Molecular characterisaton of T cell co-receptors

CD3, CD8 and CD4 in Atlantic salmon *(Salmo salar)*

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To everyones amazement

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Abstract

The commercial importance of Atlantic salmon (*Salmo salar*) in aquaculture has fuelled much research into fish health since outbreaks of infectious disease cause major financial losses. All aspects of immunology are therefore currently of great interest. The aim of this study was to extend the characterisation of T cell markers to enable antibody production and design of expression assays, to study the immune system and immune responses of salmon. Therefore, the genes and cDNAs for the T cell co-receptors CD3, CD8 and CD4 were cloned using a combination of synteny analysis and homology cloning.

CD3 in Atlantic salmon consists of three different molecules; a γδ chain, the forerunner of separate γ and δ chains in mammals, an ε chain and a ζ chain (mammals also have an η chain, which is a splice variant of the ζ gene). The translated sequences have low identities to mammalian CD3 sequences (12-34%), but they exhibit similar characteristics with single immunoglobulin-like domains in the γδ and ε genes and immunoreceptor tyrosine-based activation motifs in all cytoplasmic domains. Two copies of the CD3ζ gene were cloned, but these are considered to be alleles. The CD3ε gene has a second copy, but it is a pseudogene containing frame-shifts and stop codons and was poorly expressed compared to the intact CD3ε gene. The CD3γδ gene also appeared to be duplicated and the variants are named CD3γδ-A and CD3γδ -B. Further evidence for the homology of CD3γδ and CD3ε genes was found in their genomic orientation where the pseudo-CD3ε gene is located tail to tail with a CD3γδ gene, similar to the gene organisation in higher vertebrates.

There are two CD8 genes in Atlantic salmon, CD8α and CD8β. The synteny of these genes in humans is conserved with the publically available fugu genome sequence, allowing the subsequent identification of the CD8β gene in Atlantic salmon. There are two main transcripts and two alternative transcripts which could result in a putative truncated cytoplasmic domain in CD8α and a severely truncated version of CD8β. The main CD8α and CD8β transcripts were also cloned and sequenced in brown trout (and

CD8β in rainbow trout, since only CD8α had been cloned previously). Despite low sequence identity (16-17%) to mammals, these molecules are considered homologs encoding a single immunoglobulin-like extracellular domain anchored by a stalk with many putative glycosylation motifs. The single pass transmembrane domains are followed by short cytoplasmic domains. Unlike mammalian CD8α, the salmon molecule does not contain a CXC, Lck binding, motif in its cytoplasmic domain. This is also true for other published teleost sequences. However, both CD8α and CD8β have motifs in their cytoplasmic domains that are highly conserved between teleosts, implying functional significance.

The cloning of CD4 was more complex since two similar molecules were found, type 1 and type 2, containing four and two extracellular immunoglobulin-like domains respectively. These genes were first identified next to each other on a fugu scaffold sequence, showing synteny to CD4 on the human genome. CD4-2 is duplicated with CD4-2a and CD4-2b sub-types, which together with the duplicated CD3 genes illustrates a theme throughout this study: the tetraploid nature of the salmon genome. All the CD4 molecules identified have a Lck binding motif in their cytoplasmic domain and although sequence identities to mammals were low (13-15%) they are homologous to mammalian CD4. At the genomic level all these CD4 genes have the code for the first immunoglobulin-like domain split between two exons, which is a hallmark of CD4 molecules from higher vertebrates. Although two slightly different genomic fragments were isolated for salmon CD4-1, only one cDNA was cloned. In rainbow trout two genes were identified for the CD4-1 gene on a Southern blot, but a second distinct cDNA sequence was not detected.

The expression of all these T cell markers is highest in the thymus, when measured using TaqMan assays and RT-qPCR, with the exception of CD4-2a. There was also significant expression in other immunological tissues such as head-kidney, spleen.

Characterization of these molecules has led to the production of a sensitive and specific antibody against a CD3ε cytoplasmic peptide, which has been used to show the distribution of T cells in Atlantic salmon tissues using immunohistochemistry. Large

numbers of CD3ε expressing cells could be seen in thymus and a lymphoid tissue in gills. A significant amount of CD3ε expression was also seen in head kidney and spleen. CD3ε positive cells were also observed scattered between enterocytes in the hind gut. RT-qPCR of 11 different T cell genes performed on laser capture microdissected material from thymus, gill and hind-gut reflected the CD3ε expression seen using immunohistochemistry.

Until recently it was not possible to isolate T cells other than indirectly as immunoglobulin negative cells, due to a lack of reagents. This study paves the way for the development of more antibodies and for the positive isolation of sub-populations of leucocytes, allowing further elucidation of immune mechanisms and cellular studies in Atlantic salmon.

List of publications

Paper I

Y. Liu, L. J. Moore, E. O. Koppang and I. Hordvik. Characterization of the CD3ζ, CD3γδ and CD3ε subunits of the T cell receptor complex in Atlantic salmon. Dev Comp Immunol 2008;32 (1):26-35.

Paper II

L. J. Moore, T. Somamoto, K. K. Lie, J. M. Dijkstra and I. Hordvik. Characterisation of salmon and trout CD8α and CD8β. Mol Immunol 2005;42(10):1225-34.

Paper III

L. J. Moore, J. M. Dijkstra, E.O. Koppang and I. Hordvik. CD4 Homologues in Atlantic Salmon. Fish and Shellfish Immunology 2009; 26: 10-18

Paper IV

L .J. Moore, E. O. Koppang, U. Fischer, M. Tranulis and I. Hordvik. Distribution of T cells in Atlantic salmon tissue using an antibody to CD3ε. (manuscript)

Abbreviations

AMP	antimicrobial peptide
BCR	B cell receptor
CD	cluster of differentiation
CpG	unmethylated cytosine guanine dinucleotides
CYT	cytoplasmic
cDNA	complementary deoxyribonucleic acid
EST	expressed sequence tag
EX	extracellular
FACS	fluorescent activated cell sorting
FREPs	fibrinogen related proteins
IEL	intraepithelial lymphocyte
Ig	immunoglobulin
IgSF	immunoglobulin super family
GALT	gut associated lymphoid tissue
ITAM	immunoreceptor tyrosine-based activation motif
Lck/*lck*	p56 lck, T cell specific tyrosine kinase/Lck gene
LCM	laser capture microdissection
LLR	leucine rich repeat
MALT	mucosal associated lymphoid tissue
MHC	major histocompatibility complex
Mya	million years ago
PAMP	pathogen associated molecular pattern
PCR	polymerase chain reaction

PRR	pattern recognition receptor
RACE	rapid amplification of cDNA ends
RT-qPCR	reversed transcription quantitative PCR
rag	recombination activation gene
(m)RNA	(messenger) ribonucleic acid
TCR	T cell receptor
TLR	toll-like receptor
TM	transmembrane
VLR	variable lymphocyte receptor
WGD	whole genome duplication

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Introduction

The farming of Atlantic salmon (*Salmo salar*) in Norway is a major industry comprising 40% of total seafood exported and earning over 17 billion Norwegian kroner in 2007 (Facts about Fisheries and Aquaculture 2007). However, the intensive nature of commercial production means that infection with a wide variety of pathogens (viral, bacterial, fungal and parasitic) can result in severe financial losses during outbreaks of infectious disease. The study of fish immunology is essential to the continued development of the fish farming industry, both as a basic science and as an application for vaccine production. The increasing intensification of this industry and its globalization will only serve to increase the need to study all means of maintaining and improving fish health. Research into the cause and control of infectious disease has thus been the focus of several research programmes in recent years.

The most cost effective way to control disease is prevention and this is achieved by for example testing brood stock and by vaccination. The introduction of successful vaccines requires knowledge of the immune response which is relatively poorly characterised in Atlantic salmon. Additionally, regarding comparative immunology, salmon together with other teleosts represent a fascinating evolutionary time-point when the classical adaptive immune system first evolved.

The immune response includes not only the better known B cell antibody response, but also the T cell cytotoxic responses, which is often more important during virus infections. The key to understanding the immune response is to be able to measure it, during for example infection studies and vaccine trials. In order to make these measurements information about the major players in the immune response is required. A study of the major T cell markers not only provides information about the immunology of Atlantic salmon, but can in addition be used to produce antibodies for measuring the immune response and for designing gene expression assays.

1. Background

1.1 Atlantic Salmon (*Salmo salar*)

Atlantic salmon is in the Salmo genus with brown trout (Salmo trutta) and belongs to the family Salmonidae, which also includes rainbow trout (*Oncorhynchus mykiss*) and several species of Pacific salmon. The species name salar is from Latin meaning the leaper. Wild salmon are well known for their amazing agility, leaping up waterfalls to return to their native spawning ground to breed.

Within the teleosts, the evolutionary branch leading to Atlantic salmon evolved relatively early after the lobe and ray finned fish branches split (Fig. 1).

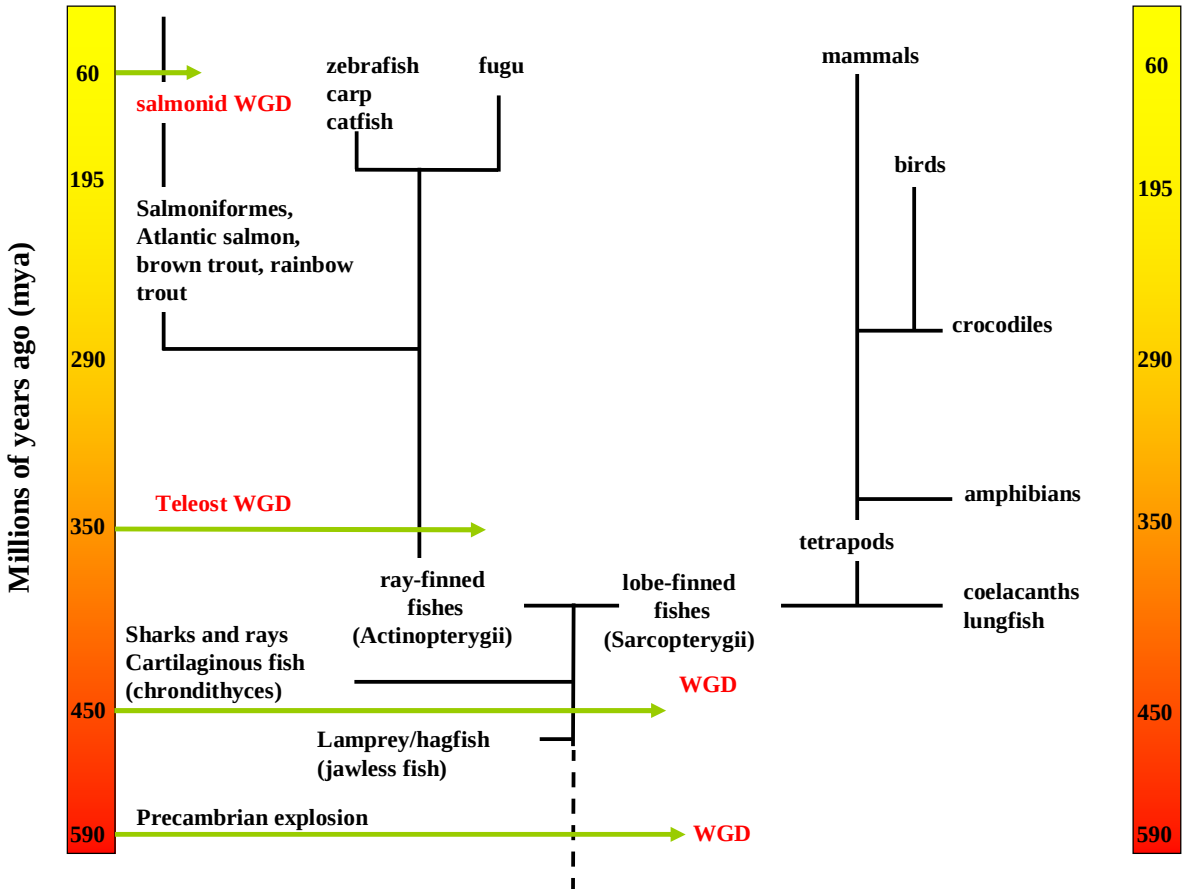

Figure 1. Simplified evolutionary tree showing the whole genome duplications (WGD) calculated to have taken place in the evolution of salmonids.

