the skin, while Salsal PIgR 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 \textit{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].

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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 (Brandtzæg, 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) (Brandtzæg, 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 (\textit{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 (\textit{Takifugu rubripes}), carp (\textit{Cyprinus carpio}) and orange-spotted grouper (\textit{Epinephelus coioides}) (Feng et al., 2009; Hamuro et al., 2007; Rombout et al., 2008).
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 plgR contains several immunoglobulin superfamilies (IgSF) variable (V) domains (V-LIKE-DOMAIN in IgSF other than immunoglobulin [IG] and T cell receptor [TR], respectively). In contrast to the mammalian and avian plgR containing five and four IgSF V domains respectively (Kaetzel, 2005; Norderhaug et al., 1999; Wieland et al., 2004), the extracellular portion of the teleost plgR contains only two V domains (Feng et al., 2009; Hamuro et al., 2007; Rombout et al., 2008; Zhang et al., 2010). Although plgR of non-mammalian vertebrates do not contain the conserved mammalian Ig binding loops (BC, CC, and FG), plgR associated with plgA 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 plgA (Kuhn and Kraehenbuhl, 1981). There is evidence that a two-domain containing teleost plgR may function similarly (Feng et al., 2009; Hamuro et al., 2007; Rombout et al., 2008; Zhang et al., 2010). A dedicated aspect of putative teleost plgRs is that in addition to being shorter, they share very low amino acid sequence identity to plgR 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 plgR-like sequences in GenBank, two clones for plgR 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 plgR (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 plgR, 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 plgR, 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 plgR has in mucosal defenses, this study was initiated to clone and characterize a possible plgR homolog in Atlantic salmon. Two plgR-like cDNAs and genes of Atlantic salmon (Salsal plgR and Salsal plgRL) were identified, revealing their genomic organization, expression, similarity to known plgR 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 of that to Salsal plgR and Salsal plgRL RT-qPCR was used to analyze the relative abundance of transcripts encoding for Salsal plgR, Salsal plgRL and CLM proteins in skin, spleen, head kidney, hindgut, gill, and liver of healthy fish. To further highlight the role of Salsal plgR, Salsal plgRL, 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 plgR 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 plgR and Salsal plgRL.

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 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 plgR and Salsal plgRL

Two putative full-length Atlantic salmon plgR 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 sequencer apparatus (Applied Biosystems). Sequences were assembled using NTI Vector 6.0 software and intron/exon regions deduced from alignments of genomic and cDNA sequences. cDNA and genomic sequences were submitted to GenBank (Salsal plgR: GQ892056, GQ892057; Salsal plgRL: 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://www.cbs.dtu.dk/services/SignalP/) and SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/) softwares. Potential N-glycosylation sites were predicted by the online tool: http://www.cbs.dtu.dk/services/NetOGlyc/ (Julienius 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
TaqMan® based assay (Applied Biosystems). The PCR reaction mix contained TaqMan® Universal Master mix (2×), 900 nM each of 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−ΔΔCT (healthy tissues) and −ΔΔCT 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 analyses of Salsal plgR and Salsal plgRL

Modeling and structure comparison of Salsal plgR and Salsal plgRL [D1] and [D2] was done using chain F of [D1] of human plgR (PDB 1xed) with structural resolution of 2.9 Å, angstrom (Hamuro et al., 2004). 1xed was found to be a good template (Identity ~25%, Similarity ~38% on average) for modeling using BLAST (www.ncbi.nlm.nih.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 plgR-like sequences was aligned with the human genome database.

3. Results

3.1. plgR cloning and sequence analysis

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

The Salsal plgR 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 plgRL 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 plgR 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 plgR sequence, Salsal plgR contains tandem repeats of 12 additional codons (SVSP2) in the same region. Our reported Salsal plgR gene, assembled from many overlapping fragments, is 5.1 kb, composed of eight exons and seven introns; while the Salsal plgRL gene is 2670 bp long, with 6 exons and 5 introns (Fig. 1). The number of introns and the exon/intron pattern of the Salsal plgR 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 plgRL 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 plgR and Salsal plgRL sequences have an identity index of only 30%. Salsal plgR is more similar to teleost plgR, exhibiting amino acid sequence identity indices of 43–52% to fugu, zebrafish and grouper (Supplementary Table 2). Salsal plgR has 29–38% amino acid identity to the published teleost plgR. Salsal plgRL is very similar to the reported putative plgR polypeptides (thus termed plgR-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. CxxC motif). It also forms phylogenetic clusters together with teleost plgR.