The lobe-finned fishes evolved into tetrapods and land animals; amphibians, birds and mammals including humans. The ray-finned fishes eventually evolved into the teleosts or modern bony fish. There are now over 20,000 teleost species making up over half of all known living vertebrates. All vertebrates appear to have undergone whole genome duplications (WGD) twice in their ancestry. Teleosts have probably experienced an additional, teleost specific, WGD since they split from the tetrapod line of evolution (Dehal and Boore, 2005; Meyer and Schartl, 1999). Salmonids as a family have had a fourth genome duplication event approximately 60 mya (million years ago). Since this time they have been returning to a diploid state (Allendorf FW, 1984; Hordvik, 1998; Shiina et al., 2005). The remaining tetraploidy renders this species a challenge with regard to cloning and identifying new genes.

1.1.1 Life cycle

Life for Atlantic salmon begins in autumn, in streams where the females lay their eggs in gravel nests in cool stream beds in the northern hemisphere. After fertilization the eggs remain buried in gravel until the following spring. The newly hatched alevins live off the remaining yolk sac for about a month before developing into fry which feed mostly on small invertebrates.

At this stage the young salmon may develop into parr and migrate to the open sea after smolting or they may live in fresh water stream for a few years. Some landlocked salmon live their entire lives in fresh water lakes, mostly found in North America. However, most salmon are anadromous, living in fresh and saltwater at different stages of their life-cycle. The length of time spent in fresh water is largely governed by water temperature. One year old salmon may migrate to sea from southern England or northern Portugal, but those living in more northerly areas may stay up to four or five years before smolting and migrating. Once in the open sea they develop rapidly, but will unerringly return to their native streams to spawn. Atlantic salmon are iteroparous

meaning they can recover after spawning to migrate and spawn again several times, unlike their Pacific cousins Coho and sockeye salmon which usually die after spawning.

1.1.2 Aquaculture and fish health

In aquaculture life for Atlantic salmon is somewhat different. Broodstock fish are stripped and their eggs are fertilized. The fry are reared in tanks on land for 12-20 months until they have reached the smolt stage, when they are transferred to open sea cages. Here they grow and mature at sea for up to two years. Compared to their wild cousins salmon reared in aquaculture facilities enjoy good survival rates through the smolt stage and up to maturity. Smoltification involves changes in metabolism in preparation for life at sea and the young salmon, both wild and farmed, are particularly vulnerable to diseases at this stage.

Farming of Atlantic salmon began in Norway in the 1970s due to an increasing market demand for this succulent, exotically coloured flesh, which is the result of the crustaceans wild salmon feed on in the open sea. More recently Atlantic salmon fillet has scored 89 out of 100 on an Overall Nutritional Quality Index list compiled by Yale University. There are only a few countries that are suitable for rearing Atlantic salmon, including Norway, Scotland, Ireland, Canada and Chile. This is largely due to the water temperature and to some extent reflects the range of wild Atlantic salmon.

Norway is the world leader in Atlantic salmon production and is responsible for creating a huge and growing world market for this fish. In 2007 over 700,000 tonnes of farmed salmon was produced in Norway and since over 80% is exported, financial losses incurred due to disease have severe financial implications for the Norwegian aquaculture industry (Chinabut and Puttinaowarat, 2005). The intensive nature of aquaculture means that infectious disease can spread rapidly within fish farms. In addition, the open nets and water currents may spread disease horizontally to neighbouring farms and wild fish in the area. More controversial is the possibility of vertical spreading of disease from brood-stock and eggs, which can be transported

many hundreds of kilometres and infect previously disease free areas (Vike, 2009). Much has already been achieved in the control of infectious disease in the industry. For example, the use of antibiotics has crashed since the introduction of effective oil-adjuvant vaccines against several bacterial diseases (Sommerset et al., 2005). However, viral diseases such as infectious salmon anaemia and pancreas disease still cause huge losses and parasites such as salmon lice continue to be a problem. A thorough understanding of the immune response is necessary to develop effective vaccines to tackle these diseases. In addition, the means to measure the immune response during vaccine trials is needed.

The farming of Atlantic salmon in Norway is responsible for significant export earnings with capacity for expansion if it can remain competitive. Thus, research into all aspects of salmon farming has been prioritised including basic immunology under HAVBRUK programmes such as “Production of aquatic organisms (2000-2005)” and “An industry in growth (2005-2008)” in conjunction with “Functional genomics (FUGE)” and the “Food programme: Norwegian food from fjord to fork”. Finally, on a humanitarian note an increase in the global production of fish through aquaculture can make a serious contribution to the need for future protein for a growing global population.

1.2 The comparative mammalian immune system

It is impossible to describe the immune system of teleosts without reference to that of humans and mice where the field of immunology is much better studied and characterised. The immune system of these higher vertebrates consists of primary and secondary lymphoid organs with distinct compartments and morphology that are served by two vascular systems containing blood and lymph. The thymus and bone marrow constitute the primary lymphoid organs while the spleen, lymph nodes, and mucosal associated lymphoid tissue (MALT) comprise the secondary lymphoid organs. Primary organs are responsible for stocking the immune system with all types of capable

lymphoid cells. The secondary tissues are responsible for the cell-mediated and antibody responses as well as the rapid, innate immune response.

Bone marrow is a proliferative haematopoietic tissue responsible for the production of all types of blood cells from pluripotent stem cells. Common myeloid progenitor cells give rise to granulocytes, basophils and eosinophils, as well as erythocytes and the platelet precursors, megakaryocytes. A common lymphoid progenitor produces precursor cells for natural killer cells (NK cells) and lymphocyte precursors. The different types of dendritic cells are derived from both of these progenitor lines. There are two main types of lymphocytes; T and B cells. The “T” reflects the thymic origin of these cells as opposed to B cells which originate from the bone marrow in mammals. The “B” is not actually derived from bone marrow, but from a gut associated organ, the bursa of Fabricius in birds where the origin of these cells was first described (Ribatti et al., 2006). The thymus is populated by bone marrow derived pre-T cells and it is here they undergo positive and negative selection for self and non-self recognition so they are ready to productively interact with pathogens as circulating naïve T cells. Similarly, naïve B cells are produced in the bone marrow and populate the lymph nodes, spleen, MALT and are found in the circulation. Spleen and lymph nodes together with tonsils and MALT constitute secondary lymphoid tissue responsible for trapping antigen and instigating immune responses.

1.3 The immune system of teleosts

1.3.1 Thymus

The thymus in higher vertebrates has a central role in the immune system, producing self-tolerant T cells that will only recognise foreign antigens for cell mediated immune responses. In teleosts, it is usually a bi-lobed organ situated above the gill arches (Fig. 2). In many species it has distinct zones; an outer cortex and an inner medulla. The cortex contains a higher percentage of T cells than the medulla which also contains a greater amount of epithelial tissue. These zones are not equally well demarcated in all

teleosts and it can be difficult to dissect and visualize this organ microscopically. The thymus involutes with age showing decreasing apoptotic activity but changes in size can occur seasonally as well as in response to hormones (Abelli, 1998; Nakanishi, 1986). As a result, there is extensive variation not only between different teleosts, but also within a single species (Bowden et al., 2005). However in nearly all teleosts the thymus epithelium is continuous with that of the gill chamber which may be significant regarding antigen availability during T cell development. The thymus is the first organ ontogenetically to become actively lymphoid although the timing is species dependent (Laird, 1978; Manning, 1994). The zebrafish thymus is active about three days post fertilization when *lck* and *rag* are presnet (Langenau et al., 2004). The thymus is also the first lymphoid organ to appear phylogenetically, occurring in cartilaginous fish (sharks and skates) after the proposed second round of WGD approximately 450 mya (Fig.1).

T cells visualized by the presence of CD8α and TCRβ mRNAs using *in situ* hybridization began to show a medulla/cortex boundary from seven weeks post hatching in sea bass *(Dicentrarchus labrax).* In one year old sea bass the cortex was shown to be almost 100% T cells with cords of T cells extending into the medulla (Picchietti et al., 2008). In Atlantic salmon the thymus can be diffuse. The apparent diversity of thymic construction in teleosts does not however indicate a lack of functional capability (Zapata, 1996).

1.3.2 Head kidney

Teleosts have no bone marrow but use part of their kidney for haematopoietic functions. B cells derive from the head kidney or pronephros in teleosts which is the anterior part of a long organ responsible for many functions, including that of a mammalian kidney (Fig. 2). The mid/hind or trunk kidney consists mostly of glomerular tissue associated with excretion (Fig. 2). Conversely, the mammalian kidney retains haematopoetic responsibilities with the production of the cytokine erythropoietin, responsible for regulating red blood cell production from mammalian bone marrow. In teleosts the head kidney functions as both a primary and secondary

lymphoid organ. It shares some morphology with bone marrow of higher vertebrates and is the site of haematopoiesis in teleosts. B cell precursors and plasma cells can be found in the anterior kidney (Zwollo et al., 2005).

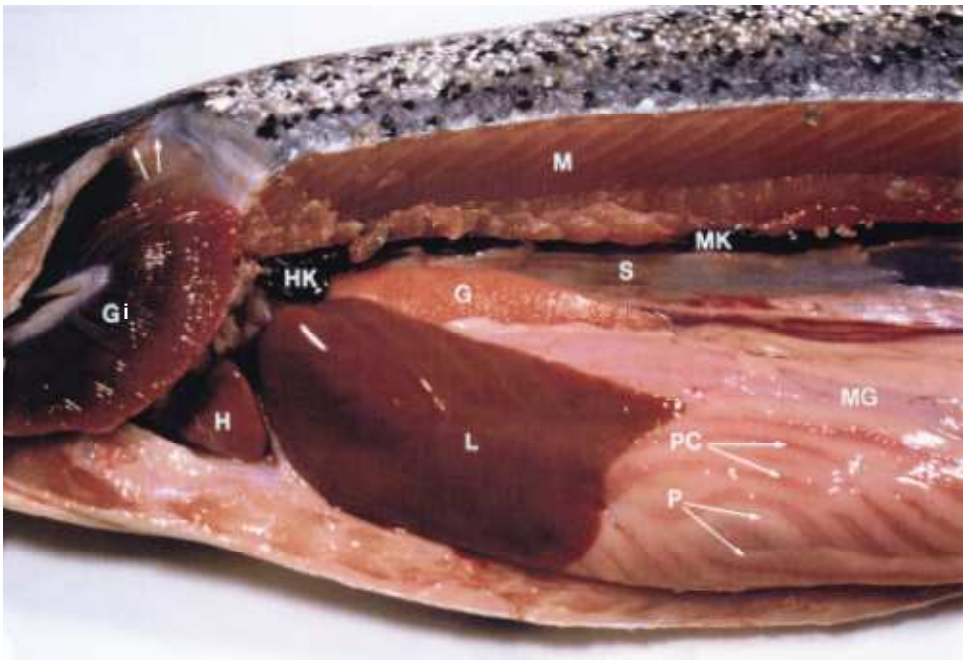

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Figure 2. Organs of Atlantic salmon: white arrows above the gills = thymus, Gi = gills, H = heart, HK = head kidney, M = muscle, MK = mid-kidney, S = swimbladder, G = gonads, L = liver, P and arrows = pylorus and PC and arrows = pancreas

As a secondary lymphoid organ the head kidney contains high numbers of melanomacrophages, which may have functions similar to lymph nodes in mammals (Agius and Roberts, 2003). The melanomacrophages are closely associated with lymphocytes and macrophages at antigen trapping sites. In Atlantic salmon melanomacrophage centres are more diffuse, but the morphology of these cells changes during infection. Together with the spleen the head kidney is a vascular organ capable of surveillance and trapping antigens arriving in the blood. Partially activated B cells can also be found in the posterior part of the kidney (Zwollo et al., 2005).

1.3.3 Spleen

The spleen is a secondary lymphoid organ having similar functions to the head kidney with regard to antigen trapping and sequestration of blood-borne substances (Fig.3). All vertebrate secondary lymphoid organs are designed to trap/filter substances in a way that allows lymphoid cells to react with them. Red and white pulp areas can be seen in teleosts but the distinction between these areas is not always clear. Melanomacrophage centres are present for clearance of ingested material and can be surrounded by immunoglobulin positive cells especially after immunisation. Proliferation of granular cells has also been observed after immunisation in association with ellipsoids and melanomacrophage centres (Van Muiswinkel et al., 1991). Interestingly, clustering of rag expression has been identified in the spleen of rainbow trout using in situ hybridization, suggestive of germinal centre formation but this has not been confirmed (Hansen and Zapata, 1998).

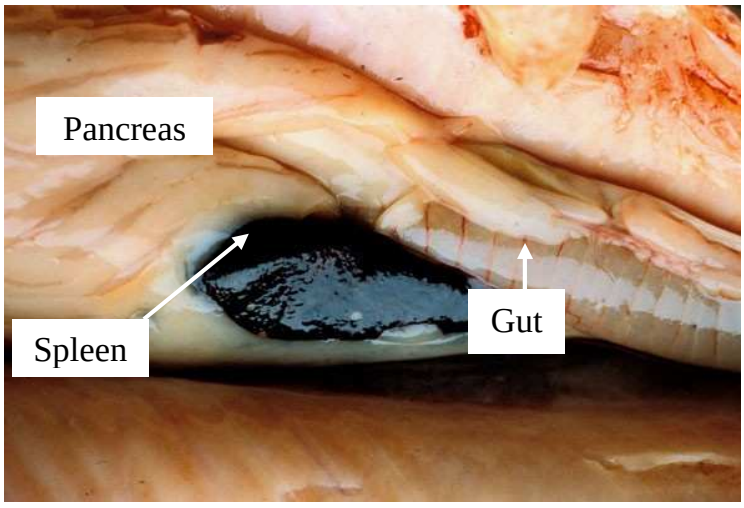

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Figure 3. Position of spleen in Atlantic salmon

1.3.4 Mucosal associated lymphoid tissue (MALT)

The gills, skin and gut form the first line of defence against invading pathogens with their layer of mucus containing antimicrobial peptides (AMPs) and enzymes for use during the immediate innate immune response. Once this surface is breached the adaptive immune response is triggered. The innate immune response continues to be of paramount importance until specific adaptive responses are achieved, such as sufficient specific antibodies and/or effective cytotoxic T cells.

Gills

The respiratory function of the gills ensures that they constantly sample the environment and thus are excellently placed to encounter invading pathogens (Fig. 2). MHC class II expressing cells have been identified in gills and their numbers increase during amoebic gill disease indicating that the gills are active immunologically (Koppang et al., 2003; Morrison et al., 2006). Recently, a lymphoid tissue at the base of the gill arch has been reported that exhibits localised CD3, TCR and MHC class II expression (Haugarvoll, 2008).

Hind gut

Peyer's patches and mesenteric lymph nodes, present in mammals as centres of organised lymphoid tissue, have not been identified in teleosts. However, the gut associated lymphoid tissue (GALT) together with the spleen is the most important secondary lymphoid tissue in teleosts. Loosely associated lymphoid aggregates appear in all parts of the gut but the hind gut appears to specialise in the trapping and processing of antigens (Press and Evensen, 1999). Enterocytes lining the gut appear to perform similar functions as mammalian M cells (Rombout et al., 1993).

The intraepithelial leucocytes (IELs) isolated from teleost gut mucosa seem to be mostly T cells expressing TCRβ, TCRγ, CD3, CD4 and CD8, but not IgM (Bernard et al., 2006b; Picchietti et al., 1997; Rombout et al., 1993). The gut lumen and lamina

propria, underlying the epithelial layer, contain the greatest numbers of lymphoid cells, including macrophages, mast cells, plasma cells and granulocytes as well as T cells.

Skin

The protective mucous layer bathing the skin of teleosts contains many compounds capable of taking part in the innate immune response, such as; lectins, lysozyme, complement proteins, and AMPs. Differences in the composition of this mucus may account for different susceptibilities to disease not only between species, but even within a given species, particularly during for example smoltification (Palaksha et al., 2008). The intensive nature of aquaculture could easily compromise this important physical barrier by causing abrasion of mucus from skin surfaces. This indicates the need for preventative measures such as control of fish densities and vaccination in farmed populations (Suomalainen et al., 2005; Svendsen, 1997).

1.3.5 Lymph

The teleost lymphatic system has been described as containing no erythrocytes and in some species a parallel blood circulation has been reported. In zebrafish a lymphatic system has been described both on a morphological and molecular basis with an ontogeny similar to mammals. Morphologically the vessels identified are blind ended, they drain the interstitial compartment and contain no red blood cells. On a molecular level the thoracic duct equivalent identified in zebrafish expressed markers of the lymphatic endothelium seen in mammals (Kuchler et al., 2006; Yaniv et al., 2006).

1.3.6 Immune cells

In teleosts, leucocytes can be classified by for example density gradient centrifugation, microscopy and FACS analysis. Morphologically lymphocytes appear small and round with only peripheral cytoplasm and can be difficult to distinguish from monocytes. Thrombocytes (nucleated platelet-like cells) can appear elliptical and they are easily differentiated from other leucocytes, but they become spherical in solution. Neutrophils usually have a characteristic lobed nucleus. Both the number and morphology of

leucocytes can vary not only between different teleost species but also due to various stressors such as infection and water quality (Tierney, 2004). Immune cells can also be classified using their CD markers, but to a lesser extent due mostly to a lack of reagents to identify these markers. Monoclonal antibodies against teleost IgM are able to distinguish leucocytes as either being IgM+ or IgM– populations. These antibodies are of course species specific and directed mostly against the heavy chain (Pettersen et al., 2000; Thuvander et al., 1990). Thrombocytes have been specifically identified using a monoclonal antibody (Kollner et al., 2004). In addition, teleosts have granulocytes, monocytes and macrophages which have also been distinguished from one another in some species by using monoclonal antibodies (Pettersen, 2003). Dendritic cells, central to the immune response in higher vertebrates, have not been unequivocally identified in teleosts although morphological studies have reported some candidates (Lovy et al., 2006). Lymphocytes can be divided into CD4 and CD8 positive sub-sets but there is a lack of antibodies capable of achieving this separation satisfactorily. It is important to note that many immune cells change their surface markers during activation and differentiation and that the same cell can be categorised differently over the course of its life-time as a naïve, activated or memory cell. Examples of this include the fact that CTLA-4 is only expressed on activated T cells and the lack of surface Ig expression on fully differentiated B cells (plasma cells).

The leucocytes inhabiting the intestinal tract in rainbow trout have been investigated and interestingly, they appear somewhat different to those described in mammals. In mammals the antigen specificity of intraepithelial lymphocytes (IELs) is restricted, which is thought to be due to many cycles of activation and re-circulation, whereas in trout a much more diverse population of cells was present that responded to a systemic infection (Bernard et al., 2006b).

Mast cells or eosinophilic granular cells have been identified in teleosts and although their tissue distribution is usually similar within families they have diverse morphology regarding the presence of basophilic or acidophilic granules. However all these cells de-granulate in response to inflammation and are found in high numbers at sites of chronic inflammation, a response similar to that of mast cells in higher vertebrates

(Reite and Evensen, 2006). Rodlet cells are considered be a teleost phenomenon and are found in epithelia, accumulating in upper epidermal layers in response to stressors, such as pollution or temperature changes (Iger and Abraham, 1997). They appear to be important in parasitic infections where, in response to stress or noxious agents, they mature and their secretions contribute to the eradication of the parasite (Leino, 1996; Reite and Evensen, 2006).

1.4 The immune response of teleosts

Evolutionarily, salmon and other teleosts are a long way from mammals and relatively little is known about their immune responses. However, they are poised at a very interesting time point in evolution when classical adaptive immunity first evolved. Innate immune mechanisms are present in all multi-cellular organisms, but only vertebrates have adaptive immunity that can anticipate, specify and regulate the immune response. For this reason teleosts represent an interesting model group. The use of teleosts as model species has developed in different fields. The zebrafish has become a model for gene expression after the limitations of simpler models such as *Drosphilia melanogaster* and *Caenorhabditis elegans* were realised. More recently zebrafish has become a model species for comparative immunology and even as a model for human disease (Meeker and Trede, 2008). The pufferfishes *Takifugu rubripes* and *Tetraodon nigroviridis* are useful model species because of their compact genome size (Brenner et al., 1993). Draft sequences of the whole genomes are publically available for both the pufferfishes and zebrafish. This provides valuable linkage information between genes (synteny), which is useful when sequence similarity between teleosts and mammals is not sufficient for gene identification. The interest in Atlantic salmon and rainbow trout as model species is mostly commercial, since in other respects their tetraploid genomes render them a challenge as a model organism.

In general, the immune response can be divided into an innate, germline encoded, immediate response and an adaptive, recombinatorial, specific but latent response. In addition, they can each be divided into a humoral (soluble) and cellular part. However,

there is considerable interdependence of the two types of responses. For example, macrophages, which are important cells in the innate response, have pattern recognition receptors (PRRs) that recognise pathogen associated molecular patterns (PAMPs) on antigens. Macrophages also take an active part in the adaptive response acting as antigen presenting cells and clearing opsonised pathogens.T cells are usually classified as part of the adaptive response, but it has been suggested that T cells bearing a γδTCR can act as PRRs in mammals, which is a hallmark of the innate response (Konigshofer and Chien, 2006). The myriad of cytokines produced by T cells and macrophages can act on both the innate and the adaptive immune system. The complement system comprises a group of soluble circulating proteases, acting in concert in an amplifying cascade to help eliminate pathogens. It also functions in a multi-disciplinary fashion acting with antibodies via the classical pathway in the adaptive response, and independently of antibodies via the lectin pathway in the innate response.

1.4.1 Innate immune response

Innate responses rely on proteins and receptors encoded by germ-line DNA that recognise common molecular patterns not normally found on cell surfaces of multi-cellular organisms. These PRRs recognise PAMPs, such as the lipopolysaccharide of bacterial cell-walls, dsRNA (viruses) and bacterial DNA. Many of these PRRs are toll-like receptors. Toll receptors, first identified in the fruit fly (*Drosphilia melanogaster*), comprise six different families of receptors each binding to a general class of PAMPs (Lemaitre, 2004; Takeda and Akira, 2003). In addition, host cell DNA can be a target as in the autoimmune disease, systemic lupus erythematosus. Antigens not normally exposed on cell surfaces, due to perhaps necrosis, inflammation or cancer can also signal danger and activate the innate immune response (Matzinger, 2002) .

Innate immune mechanisms in teleosts appear to have greater significance and more diversity than in higher vertebrates. There are for example, several C3 molecules in the teleost complement system where mammals have only one and they are differentially

regulated (Lovoll et al., 2007; Nakao et al., 2003). The innate immune response is possibly less temperature dependant than the adaptive response where it can take up to 12 weeks to mount an effective antibody response in cold water species and up to six weeks in Atlantic salmon (Ellis, 2001). Many pathogens can prove fatal within days of infection if not re-buffed by an active innate immunity that is activated immediately. In mammals, the adaptive immune response has the more important role but it is the innate response that starts the process. This illustrated in the Severe Combined Immuno-Deficient (SCID) mouse model whose lack of adaptive immunity causes recurrent infection leading to death. Recently, experimental infection of sea bass with the monogean parasite *Diplectanum aequans* showed increases in IL-1β and TGF-β gene expression but not for TCR-β suggesting only an innate response was triggered (Faliex et al., 2008).

1.4.2 Adaptive immune response

The adaptive immune response evolved with the earliest vertebrates creating a capacity to neutralise an enormous range of antigens by recombining germ-line DNA into an almost infinite number of different receptors. This involves combining variable, diversity and joining (V, D, J) gene segments present in both immunoglobulin and TCR gene complexes. The TCR and antibody molecules are the key receptors involved in the adaptive response. At the crux of this recombination system are the recombinase activation genes *rag1* and *rag2* that encode enzymes acting at recombination sequence sites (RSS) (Schatz et al., 1989). The genes encoding these enzymes first appeared between the agnathans (jawless fish represented today by hagfish and lamprey) and the chrondricthyes (sharks/rays) (Fig. 1). However, agnathans do have antigen receptors derived from germ-line rearrangements of leucine rich repeat modules (LRRs). These modules encode cell surface variable lymphocyte receptors (VLRs) (Pancer et al., 2004; Pancer et al., 2005). But there is no evidence so far of ancestral *rag*, immunoglobulin, TCR or MHC genes in these early vertebrates. Thus, chrondricthyes/ osteichthyes provide the earliest genetic model for the adaptive immune system in

higher vertebrates and as such, several teleosts have become model species, including zebrafish and fugu. The LRR sequences of agnathans occur in all other vertebrates as toll-like receptors (TLRs) which are an integral part of the innate immune system. The question of why adaptive immunity evolved is an interesting one. It has been proposed that an explosion in the number of pathogens approximately 400 mya caused the necessity to evolve an adaptive response, but plants and invertebrates today manage very well without adaptive immunity. Thus, it is possible that the adaptive immune system evolved for a different reason than defence (Stewart, 1992). The distinction of self and non-self or more specifically the ability to be able to identify danger would have been critical for multi-cellular organisms and could have driven the evolution of variable region molecules (Rinkevich, 1999; Stewart, 1992). In support of the theory that the adaptive immune system evolved as a defence mechanism, is the fact that since pathogens can evolve faster than vertebrates with doubling times of minutes not years then an equally efficient system of defence had to evolve to meet this challenge. Alternatively, jawed vertebrates that are able to eat almost anything would have required a mechanism to defend themselves against damage to the mucosal surface of their gut and ingestion of pathogens (Matsunaga and Rahman, 1998).