Like other teleost plgR sequences, Salsal plgR and Salsal plgRL show low amino acid identity with plgRs of higher vertebrates (Salsal plgR has 15–18% identity whereas Salsal plgRL has 17–21% identity). The same holds true for the transmembrane (TM) and cytoplasmic (CY) regions. However, the TM region of Salsal plgRL and grouper plgR exhibits ~31% identity to TM of human plgR (Supplementary Table 2). The two V domains of Salsal plgRL and Salsal plgR show the highest amino acid identity to [D1] of human, which also holds true for most of the other teleosts (Supplementary Table 2). While KsWC (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 plgR, DgWYWC motifs (positions 98–104) are highly conserved in [D1] and [D2] of teleost, and [D5] of higher vertebrates plgR (Fig. 2). In human plgR and FcμR, and partly in teleost plgR, there is a conserved region (G...CXV...KWy(positions 16–41)) predicted to be the binding site for IgA/IgM (Fig. 2). Within the teleost plgR, 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 plgR 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 plgR (Hamburger et al., 2004), 4 of them are conserved in
Fig. 1. Genomic organization of the Salsal plgR and Salsal plgRL 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 plgR, 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 plgRL, 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, cystolic region. Human (Homsap) and Grouper (Epicoi) plgR are displayed for comparison (not to scale).

teleost plgR (Fig. 2). In Salsal plgR and Salsal plgRL, 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 plgR and salmon CLM.

A prediction for functional sites on the protein sequences of Salsal plgR and Salsal plgRL 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 plgR 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 plgR domains and species are indicated by (*). The positions of fully conserved cysteine residues are in bold and shaded light grey. The cysteine residues within [D5] of mammalian plgR responsible for covalent IgA binding are shaded dark grey. All other important conserved amino acids are shown by white text in black background. DcGWWC motif (positions 98–104), highly conserved motifs [D1] and [D2] of teleost plgR, and [D5] of other vertebrates, are in bold and underlined. CDR-like loops (BC, CC’ and FG) are shown below the sequence. Accession numbers for plgR are: Gr. Epinephelus coioides (ACV91678.1); Fu. Tocalypse rubripes (AB176685); ZI. Danio rerio (NP_001361); Hu. Homo sapiens (NM_002264); rat, Rattus norvegicus (NP_056855); Bo. Bos taurus (NM_174143); Ch. Gallus gallus (AY233381); and Xe. Xenopus laevis (EF075876). Sequences of the Atlantic salmon CMBF35-like molecules (CLM), obtained from the nucleotide database, are CLM1 (NM_0011446657), CLM7 (NM_001146562), and CLM8 (NM_001134362). Ss-1, Salsal plgR; Ss-2, Salsal plgRL; [D1], Domain 1; [D2], Domain 2; [D5], Domain 5.
component during transcytosis. This region in Salsal plgR spans from position 230–270 and include mainly phosphorylation, and glycosaminoglycan attachment sites. In Salsal plgRL, 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 plgR and Salsal plgRL. This is in contrast to salmon CLM where this instability motif is predicted to be distributed all over the sequence. In human plgR 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 plgR and Salsal plgRL (Fig. 5), allowing an extended stalk structure like for example in salmon CD8 (Moore et al., 2005).
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 Cx3C 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 (Fcc/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).
3.4. Analysis for closely related genes

BLASTP search using Salsal plgR and Salsal plgRL as queries revealed similarity to CMRF35-like molecules (CLM7 and CLM1 respectively) next to a series of plgR (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 plgR (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 plgR, having a C\(\text{X}_3\)C motif in [D1], while [D2] of CLM1 and CLM7 have a C\(\text{X}_5\)C motif. CLM8 contains only one V domain (Fig. 4), and is similar to MDIR of Raja eglanteria and CLM8 of rats. In CLM [D1], the cysteine C104 (2nd-CYS) is replaced by glycine. The C\(\text{X}_5\)C motif makes these molecules more similar to plgR than NILT where \(x \approx 3\), in most cases (Ostergaard et al., 2010; Stet et al., 2005). Moreover, in database searches for CLM related sequences in teleosts, plgR are in the top 10 BLASTP hits (Supplementary Table 5). Salmon CLM1 and CLM7 show amino acid sequence identity of 24–27% to teleost plgR, and ~20% to NILT. Teleost plgR 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 plgR 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 plgR and Salsal plgRL, the connecting region is predicted to be heavily O-glycosylated, and CLM1 contains none (Fig. 5).