Antibody mediated immunity

The mammalian adaptive immunity is characterised by the production of antigen-specific antibodies comprising five classes; IgM, IgG, IgA, IgD and IgE. IgM and IgD can be co-expressed in humans whereas the successive expression of IgG, IgA, and IgE is regulated by class switching. In teleosts, IgM and a molecule homologous to IgD have been isolated but class switching has never been observed. More recently IgT and IgZ have been cloned from trout and zebrafish respectively (Danilova et al., 2005; Hansen et al., 2005). Although IgT and IgZ group together in phylogenetic trees further investigation is required before it is known if they are orthologs.

In mammals antibodies are produced when a B cell is activated by the interaction of its membrane bound immunoglobulin (IgM or IgD) with an antigen. IgM is produced as a primary response and the same antigen specificity is expressed by IgG in the secondary

response by class switching. There is no IgG equivalent in fish, although this is the most significant antibody class in mammals with several subclasses and the highest serum concentration. Antibody production is both slower and poorer in teleosts compared to mammals with less affinity and memory (Cain et al., 2002; Kaattari et al., 2002).

Cell mediated immunity

In mammals, cell mediated immunity is characterised by the production of specific cytotoxic cells from naïve T cells. These cells engage and eliminate infected cells presenting the antigen they are primed for. These cytotoxic T (Tc) cells are activated by their T cell receptor recognising peptides displayed on MHC class I molecules and they express CD8 as a co-receptor (Fig. 4). Similarly T cells recognising peptides displayed by MHC class II molecules are T helper (Th) cells and express CD4 as a co-receptor (Fig. 4). Th cells secrete many different cytokines when activated that regulate and propagate the immune response including antibody production from B cells. In mammals and teleosts the T cell receptor is made up of αβ or γδ chains which, due to very short CYT domains, require the CD3 complex for T cell activation (Figs.4 and 5). All known CD3 molecules have a negative amino acid residue in their TM domain. In mammals, CD3 molecules interact with positively charged lysine and arginine residues in the TM domains of the TCR. This anchors the TCR/CD3 complex together (Call et al., 2002; Shelton et al., 2001). The CYT domains of the CD3 complex have several immunoreceptor tyrosine-based activation motifs (ITAMs) for downstream signalling (Figs. 4 and 5). Antigen is presented to T cells by the polymorphic MHC class I or class II molecules. MHC class I molecules are expressed on almost all cells in the body and present endogenous antigens (viral, cancer antigens) to T cells bearing CD8 as a co-receptor. MHC class II molecules are primarily expressed on antigen presenting cells (dendritic cells, macrophages and B cells) and usually present exogenous, particulate antigen to T cells bearing CD4 as a co-receptor.

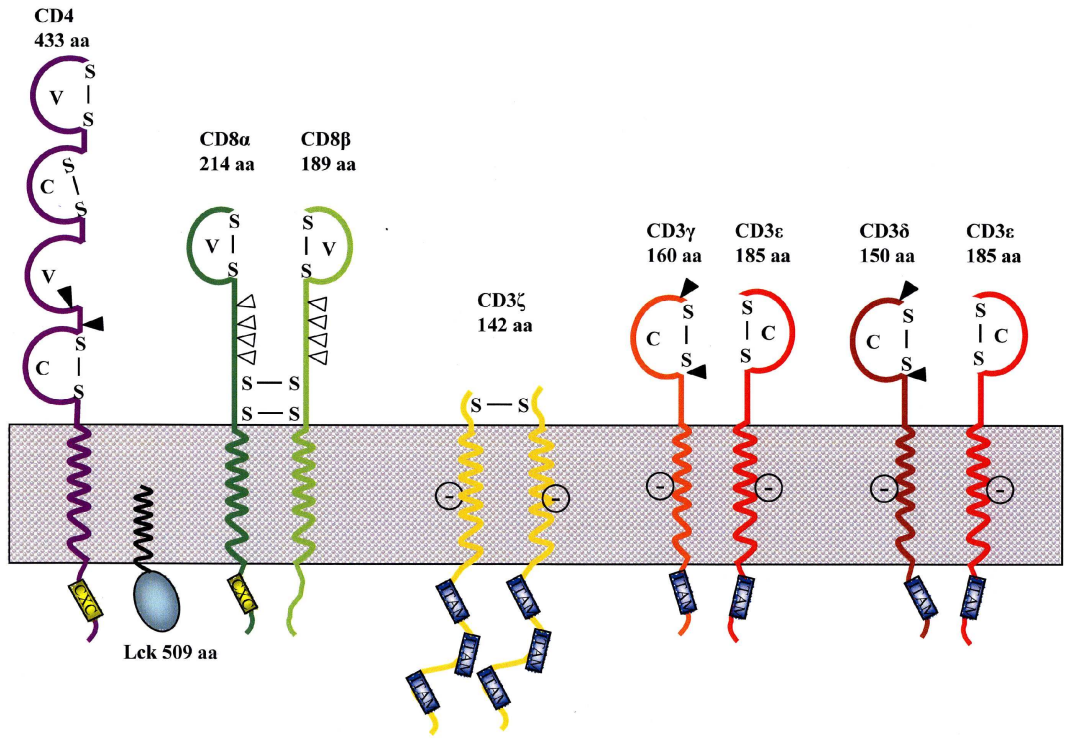
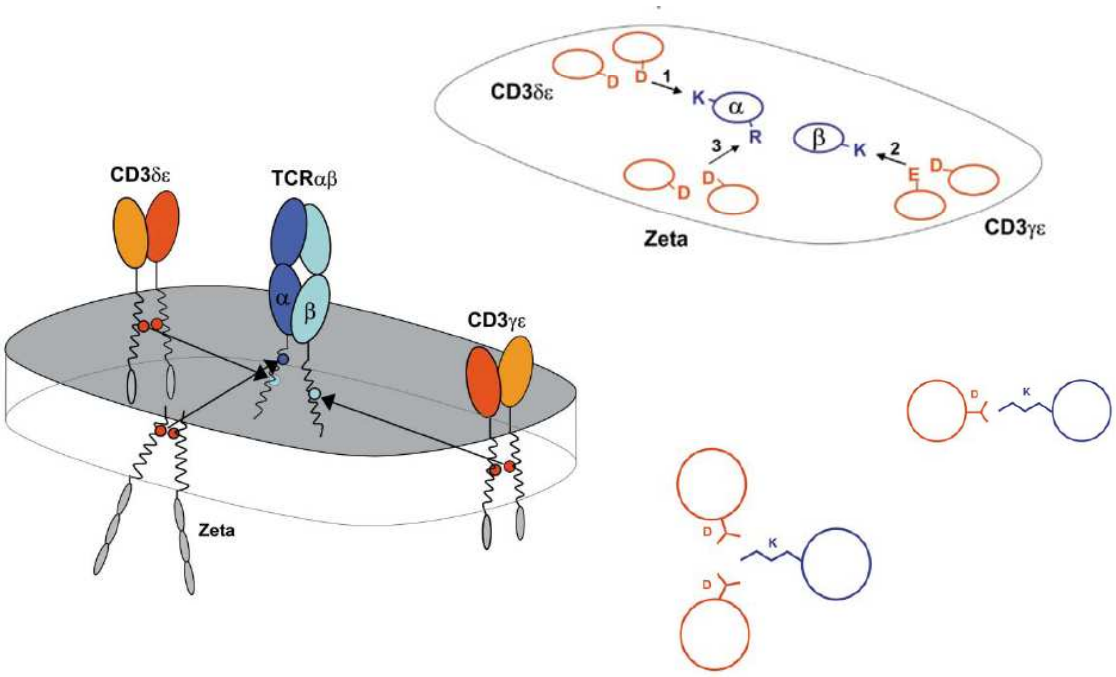


Figure 4. Human T cell co-receptors; CD4, CD8 and CD3 showing, IgSf domains (variable, V or constant, C) with cysteine double bonds (S-S) including an atypical cysteine double bond in CD4, O (△) and N (▲) linked glycosylation, cytoplasmic activation motifs and charged TM residues for anchoring the TCR complex. Lck is myristylated ( ) and anchored to the inner leaflet of the cell membrane.

The involvement of these CD4 and CD8 co-receptors in antigen recognition causes the protein kinase Lck, which is bound to their respective CYT domain to phosphorylate neighbouring CD3ζ ITAMs that in turn recruit ZAP-70 (**z**eta(ζ) chain **a**ssociated **p**rotein of 70kDa). Further downstream phosphorylation reactions result in altered gene expression. The presence of some of these downstream mediators has been verified in zebrafish (Yoder et al., 2007). This is the first of two signals necessary for T cell activation. The second signal involves the interaction of B7 (CD80 and CD86) on the antigen presenting cell and CD28 on T cells. In CD8/Tc cells this activation cascade leads to a cytokine release and differentiation of T cells into cytotoxic T lymphocytes

(CTLs) that kill infected/altered cells. Cytotoxicity has been well documented in teleosts (Fischer et al., 2003; Fischer et al., 2006; Nakanishi et al., 2002; Shen et al., 2002; Somamoto et al., 2006; Utke et al., 2007; Zhou et al., 2001). In CD4/Th cells this second signal stimulates the production of cytokines which can help B cells to produce antibodies, act on the complement cascade and help naïve T cells differentiate into CTLs. Co-stimulatory, CD28 and the subsequently expressed, inhibitory CTLA-4 (CD152), which are involved in this second signal have been identified in teleosts (Bernard et al., 2007; Bernard et al., 2006a). Many cytokines and members of the complement cascade have also been identified in teleosts (Boshra et al., 2006; Li et al., 2007; Nakao et al., 2003; Robertsen, 2006; Zou et al., 2005).



Modified from Call *et.al,* Cell Vol 111, 2002

Figure 5. Formation and anchoring of the TCR/CD3 complex in mammalian cell membranes. Lysine (K) and argine (R) in the TM of the TCR associates with aspartic acid (D) or glutamic acid (E) in the TM of CD3. CYT domains contain ITAMs ( )

1.4.3 Vaccination

Much of the research carried out within the field of fish immunology is aimed at improving vaccination regimes and developing new vaccines against emerging diseases. The effective vaccines against bacterial diseases introduced in the 1990s are mostly inactivated bacterial preparations and can be administered by immersion. Vaccine research now includes the development of recombinant subunit vaccines and DNA vaccination, which are more useful against viral diseases and avoid the safety issues surrounding the use of live virus vaccines. The biggest challenge for vaccine production is cost effectiveness since teleosts need relatively large doses of antigen compared to similar vaccines in mammals. Many teleost species are particularly vulnerable to disease during their early life stages when vaccination maybe ineffective or lead to tolerance instead of immunity. However, immuno-stimulants as feed supplements can be an efficient way to stimulate the innate immune system which is functional only hours after hatching in carp and zebrafish but almost certainly takes longer in colder water species such as salmonids (Kumari and Sahoo, 2006; Pedersen et al., 2006; Salinas et al., 2004). Adjuvant added to vaccines should ideally help with antigen presentation, stimulation of the innate response and provide a second signal for lymphocyte activation (Secombes, 2008). However, it seems the addition of adjuvants is not without drawbacks. One of the most commonly used adjuvants, mineral oil, has shown an alarming association between vaccination and autoimmune disease in farmed Atlantic salmon (Koppang et al., 2008). DNA fragments containing CpG motifs (imitating bacterial DNA) have been shown to non-specifically stimulate the immune response to viral antigens and could prove useful as an adjuvant (Jorgensen et al., 2003). The use of cytokines such as IL-12 and IL-8 as vaccine adjuvants or co-stimulants has been discussed (Secombes, 2008). The cost of these types of adjuvant will of course be a problem in large scale vaccination, but chimeric plasmids carrying both adjuvant and antigen followed by DNA vaccination is a possibility. In mice intracellular stimulation of NFκ-β (an early indicator of T cell activation) has been successful in producing a desirable Th1 response, with increased IgG2a, IFNγ and T

cell proliferation (Andreakos et al., 2006). DNA vaccination has already been successfully tested in fish and the flexibility and production cost of this antigen preparation method makes it a field of active research. Vaccination of rainbow trout with a heterologous plasmid DNA was equally efficacious on challenge as a plasmid expressing the specific protein but this was a short-lived, non-specific, anti-viral response (Lorenzen et al., 2002).

1.5 Clusters of differentiation (CD markers)

Within the context of this study, markers refer to CD or clusters of differentiation. These describe a system used originally for identifying cell surface molecules on human leucocytes. The CD nomenclature was first devised and published at the 1st International Workshop and Conference on Human Leucocyte Differentiation Antigens (HLDA), in 1982. Initially, it was an attempt to categorize the myriad of monoclonal antibodies directed against the numerous molecules found on the surface of human leucocytes but has since been expanded to include other cell types. Each new CD molecule has to be recognized by at least two monoclonal antibodies that bind to the molecule. There are now 339 CD markers registered as updated after the 8th HLDA International Workshop (Vidal-Laliena et al., 2005).

In teleosts, such as Atlantic salmon, a comparative approach is used and the situation is reversed. Using similarities conserved in the sequences of many of the known CD molecules in mammals and more closely related species, such as chicken and *Xenopus laevis*, DNA databases are searched, transcripts (ESTs) identified and the homologs to human CD markers characterised. Antibodies can subsequently be raised against proteins or peptides whose sequences are deduced from the translated nucleotide sequences. This reversal of CD marker research in teleosts is due to the relative ease with which DNA sequence information is produced and DNA is handled compared to proteins. This has lead to an enormous, publicly available resource of sequence information from genes and ESTs and the concomitant increase in cloning of teleost

markers (Randelli et al., 2008). Key homologous T cell markers in teleosts are presented in appendix C.. It is now also more common in human studies to have cloned the relevant protein before monoclonals have been produced using transfected cells or recombinant protein as an antigen (Vidal-Laliena et al., 2005).

Many CD markers are receptors or ligands, responsible for cell signalling, initiating cascades of events leading to changes in gene expression, others are cell adhesion molecules. The protein expression profile personified by the presence, absence and concentration of CD markers defines the differentiation stage of the cell.

Some of the most significant CD markers include those defining major lymphocyte sub-populations. All mammalian lymphocytes express CD45 and B and T cell populations are defined by their B and T- cell receptors respectively (BCR and TCR). The BCR complex is comprised of IgM and IgD molecules together with the heterodimeric co-adaptor, signalling moiety Igα/Igβ (CD79a and CD79b). The TCR is more complex as described above.

Lymphocytes interact with MHC molecules that are responsible for presenting peptide antigens. MHC class I molecules are present on almost all cells (not brain, cornea or gonads) and MHC class II are present on professional antigen presenting cells such as dendritic cells, B cells and macrophages in varying amounts depending on their activation state. MHC molecules are best known for their polymorphic properties and both maternal and paternal alleles are co-expressed. MHC class I and class II homologs have been cloned in many teleost species (Antao et al., 1999; Aoyagi, 2002; Dijkstra et al., 2007; Grimholt et al., 1994; Hordvik et al., 1993; Okamura et al., 1997; Shum et al., 2001). The genomic arrangement of these genes is different between species. In humans it is an extensive complex on chromosome 6 spanning 4000 kilobases containing all the MHC class I and class II genes and many other associated proteins for processing and displaying antigens hence the term **M**ajor **H**istocompatibility **C**omplex. In chickens this complex is much smaller and simpler than in mammals, “the minimal essential MHC” (Kaufman, 2000; Kaufman et al., 1995). In teleosts, MHC class I and MHC class II are coded for on different chromosomes. There is evidence of

the teleost WGD and the salmonid specific duplication in the synteny of these regions (Palti et al., 2007; Shiina et al., 2005). However, the name MHC remains a useful label since these molecules perform similar functions in all species. The cloning of these genes before the outset of this study provided strong indications that Atlantic salmon possessed equivalent molecules to both CD4 and CD8 in mammals.

Lck is a T cell specific protein tyrosine kinase and as such can also be a useful marker of T cells. It is present in the cytoplasm of T cells and interacts with the CYT domain of both CD4 and CD8. The activation of Lck constitutes the first signal in a cascade leading to T cell activation. It is one of many protein tyrosine kinases having src homology, SH2 and SH3 domains, in addition to a highly conserved kinase domain near the C terminal. The N terminal is myristylated and in this way anchored to the inner leaflet of the cell membrane (Fig. 4). Lck in teleosts was first reported by Brenner and co-workers in fugu who identified a dual promotor similar to that found in mammals (Brenner et al., 2002). In zebrafish a GFP reporter coupled to the *lck* gene has been used to investigate T cell development (Langenau et al., 2004). More recently, two transcripts, Lck 1 and Lck 2 have been identified in rainbow trout (Laing et al., 2007).

Lymphocyte activation gene (LAG-3 or CD223) is expressed on activated T cells and natural killer cells and contains four EX Ig domains, similar to CD4. It has only one V-type domain, the other three EX domains being C-type domains and no lck binding motif in its CYT domain (Triebel et al., 1990).

2. Aims of this study

This research was initially funded by the Norwegian Research Council's Functional Genomics programme (FUGE) within a project whose mandate included the study of disease related genes in Atlantic salmon.

The primary aim of this study was to clone and characterise key molecules and receptors on T cells in Atlantic salmon. In this regard the cloning and characterisation of Atlantic salmon CD3, CD8 and CD4 transcripts and genes was undertaken.

A secondary aim of this project was to use the acquired sequence information to make new tools, which would allow further characterisation and measurement of the immune system and the immune response in Atlantic salmon. To this end a polyclonal antibody was produced against CD3ε and used in paper IV. Further work to produce monoclonal antibodies against CD3, CD8, and CD4 is underway.

3. Summary of results

The transcripts and genes encoding Atlantic salmon homologs to mammalian CD3, CD8 and CD4 have been cloned, sequenced and partially characterised. These results comprise DNA sequences of 16 cDNAs and 11 genes. Accession numbers for all these sequences are presented in appendix A. In addition, studies with an antibody raised against a peptide within CD3ε have illuminated the distribution of T cells in Atlantic salmon tissues.

Paper I

Y. Liu, L. J. Moore, E. O. Koppang and I. Hordvik. Characterization of the CD3ζ, CD3γδ and CD3ε subunits of the T cell receptor complex in Atlantic salmon. Dev Comp Immunol 2008;32 (1):26-35.

Three different CD3 transcripts were sequenced; CD3ε, CD3γδ and CD3ζ. The CD3ε/CD3γδ locus appeared to be duplicated and one of the CD3ε genes proved to be a pseudogene located tail to tail with one of the CD3γδ genes. The CD3γδ gene is considered the forerunner of the separate CD3γ and CD3δ genes in mammals. The two slightly different CD3γδ genes were labelled A and B and like the intact CD3ε they have one ITAM in their CYT domains. The CD3ζ gene was the most similar to its mammalian homolog with only a short extracellular domain and three ITAMs in a relatively long CYT domain. Two slightly different but intact CD3ζ genes were found (CD3ζ -1 and CD3ζ -2), but since they were not equally represented in GenBank it is possible that they are alleles. Expression of all the CD3 molecules was highest in the thymus for all these transcripts when measured using RT-qPCR.

Paper II

L. J. Moore, T. Somamoto, K. K. Lie, J. M. Dijkstra and I. Hordvik. Characterisation of salmon and trout CD8α and CD8β. Mol Immunol 2005;42(10):1225-34.

The cDNAs for Atlantic salmon and brown trout CD8α and CD8β were sequenced together with that of CD8β of rainbow trout (the CD8α sequence had been published previously). In common with mammals these molecules have an Ig-like domain and a stalk region with multiple glycosylation motifs extracellularly and a TM domain followed by a short CYT domain. Each domain was encoded by a single exon apart from the CYT domain, the latter part of which was encoded together with the 3'UTR. An extra intron in the 3'UTR of Atlantic salmon CD8α can be spliced out which might result in a variant with a truncated CYT domain. The Lck binding motif (CXC) in mammalian CD8α is not present in the CYT domain of salmon CD8α. The genes for CD8α and CD8β were also sequenced. Gene expression in seven different Atlantic salmon tissues was examined using RT-qPCR and showed the highest expression in the thymus for all transcripts

Paper III

L. J. Moore, J. M. Dijkstra, E.O. Koppang and I. Hordvik. CD4 Homologues in Atlantic Salmon. Fish and Shellfish Immunology 2009; 26:10-18

Atlantic salmon have two similar CD4 molecules, CD4-1 and CD4-2 with four and two Ig domains respectively. CD4-2 also had two sub-types CD4-2a and CD4-b. CD4-2 was found adjacent to the CD4-1 gene using synteny analysis in the publicly available fugu genome database. All molecules contained a Lck binding motif in the CYT domain. The genes for these molecules were also sequenced and the pattern of their intron/exon boundaries further underlined their homology to mammalian CD4 genes. This included the code for the first Ig-like domain for both CD4-1 and CD4-2 being split between two exons which is a characteristic of mammalian CD4 molecules.

Southern analysis of genomic DNA from two homozygous strains of rainbow trout indicates that CD4-1 is duplicated although only one transcript was cloned at the cDNA level. Expression estimated using RT-qPCR in several tissues showed the highest expression was in the thymus, except for CD4-2a which was more highly expressed in the spleen.

Paper IV

L.J.Moore, E.O. Koppang, U. Fischer, M. Tranulis and I. Hordvik. Distribution of T cells in Atlantic salmon tissue using an antibody to CD3ε. (manuscript)

A polyclonal antibody raised against a relatively conserved peptide in the CYT domain of CD3ε was evaluated regarding its sensitivity and specificity using western blot and FACS analysis. Western blot of transfected cell lysates and salmon tissue extracts showed a band of 19-20 kD. The strongest signal was in thymus followed by gills, with weak signals in spleen and leucocytes. The antibody was then used to identify the CD3ε protein in different salmon tissues using immunohistochemistry. The highest concentration of CD3ε expressing cells was in the thymus followed by a lymphoid tissue at the base of the gill arch. Spleen and head kidney had scattered positive cells and the hind gut showed a few positive cells between the enterocytes. Laser capture microdissection of positive tissues followed by RT-qPCR showed that the gene expression of CD3ε together with the expression of TCR, CD4, CD8, Ig, MHC and Lck reflected that seen in immunohistochemistry.

4. Discussion

The T cell markers presented in this thesis constitute co-receptors and adapter molecules for the TCR in Atlantic salmon, where they define immune cells and take part in initiating immune responses. Previous work in our laboratory has involved cloning and characterising key immune molecules in Atlantic salmon, such as immunoglobulin, MHC and T cell receptor molecules (Hordvik, 2002; Hordvik et al., 1997; Hordvik et al., 1993; Hordvik et al., 1996; Hordvik et al., 1999; Hordvik et al., 1992). During the course of the present study the GRASP consortium/Dr. B. Koop and his group has sequenced the entire loci for the α, β, γ and δ TCR genes in Atlantic salmon (Yazawa et al., 2008a; Yazawa et al., 2008b). T cell markers in teleosts have recently been included in a review (Randelli et al., 2008).

4.1 Cloning strategies

The cloning of the first immune genes in teleosts, namely immunoglobulin, TCR and MHC genes were identified by immunoscreening, cross-hybridization and PCR with degenerate primers. Characteristic features such as their recombined variable segments and their pattern of polymorphic residues confirmed their identities as mammalian homologs. The present cloning of CD3, CD8 and CD4 in Atlantic salmon was primarily based on PCR homology cloning and synteny analysis. The sequences used to design primers were identified from different sources. If other salmonid sequences were published, as was the case for CD8α, degenerated primers were unnecessary since these sequences are typically over 90% identical to Atlantic salmon (Hansen and Strassburger, 2000). Alternatively, sequences from other closely related species may be used as a search in BLAST to identify Atlantic salmon ESTs from which primers could be designed. This strategy was successful during the cloning of CD3γδ and CD3ζ using

flounder and *Xenopus laevis* sequences as BLAST queries. Finally, if no matches in salmonid ESTs are found, sequences from more distantly related teleosts may amplify useful products during PCR with or without degenerate primer design. **CO**nsensus-**DE**generate **H**ybrid **O**ligonucleotide **P**rimers or CODEHOP is a programme designed for degenerate primer design for PCR homology cloning of distantly related genes (Rose et al., 2003; Rose et al., 1998). When cloning CD3ε from salmon, degenerated primers were designed from published fugu and flounder sequences.