3.5. Differential expression of plgR and CLM

RT-qPCR analysis revealed that Salsal plgR and Salsal plgRL are expressed in skin, gills, hindgut, spleen, and head kidney (Fig. 7A). Salsal plgR transcripts, even though somewhat lower than that in lymphoid tissues, are up to 10 fold higher than Salsal plgRL and CLM in skin, while Salsal plgRL transcripts are abundant in gills (Fig. 7A). Both Salsal plgRL 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 plgR homolog in Atlantic salmon. Accordingly, we first cloned a salmon counterpart of other teleost plgRs (Salsal plgR). A BLAST search using this sequence as query resulted in ESTs in salmon encoding for another molecule similar to Salsal plgR, with 29–38% amino acid identity to teleost plgR, and ~20% identity to mammalian plgR. We then cloned the full length cDNA and gene of this molecule (Salsal plgRL). When both sequences were subjected to BLAST searches against the salmon database, the plgR 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 plgR (Green et al., 1998). Salsal plgR and Salsal plgRL 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 plgR and Salsal plgRL are expressed in mucosal and lymphoid organs of Atlantic salmon, and also positively respond to...
infection in skin by *L. salmonis*. It is worth noting that plgR expression in salmon and other teleosts is not exclusive to mucosal tissues, plgR 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 plgR 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 plgR (Rombout et al., 2008). In the skin and gut, as there are relatively few cells expressing plgR, plgR expression is dependent on the tissue section sampled. While most of the epithelial cells in fugu skin expressed plgR, 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 plgR 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 −ΔΔCt method with EF1A as internal reference gene, and in relation to uninfected control. Data are mean−ΔΔCt ± SE. Significant differences from control (n = 3, P < 0.05) are indicated with *.
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 pgR and Salsal pgRL are similar to teleost pgR studied so far by having two V domains, while mammalian and avian pgR 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 pgR and Salsal pgRL domains are structurally equivalent to [D1] of human pgR (Fig. 6). [D1] of human pgR 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 pgR non-covalently interacts with polymeric Ig through its exposed CDR-like loops (BC, CC' and FG). In teleosts, these loops are not conserved. However, 3D structural prediction of Salsal pgR and Salsal pgRL showed that these loops are exposed (Fig. 6), suggesting that they may have Ig binding roles.

The ability of pgR to bind polymeric Ig and mediate their transcytosis depends on the domain structure of pgR and the nature of Ig (Kaelzel et al., 1994; Norderhaug et al., 1999). Generally, ligand binding requires Ig polymerization. Immune complexes containing only monomeric Ig are not transported (Kaelzel et al., 1994). Whereas most mammalian Ig isotypes occur solely in a monomeric (IgC, 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 pgR (Braathen et al., 2007; Brandtzæg and Prydz, 1984). In teleosts, J chain containing antibodies have not been reported. In the human pgR 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 (Nativg 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 very low; estimated to be below 15 μg/ml in healthy fish (Hatten et al., 2001). The amounts of IgM in mammalian secretions exceeds the total daily production of IgG (Brandtzæg, 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 pgR on human IgM overlaps with that of FceRI/µR (Ghumra et al., 2009). FceRI/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 FceRI indicates that these molecules are distantly related to Salsal pgR or CLM. Despite having low amino acid sequence identity to the pgR, the FcRI has a conserved IgA/IgM binding motif (Shibuya et al., 2000), which is also partly preserved in salmon and other pgRs. Within the teleost pgR, 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 CD300L, which is shown to interact with IgA2 and IgM (Takatsu et al., 2006). A recent study reported that IgM can bind to FceRI 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 pgR

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**Table 2**

BLASTP hits of Salsal pgR and Salsal pgRL (excluding zebrafish sequences).^1^<br>