CD4 identification was more demanding, but examination of the fugu genome revealed two genes on scaffold 627 with similarity to CD4 in mammals (Dijkstra et al., 2006). The salmon genes for CD4 and CD8β were both found using synteny analysis as discussed further below.

4.2 Sequence analysis

PCR amplification products were cloned and plasmids purified before sequencing. Sequencing several clones when possible and in both directions added to the fidelity of the final sequence. When discussing sequence identity these nucleotide sequences have been translated using ExPASy (**Ex**pert **P**rotein **A**nalysis **Sy**stem) and the order of their amino acids compared. Most molecules in this thesis had low identities even compared to other teleosts. This is partly due to them containing Ig domains which are unusually divergent as discussed below. However, sequence identity or similarity is only the first of many parameters that can be used to determine homology. Thus, we were able to prove homology using other techniques such as conservation of motifs and gene organisation.

The NCBI glossary states that similarity is “the extent to which nucleotide or protein sequences are related. The extent of similarity between two sequences can be based on percent sequence identity and/or conservation”. In this respect the terms identity and similarity are often used interchangeably and refer to the % of the residues that match

exactly between the sequences being compared. This % gives information about the substitution rate between the sequences being compared. If a similarity value is based on “conservation” this, again according to NCBI glossary, refers to the conservation of the physico-chemical properties of the amino acid. This similarity % is more generous or useful when homology is being sought in divergent sequences. The Clustal programmes most commonly used for sequence alignment can provide information on both identical and conserved amino acid matches. However, concluding sequences are homologous needs further support such as conserved domains/motifs or functional data.

The translated sequences in this study were all identified as membrane proteins with a signal sequence and Ig domains, with the exception of CD3ζ (Fig. 6). Alignment of cDNAs with their corresponding genomic sequences showed a preponderance of a one domain per exon pattern with the exception of CYT domains. The second part of the CYT domains for CD4 and CD8 were coded for together with the 3'UTR (Papers II and III). The exon boundaries in the CYT domains of CD3 occurred within the ITAMs (Paper I).

In addition, the first Ig domain in both teleost CD4 molecules was split between two exons, a fact which helped prove their homology since this is similar in CD4 from higher vertebrates as well as other teleost CD4s (Paper III).

4.3 Atlantic salmon CD3, CD4 and CD8

Three different CD3 molecules have been characterised in teleosts; ζ, ε and a single γ/δ molecule, the forerunner of the γ and δ molecules in mammals (Paper I and references therein). CD3ζ, now also named CD247, has a very short EX domain and each of the three ITAMs in the CYT domain are encoded by a different exon. This pattern of intron/exon boundaries centred in essential signalling motifs was not followed in CD4 whose putative Lck binding motif was coded for within the CYT domain exon.

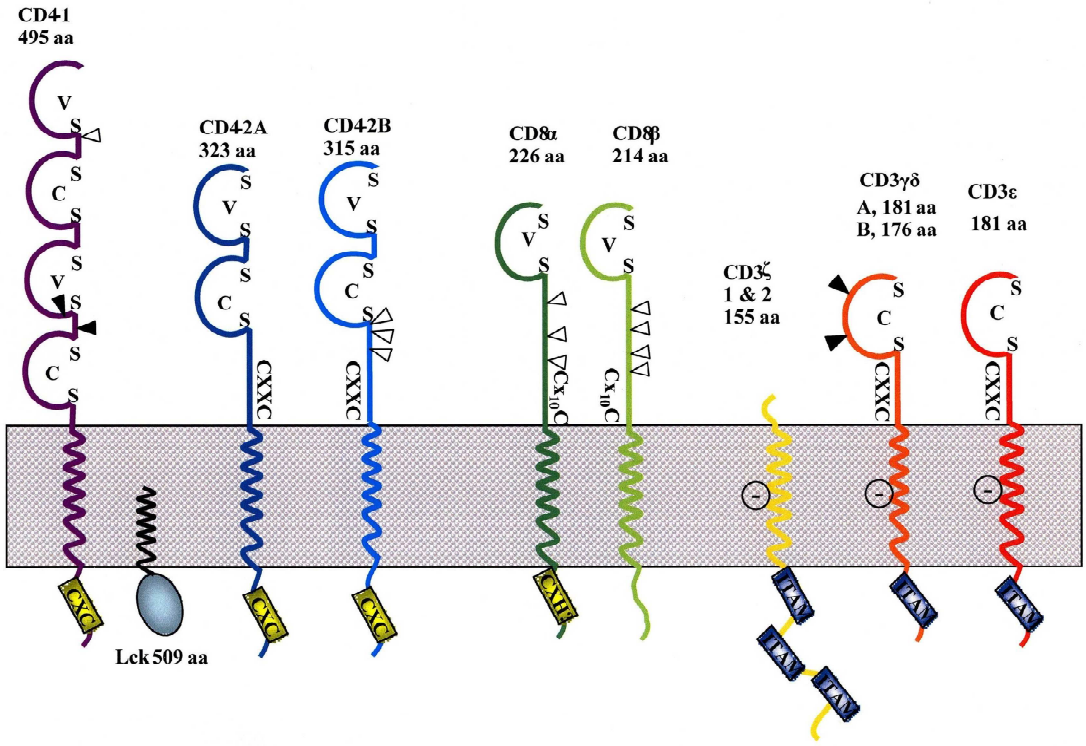


Figure 6. Teleost T cell markers showing variable (V) and constant (C) Ig-like domains, O (△) and N (▲) linked glycosylation, cytoplasmic activation motifs and cysteines (S) for possible dimerisation. Lck has a myristylation motif and could be anchored to the cell membrane (WWW)

The two forms of CD3ζ in salmon that were cloned were considered allelic variants since CD3ζ-2 represented only 10% of the CD3ζ ESTs in GenBank and the overall identity was high resulting in only one amino acid difference between the two molecules. In addition to the published salmon and zebrafish CD3ζ sequences, ESTs are available for rainbow trout and catfish (CA344798 and DQ114900). Zebrafish also have two CD3ζ transcripts encoded by genes on different chromosomes and thus, in contrast to the Atlantic salmon CD3ζ, may represent gene duplication (Yoder et al., 2007). Interestingly, Yoder and co-workers were only able to identify the first exon of CD3ζ in the current release of the zebrafish genome database on chromosome 1

indicating that zebrafish also have a rather long intron between the first and second exons as is the case for mammals. We were similarly unable to amplify this intron when sequencing the CD3ζ gene in salmon (Paper I). In zebrafish the second CD3ζ-L (CD247-L) gene can be found on chromosome 9 (www.ncbi.nlm.nih.gov/ projects/mapview, zebrafish version 7).

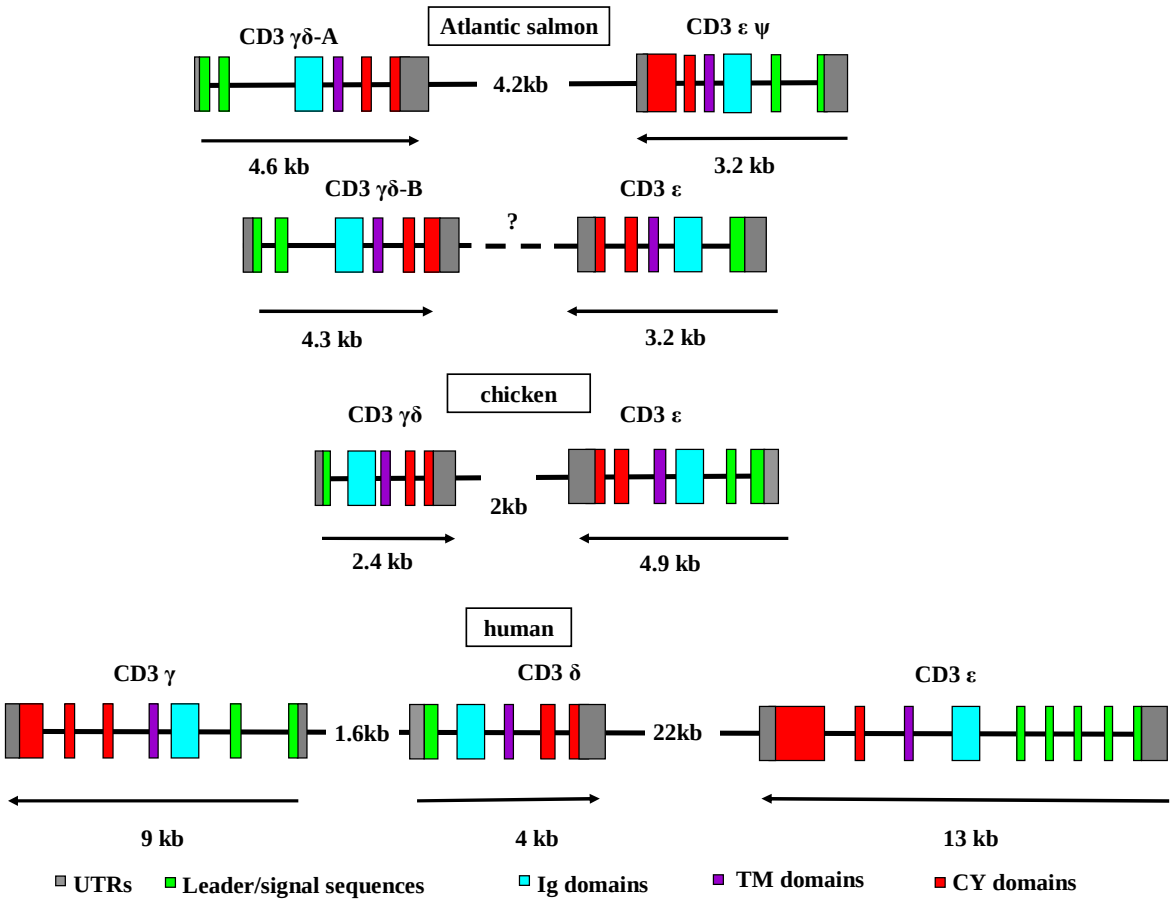


Figure 7. Comparison of CD3 genes in salmon, chicken and human, showing gene orientation, exon organisation and duplication of salmon CD3 genes.

CD3ε and CD3γδ are similar to each other with single Ig-like domains and shorter CYT domains containing one ITAM which was similarly divided onto two exons. The CD3γδ gene appears duplicated with A and B sub-types differing by only 1.2% of nucleotides, but resulting in seven amino acid substitutions and a five residue insertion

in the EX domain of CD3γδ-A (Paper I). CD3ε also had two variants, but one was a pseudogene exhibiting several frame shifts and an elongated CYT domain and both transcripts are poorly represented in GenBank (Fig.7). Pseudo-CD3ε and a CD3γδ gene were co-localised in Atlantic salmon, tail to tail with each other in a similar orientation as mammalian CD3 molecules (Fig. 7).

CD8α in rainbow trout had been identified before the onset of this thesis using highly degenerate primers to amplify Ig-like domains from a rainbow trout thymic cDNA library (Hansen and Strassburger, 2000).

Subsequent to the cloning of CD8α and CD8β in salmonids (Paper I) transcripts for these molecules have been cloned and characterised in several other teleosts including sea bass, sea bream, carp, fugu and halibut (Buonocore et al., 2006; Patel, 2008; Randelli et al., 2006; Somamoto et al., 2005; Suetake et al., 2007; Sun XF, 2007). These two CD8 molecules are expressed as homodimers (CD8αα) or heterodimers (CD8αβ) in mammals. The CD8αα homodimer is mainly present on intraepithelial lymphocytes (IELs) that also express γδTCRs and seems to have a separate and distinctive role in mammals compared to CD8αβ. It can be expressed transiently or constitutively on a wide variety of cells including; γδTCR and αβTCR IELs or co-expressed with CD4 or CD8αβ and MHC I or MHC II on αβTCR IELs (Leishman et al., 2002). Taken together these observations indicate that CD8αα is not a specific T cell marker and that it is the CD8β chain that confers co-receptor status on the CD8 molecule (Arcaro et al., 2001; Gangadharan and Cheroutre, 2004). The presence of CD8αα on cells is thought to play a co-repressor function to negatively regulate T cell activation (Cheroutre and Lambolez, 2008).

In fugu, CD8+ cells have been characterised in leucocyte populations using a polyclonal antibody against CD8α (Araki et al., 2008). Here CD8+ cells included lymphocytes, monocytes and thrombocytes that were negative for CD4 and were stimulated by phytohaemaglutamin. Since this result almost certainly reflects the presence of the homodimer as well as the heterodimer it's quite possible that the non-lymphocytic cells express the homodimer. The CD8 negative cells in this experiment

included lymphocytes and neutrophils that expressed CD4 and light chain genes and were stimulated by lipopolysaccharide. These data indicate that fugu CD8+ cells have similar attributes to mammalian CD8 cells including that CD8α expression is not restricted to lymphocytes. Thus, a CD8β antibody might be the best tool for studies investigating and isolating classical CD8+ T cells.

a) CYT domain CD8α

```
A.salmon         CNRIRTKRCPHHYKRQPRMAAPGQQHPIANNRLF
A.salmon(trunc)  CNRIRTKRCPHHYKRQ-----------------
br.trout         CNRIRTKRCPHHYKRQPRMAAPGQQHPIANNRLF
r.trout          CNRIRTKRCPHHYKRQPRMAAPGQQHPIANNRLF
fugu             CNRVRTRRCPHHYKRKPRGKPPGNK---------
gb.carp          CNRLRTRRCPHHYKRQPR--PAGHAKLTNNPL--
seabass          CNKIRTRRCPHHYKRKPRTMAPGKQMMTHRHV--
zebrafish        CNRLRTRRCPHHYKRQPRGPPAGHAKMPNSHF--
flounder         CNHIRTRRCPHHHRRKPRTMAPGKQMKNNTHV--
halibut          CNQIRTRRCPHHYRRKPRTMPPGKQMRNHPHV--
                 **::**:*****::*:
```

b) CYT domain CD8β

```
A salmon         FSQLPKKCRHQFAKKRPLK
br.trout         FSRLPKKCRHQFAKKRPLK
r.trout          FSRLPKKCRHQFAKKRPMR
fugu             FSRLPKKCRHRFVKKRLLT
halibut          FSRLPKKCRHNFVKMRQMP
C.carpio_        FSRLPKKCRHRFTKTNQLR
                 **:*******.*.* . :
```

Figure 8. The CYT domains of both CD8α and CD8β show very high conservation between teleosts. 8a) The conserved, blue shaded, cysteine represents the position of the CXC motif for Lck binding in the mammalian CD8α sequence. Could one or both of the pair of bold histidines also take part in this binding?

An updated alignment of available teleost sequences shows extreme conservation of the CYT domain in both CD8α and CD8β (Fig 8, light blue boxes). The area corresponding to the Lck binding motif in mammalian CD8α is totally conserved suggesting that this is important for function. Histidine is well known for its zinc binding properties in zinc finger proteins (Matthews and Sunde, 2002). Therefore, it seems plausible that one or both of these histidine residues present in CD8α (bold in Fig. 8a) could interact with zinc and engage Lck during T cell activation.

In contrast to CD8β sequences of higher vertebrates, the teleost CYT domain is also highly conserved indicating that this is also important for function (Fig. 8b).

In humans the CD8B locus is upstream of the CD8A locus and duplicated (CD8B1 and CD8B2) and although both CD8B loci genes appear functional all cDNAs are derived from the CD8B1 locus (Nakayama et al., 1992).

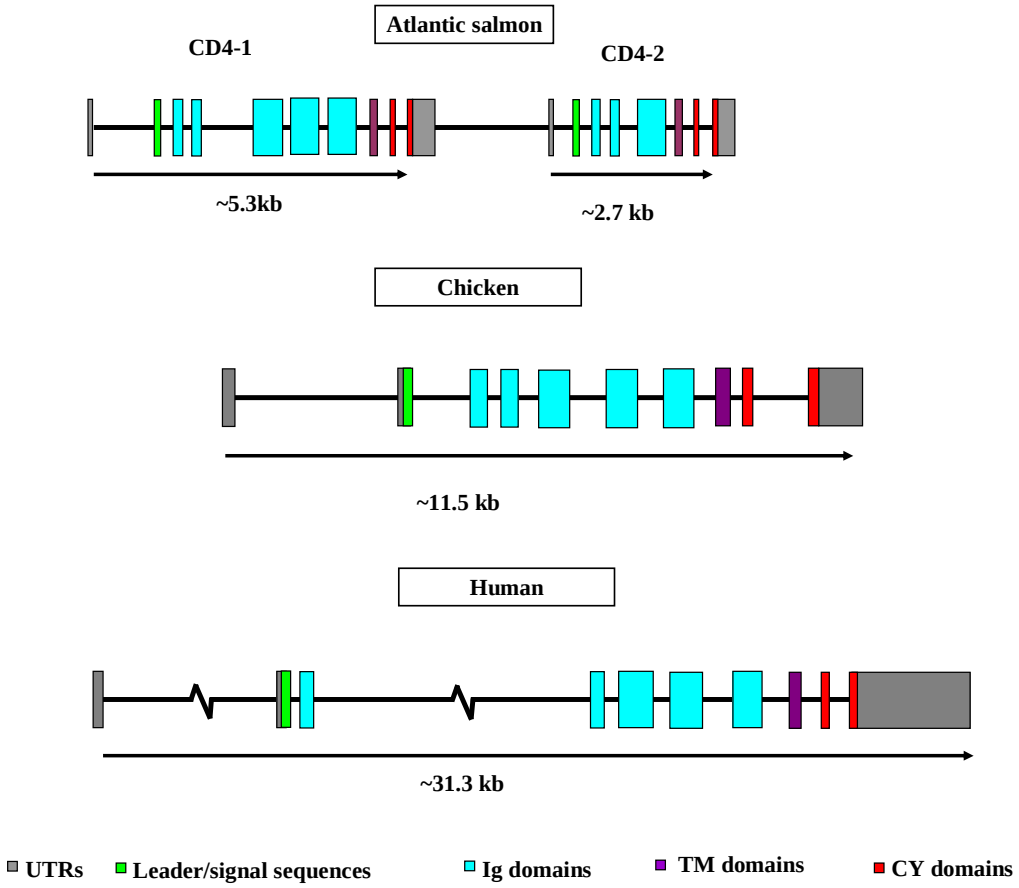


Figure 9. Comparison of CD4 genes in Atlantic salmon, chicken and human. The first Ig-like domain is encoded by two exons in all genes. The human CD4 gene is much longer than in the other species.

At the outset of this study no CD4 molecules had been characterised in teleosts. Molecules and genes have now been characterised in fugu, trout, catfish, carp, sea bass and halibut (Buonocore et al., 2008; Dijkstra et al., 2006; Edholm et al., 2007; Laing et al., 2006; Patel, 2009; Suetake et al., 2004; Sun XF, 2007) in addition to Atlantic salmon (Paper III). All teleosts investigated have a CD4 molecule with four

extracellullar domains and a CXC motif for binding Lck in their CYT domains. In addition to CD4-2 in Atlantic salmon (paper III), CD4 with only two Ig EX domains can be found in trout, carp and fugu while catfish have a quite different molecule with three EX Ig domains (Dijkstra et al., 2006; Edholm et al., 2007; Laing et al., 2006). A two domain molecule has not been identified in halibut and sea bass. These two Ig domain molecules also have the CXC, Lck binding motif in their CYT domains. The residues preceding the Lck motif form an alpha helix in human CD4 and stabilise its interaction with Lck (Kim et al., 2003). Although CD4-2 has some hydrophobic residues in this area the CD4-1 is both too short and lacking suitable amino acids to form a helix. However, CD8 also has no alpha helix capability and interacts with Lck in a different way (Kim et al., 2003). The intron/exon pattern is similar in all CD4 genes published with typical characteristics such as the code for the first Ig-like domain being split between two exons (Fig. 9). Evidence for a duplicated CD4-1 gene with two slightly different genomic fragments was not substantiated by cloning a second cDNA, but two CD4-1 genes were observed in a Southern analysis in rainbow trout.

LAG-3 which shows similarities to CD4 in mammals, has been identified *in silico* in teleosts and has four EX Ig domains, but no Lck motif in the CYT. It can cluster with CD4 molecules, including teleost CD4-1, in phylogenetic trees but its relationship to CD4 has yet to be elucidated (Laing et al., 2006).

The expression of all these T cell co-receptors was investigated using RT-qPCR in several salmon tissues. In most cases these genes were many times more highly expressed in thymus compared to other tissues. The pseudo CD3ε, as might be expected, had very low expression compared to the intact gene. In mammals, appropriate expression of CD3 chains is necessary for membrane TCR expression (Buferne et al., 1992; Geisler, 1992). The stoichiometry of non-mammalian CD3 molecules has not been addressed in teleosts but in the amphibian *Xenopus laevis* a human peptide antibody successfully identified CD3ε and was used to study the TCR complex. The finding of only two CD3 molecules associated with the TCR in this study

supports the theory that the CD3γδ molecule is the forerunner of the two separate, γ and δ molecules in mammals (Gobel et al., 2000).

In mammals, expression of the CD8α chain is necessary for expression of the CD8β chain (Fung-Leung et al., 1994). In Atlantic salmon, CD8α displayed alternative splicing with an extra intron in the 3'UTR resulting in a truncated CYT tail. A second CD8β transcript identified had a premature stop codon in the EX domain. Expression of these splice variants of CD8 have not been investigated but in humans seven different spliced variants are present. This is achieved by alternative splicing of TM and CYT exons and results in four variants that lack a TM domain and that are potentially secreted (DiSanto et al., 1993).

4.4 Synteny analysis

The conserved synteny between known (human or murine) and teleost genomes was crucial to identifying some of these genes presented in this thesis. The concept of synteny is common when researching genes and transcripts in related organisms and refers to the order and orientation of genes on chromosomes. The nucleotide sequence of many genes has been sufficiently conserved over time to allow comparison of genes with similar functions between, for example, teleosts, birds and mammals. This is true, for actin, for example, which is highly conserved throughout the animal kingdom. Synteny analysis can only be applied when the genomic sequence is available Atlantic salmon genome sequencing is in progress at: http://web.uvic.ca/cbr/grasp/, http://www.salmongenome.no/cgi-bin/sgp.cgi and http://www.abdn.ac.uk/sfirc/salmon/. For synteny and homology searches both fugu and zebrafish genomic sources are available: www.sanger.ac.uk/Projects/D_rerio, www.ensembl.org/Danio_rerio and www.fugu-sg.org. Genomic synteny was instrumental in identifying both CD8β and CD4-2 in this study. As can be seen from the figure, synteny is not absolute but relative (Fig. 10). For CD4, a match to the human gene for USP5 was found first and using

Genescan the CD4 genes were identified (Dijkstra et al., 2006). CD8β was found by matching the rainbow trout sequence for CD8α against the fugu genome database and scanning the upstream sequence since this is where the human CD8β gene lies.

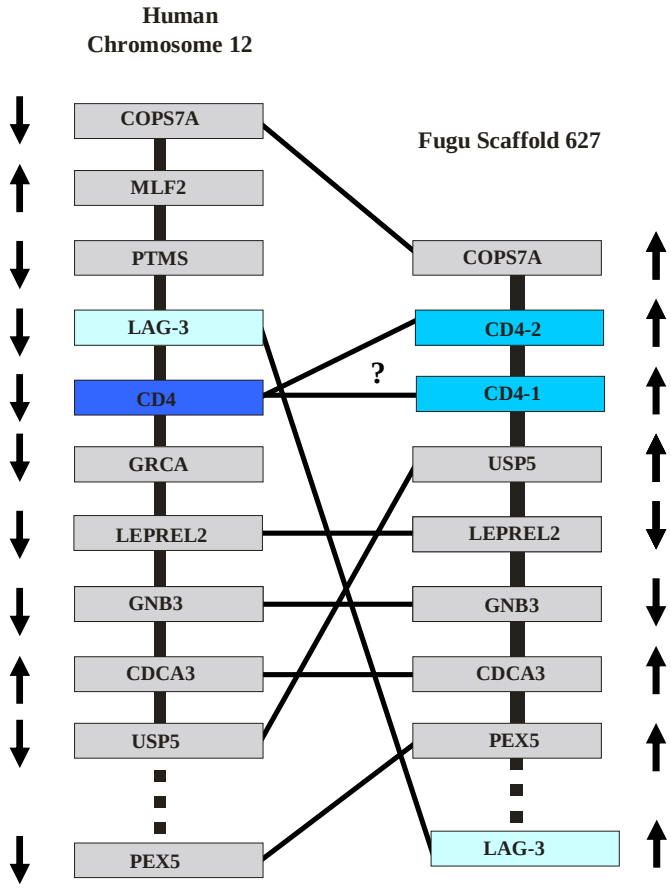


Figure 10. Synteny of genes between human and fugu genomes surrounding the CD4 gene (modified from Dijkstra et al. 2006). Synteny is not absolute but relative, both in terms of order and orientation. The relationships between the CD4s and LAG-3 has yet to be elucidated.