<table>
<thead>
<tr>
<th>Accession</th>
<th>Organism</th>
<th>Description</th>
<th>Query coverage</th>
<th>E-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADB84838.1</td>
<td>Salmo salar</td>
<td>pgR</td>
<td>100%</td>
<td>0.0</td>
</tr>
<tr>
<td>ADB81776.1</td>
<td>Oncorhynchus mykiss</td>
<td>pgR</td>
<td>100%</td>
<td>0.0</td>
</tr>
<tr>
<td>ACN41878.1</td>
<td>Epinephelus coioides</td>
<td>pgR</td>
<td>100%</td>
<td>1E−117</td>
</tr>
<tr>
<td>ADB93435.1</td>
<td>Paralichthys olivaceus</td>
<td>pgR</td>
<td>98%</td>
<td>3E−109</td>
</tr>
<tr>
<td>AB97624.1</td>
<td>Cyprinus carpio</td>
<td>pgR</td>
<td>89%</td>
<td>6E−99</td>
</tr>
<tr>
<td>AB95657.1</td>
<td>Tukifugu rubripes</td>
<td>pgR</td>
<td>92%</td>
<td>1E−91</td>
</tr>
<tr>
<td>ABQ10652.1</td>
<td>Danio rerio</td>
<td>pgR</td>
<td>80%</td>
<td>1E−88</td>
</tr>
<tr>
<td>ACN37731.1</td>
<td>Tetraodon nigroviridis</td>
<td>CLM1 precursor</td>
<td>61%</td>
<td>2E−53</td>
</tr>
<tr>
<td>ADN18014.1</td>
<td>Salmo salar</td>
<td>pIgRL</td>
<td>68%</td>
<td>8E−52</td>
</tr>
<tr>
<td>NP_00140034.1</td>
<td>Salmo salar</td>
<td>CLM1</td>
<td>57%</td>
<td>5E−27</td>
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<tr>
<td>NP_00143420.1</td>
<td>Salmo salar</td>
<td>CLM1</td>
<td>57%</td>
<td>7E−27</td>
</tr>
</tbody>
</table>

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^1^ 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.

^2^ As Danio rerio sequences comprise ~50% of the hits (mainly predicted pgR, and a number of unnamed proteins), they have been excluded from this list for simplicity.
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 Fcε/μR and interleukin genes on chromosome 1 (Kaatzel, 2005; Shibuya et al., 2000). However, mining into the genome database using Salsal plgR and Salsal pIgR 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 transport, by attaching to host and pathogenic factors (Phalipon and Cortesey, 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 (MDI3R) 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 CεC 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, FcR in catfish, and the human and mouse Fcε/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 pIgR vs human Fcε/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.

### Acknowledgements

Financial support was obtained from The Norwegian Research Council. We thank Lars Hamre for help with *L. salmonis* challenge test.

### Appendix A. Supplementary data


### References


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

Tariku Markos Tadiso1*, Aleksei Krasnov2, Stanko Skugor2, Sergey Afanasyev2,3, Ivar Hordvik1, Frank Nilsen1

**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 eggstrings 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...
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 kisutch*) 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 copepods 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
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, carboxypeptidases 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.

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**Figure 1** An overview of transcriptomic responses to lice

A: The number of differentially expressed features in skin and spleen (mean log2 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).
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, metalloeductase 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
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

<table>
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<th>Functional group, pathway</th>
<th>Features</th>
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<th>Vocabulary</th>
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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.
### Figures

#### Figure 3

**Examples of immune genes with differential expression in skin (microarray results)**. Data are mean log_{2}ER (n = 4).

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<th>D1</th>
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<th>D5</th>
<th>D10</th>
<th>D15</th>
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<td>Rhamnose-binding lectin WCL1</td>
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<td>1.03</td>
<td>1.18</td>
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</tr>
<tr>
<td>C-C chemokine receptor type 3</td>
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<td>0.49</td>
<td>0.96</td>
<td>0.22</td>
<td>0.13</td>
</tr>
<tr>
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<td>1.74</td>
<td>2.38</td>
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</tr>
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<td>SAPS domain family member 3</td>
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<td>3.37</td>
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</tr>
<tr>
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<td>Transcription factor AP-1</td>
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<td>-1.58</td>
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<tr>
<td>Nuclear factor NF-kappa-B p100 subunit</td>
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<tr>
<td>Transcription factor jun-B</td>
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<td>-1.53</td>
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<td>Ilalectin-9</td>
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<td>1.26</td>
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<td>0.26</td>
</tr>
</tbody>
</table>

#### Figure 4

**Examples of immune genes with differential expression in spleen (microarray results)**. Data are mean log_{2}ER (n = 4).

<table>
<thead>
<tr>
<th>Gene</th>
<th>D1</th>
<th>D3</th>
<th>D5</th>
<th>D10</th>
<th>D15</th>
</tr>
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<tbody>
<tr>
<td>Acute phase serum amyloid A (SAA)</td>
<td>3.57</td>
<td>-0.35</td>
<td>0.53</td>
<td>-0.69</td>
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<tr>
<td>Cathelicidin antimicrobial peptide</td>
<td>1.88</td>
<td>0.03</td>
<td>0.70</td>
<td>0.54</td>
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</tr>
<tr>
<td>Differentially regulated trout protein 1</td>
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<td>0.89</td>
<td>0.40</td>
<td>-2.17</td>
<td>-0.70</td>
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<tr>
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<td>0.00</td>
<td>0.03</td>
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<tr>
<td>Lyszyme C II precursor</td>
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<td>0.20</td>
<td>-0.78</td>
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<tr>
<td>Metalloedacase STEAP4-1</td>
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<td>-0.11</td>
<td>0.20</td>
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<td>Metalloedacase STEAP4-2</td>
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<td>1.41</td>
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<tr>
<td>Serum amyloid A</td>
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<td>0.91</td>
<td>0.49</td>
<td>-1.70</td>
<td>0.56</td>
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<tr>
<td>C-C motif chemokine 28-1</td>
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<td>0.11</td>
<td>0.80</td>
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<td>-0.96</td>
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<tr>
<td>C-C motif chemokine 28-2</td>
<td>0.14</td>
<td>0.07</td>
<td>0.77</td>
<td>1.84</td>
<td>-0.21</td>
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### Table

<table>
<thead>
<tr>
<th>Gene</th>
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<th>D3</th>
<th>D5</th>
<th>D10</th>
<th>D15</th>
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<td>-1.78</td>
<td>-0.68</td>
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<td>Gamma interferon-inducible thiol reductase</td>
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<td>-1.28</td>
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<td>0.30</td>
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<tr>
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<td>-1.29</td>
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<td>Interferon-inducible protein Gig2-like</td>
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<td>TNF decoy receptor</td>
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<tr>
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<td>Lysozyme C II</td>
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<tr>
<td>C-reactive protein</td>
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<td>0.09</td>
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<td>Myeloperoxidase precursor</td>
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<tr>
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<tr>
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<td>-0.25</td>
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<td>Matrix metalloproteinase</td>
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<td>1.62</td>
<td>0.70</td>
<td>1.59</td>
<td>1.31</td>
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<tr>
<td>Matrix metalloproteinase</td>
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<td>1.63</td>
<td>0.00</td>
<td>1.71</td>
<td>1.44</td>
</tr>
<tr>
<td>Colagenase 3 precursor</td>
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<td>1.64</td>
<td>0.21</td>
<td>1.70</td>
<td>1.72</td>
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</table>

The data illustrate the differential expression of immune genes in spleen and skin samples, highlighting the role of specific genes in immune response and inflammation processes. The findings provide insights into the complex interplay of immune molecules, such as cytokines and chemokines, which are key players in the defense against pathogens and the regulation of the immune response.
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

![Figure 5 Temporal expression changes](image-url)
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 *.

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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 summarized 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 copepodid 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 leukocytes 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 plgR, 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, plgR 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™ TRIsol® Plus kit (Invitrogen), and purified with RNeasy mini kit (QIA-GEN). 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 olignonucleotide 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 log₂-expression ratios (ER) was performed. The differentially expressed genes (DEG) were selected by criteria: log₂ER > [0.8] and p < 0.01, (sample t-test, null hypothesis log₂ER = 0) in at least one time-point. The microarray data were submitted to GEO, GSE26981 (skin) and GSE26984 (spleen).
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).
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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: -ΔΔCt = - (ΔCt\text{Test} - ΔCt\text{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.
Acknowledgements
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Competing interests
The authors declare that they have no competing interests.

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