Further help when looking for homologs is the pattern of introns and exons which is also frequently conserved during evolution including the phase at the intron/exon boundaries. This phase refers to the way a codon for a single residue is split. These characteristics and their conservation were also important in being able to define these cloned genes as homologs.

4.5 Gene Duplication

Gene duplication is another common theme occurring when studying genes and genomes, especially in teleosts. It is not uncommon when working with teleosts to find two slightly different sequences for a gene of interest, perhaps amplified and subsequently cloned and sequenced using the same primers, as occurred when cloning CD4-2. These sequences could be alleles: one from each parent expressed from a pair of chromosomes, as is probably the case for the two CD3ζ genes in this study. Alternatively, the cloning of two different genes could be due to duplicate genes, especially in Atlantic salmon whose genome is still largely tetraploid, as suggested for the two CD3γδ genes. This could be the result of a relatively recent WGD event in salmonids where the genome is still reverting to a diploid state (Allendorf FW, 1984; Hordvik, 1998; Shiina et al., 2005). For this reason many genes are still duplicated even if their transcripts are not functional, i.e.: they have become pseudogenes like the second copy of the CD3ε gene in Atlantic salmon. Whole genome duplication (WGD) has probably been an important mechanism during evolution, increasing the inheritable material available. It has been suggested a WGD before the lobe-finned and ray-finned fishes split provided sufficient genetic material for the evolution of the adaptive immune response. It can be assumed that there is not the same selective pressure on duplicated genes which allow mutations to accumulate more rapidly after such a WGD event which can of course end in gene destruction and pseudogene production. When whole genomes are duplicated synteny is conserved, whereas when single duplications occur tandem arrays are created on the same chromosome. The V, D, J and C genes in the TCRγ locus of Atlantic salmon are considered a result of tandem duplication (Yazawa et al., 2008a). The a and b forms of CD4-2 are not co-localised, but since they do not appear to be present in all teleosts, it is unlikely they are due to WGD unless it was the relatively recent salmonid specific WGD. Zebrafish have what appears to be a teleost specific duplication of *Drmt2* (a zinc finger transcription factor) that exhibits differential expression during ontogeny. The *Drmt2a* and *Drmt2b* genes are localised

on different chromosomes with *Drmt2b* showing functional diversity compared to *Drmt2a* which has structure and functions in common with other vertebrates. It is speculated that this duplication is a result of the teleost specific WGD approximately 350 mya (Zhou et al., 2008). The duplicated genes for interferon in rainbow trout have also been shown to be differentially expressed (Purcell et al., 2008). The possible differential expression of Atlantic salmon CD4-2a and CD4-2b has yet to be investigated fully. Due to the passage of time when synteny can be perturbed it is not always easy to determine whether duplicated genes have resulted from WGD or tandem duplication. Duplicated genes can quickly gather lethal mutations resulting in pseudogenes if the organism finds no (new) use for them. Alternatively, they can be lost altogether leaving no trace of the duplication event (Wolfe, 2001). The duplication events leading to the CD4 family of genes, including LAG-3, are difficult to interpret. Laing and co-workers suggested that the two domain molecule could have been duplicated to form the four domain molecule since a CD4 specific motif (WXC) occurs in both D2 and D4 (Laing et al., 2006). However the split first exon is an equally unique hallmark of CD4 molecules and is not present in D3 of the four domain molecule which makes this scenario unlikely. The consensus would now appear to be that salmonids have undergone four WGDs (Fig. 1). The strongest evidence for WGD comes from investigation of the Hox gene cluster which is singly represented in amphioxus, an ancient, surviving cephalochordate (phylogenetically the closest invertebrate to vertebrates). Already in lamprey, two or three Hox gene clusters are in evidence suggesting two WGD. Whereas all jawed vertebrates, including teleosts and humans, have at least four hox gene clusters providing evidence for a third WGD event (Elgar, 2004; Meyer and Schartl, 1999; Ohno, 1999). In appears that salmonids have undergone an additional relatively recent WGD approximately 60 mya (Allendorf FW, 1984; Shiina et al., 2005). Due to the massive amount of whole genome information now available, research into genome duplication has led to many new recent efforts to diagnose the timing and extent of whole genome duplications (Blomme et al., 2006; Panopoulou and Poustka, 2005).

4.6 Protein Domains

In addition to the gene orientation and nucleotide sequences, the amino acid translations provide another level of information with which to compare genes and study homology.

Domains in protein sequences can be predicted from translated nucleic acid sequences and are known to be highly likely to form distinct secondary structures which perform known functions. Transmembrane domains are a good example and can be relatively accurately predicted (www.cbs.dtu.dk/services/TMHMM) due to their amphiphatic nature and the many examples there are to gather data from. This domain is an alpha helix requiring four amino acids per turn, resulting in approximately 22 amino acids being necessary to transverse the lipid bilayer of animal cells. All the transcripts and translated proteins presented in this study contain single pass alpha helix TM domains. These domains were frequently the most highly conserved between species probably due to the stringent requirements of the transmembrane helix. In addition, the TM domains of the TCR/CD3 complex contain conserved amino acids for their interaction during activation (Fig. 5). This feature is also present in CD3 of teleosts (Paper I and references therein). Alpha helices also occur outside of TM domains. There is a short α helical domain in the CYT domain of CD4 preceding the Lck binding motif. This helix is somewhat better represented in CD4-2 than CD4-1 suggesting that if these CD4 molecules interact with Lck they do so in different ways (Paper III).

Another domain encountered in almost all the molecules presented in this thesis is the ubiquitous immunoglobulin domain. The immunoglobulin superfamily (IgSF) domain has a long history and has been identified in species before the evolution of adaptive immunity, where it was first described in immunoglobulins. Insects have hemolin which is a soluble protein in the hemolymph comprising one or two IgSF domains that bind to bacteria cell membranes (Sun et al., 1990). Invertebrates have a whole family of

fibrinogen related proteins (FREPs) involved in defence which also contain IgSF domains (Zhang et al., 2004).

The sequences able to form IgSF domains are extremely diverse and frequently have little in common except the canonical cysteines spaced approximately 60 residues apart and the invariable tryptophan residue that forms the core of Ig superfamily domains (Ioerger et al., 1999). Many of these domains are missing one of these trademark canonical cysteine residues indicating that it is not essential for the formation of Ig domains. However, the two anti-parallel β sheets are usually stabilized by a cysteine double bond producing a globular domain. So stable and desirable is this fold that these domains form in proteins with very dissimilar sequences. The molecules discussed in this thesis have similarities between teleost and mammals below 17% but basic predicted structures remain the same (Buonocore et al., 2006; Buonocore et al., 2008; Costani et al., 2008). This is because the only requirement for stability is the perpendicular orientation of alternating hydrophobic and hydrophilic amino acid side chains towards the inside and the outside of the domain respectively.

This domain is frequently repeated in for example Ig molecules and CD4 but single domains are also common in for example CD8, and CD3 molecules. Homology between molecules containing Ig domains can be difficult to prove since the primary sequences can be so degenerate. This is amply illustrated by the first cloning of CD3 in flounder when it was difficult to determine whether it was the ε or γδ transcript until further sequencing was completed in both flounder and other teleosts (Araki et al., 2005; Park et al., 2005; Park et al., 2001). Unfortunately, although Park and co-workers were careful not to conclude the identity in their first paper the accession number from this study remains incorrectly annotated as CD3ε in GenBank. This has resulted in two sequences from flounder called CD3ε (AB044571/2 and AB081751) and errors in subsequent papers from different groups using the GenBank reference.

Despite the low identity indices between human molecules and the teleost molecules discussed here, the predicted structures of these molecules seem to be conserved indicating the importance of these molecules in their many interactions with other

immune molecules such as MHC and signalling molecules (Buonocore et al., 2006; Buonocore et al., 2007; Buonocore et al., 2008). This rapid diversification has been investigated by comparing amino acid sequences in many Ig domains in many human and murine proteins. The rate of amino acid changing (non-synomonous) substitution is in fact greater in immune-related molecules especially if these molecules are exclusively expressed in the immune system (Hughes, 1997).

Examples of domains which are highly conserved not only in structure but also in primary sequence are Src homology (SH) domains. These occur in many protein tyrosine kinases, including Lck, the T cell specific kinase. If the unique N terminal domain in Lck, which is responsible for binding to CD4 and CD8, is excluded these molecules are 74% identical between humans and Atlantic salmon. This is because Lck contains a SH2 and SH3 domain together with a kinase domain at the C terminal part of this protein. These domains occur in many kinases involved in intracellular signalling and have diverged very little between teleosts and mammals (Laing et al., 2007). This is very different from the molecules containing Ig domains whose primary sequences have diverged a great deal as discussed above. This suggests an enzyme involved in many different signalling cascades has little room for mutations or else it becomes dysfunctional and thus the pressure for sequence maintenance is much greater.

However, the conservation of primary sequence and structure does not necessarily mean the conservation of function. Famously, crystallins of vertebrate and invertebrate eye lenses are identical to lactate dehydrogenase (LDH) where this enzyme has been co-opted for use as a structural protein without changes in sequence or duplication (Hendriks et al., 1988; Wistow et al., 1987).

4.7 Protein Motifs

Motifs are usually short, linear sequences of residues, whose function is known from data on characterised proteins. Motif identification can be helpful in homolog identification and allows assumptions to be made about function which is very useful when new gene products are being characterised. Bioinformatics has provided many useful programs in order to search for motifs for example; www.cbs.dtu.dk/ services/NetNGlyc, www.cbs.dtu.dk/services/NetOGlyc (Gupta et al., 1999; Julenius et al., 2005) used to predict glycosylation sites in mammalian proteins.

Motifs for glycosylation were examined since these sites can be conserved and provide useful information on the homology of the sequence being examined. Many molecules with immunological functions are glycosylated, including T and B cell receptors, soluble antibodies and many complement components which help form binding surfaces. Glycosylation can also help protect proteins from proteases which are present extracellularly where many immune functions take place. Even the glycocalyx present on all cells may serve to prevent unwanted binding to lectins which would trigger the innate immune response or complement activation which would cause inappropriate cell lysis (Rudd et al., 2001).

Basically, glycosylation occurs at sites in protein sequences in two different ways. O-glycosylation involves the addition of sugars to the hydroxyl group of threonine or serine residues in either the golgi apparatus or the cytoplasm. N-glycosylation is the addition of a large branched array of sugar residues in the endoplasmic reticulum to asparagine (N) which is subsequently trimmed during passage through the golgi. Motifs for O-linked glycosylation are phosphorylated if not glycosylated and this has enormous significance when considering intracellular signalling where phosphorylation is an important mechanism for regulating protein activity.

The significance of glycosylation sites is not known in teleosts, but the connecting peptide in CD8 is heavily O-glycosylated in mammals allowing it to form an extended

stalk structure. The glycosylation of the CD8β improves it's co-receptor functions allowing peptide binding at much lower concentrations than for CD8αα and also changes the avidity of MHC class I binding to CD8+ cells during development in the thymus (Moody et al., 2001; Wong J. S., 2003). Putative glycosylation sites are present, not only in Atlantic salmon CD8 (Paper II) but also in other teleost CD8 sequences making it highly likely that a similar extended structure is present in teleosts (Fig. 6).

The glycosylation site at the beginning of the D4 in the EX domain of the teleost CD4 molecules helped characterise them as CD4 homologs since it is present in all known CD4 sequences except for chicken (Paper III). Conversely, these motifs did not help in the analysis of CD3 where the presence of glycosylation sites is different in CD3γδ and CD3ε between teleost species (Paper I and references therein).

Motifs for interaction with signalling molecules such as ITAMs and the protein kinase Lck motif, have also been significant in this study. ITAMs have a consensus sequence in higher vertebrates: YxxL (x6-8) YxxL . The tyrosine residue in these motifs is phosphorylated causing recruitment of downstream signalling molecules during T cell activation. CD3ζ has three ITAMs in the CYT domain and CD3γδ and CD3ε have one. Zebrafish have one CD3ζ with three ITAMs similar to the salmon molecule and one shorter CD3ζ with only two ITAMS which is similar to the catfish CD3ζ sequence available (Yoder et al., 2007). Although the stoichiometry of CD3 in teleosts is not known, with up to ten ITAMs in the mammalian CD3 complex (Fig. 4 and 5), an amplification of the activation signal occurs at this point leading to interaction with many molecules of the next signal transduction component (Shores et al., 1994; Shores et al., 1997). This feature is a hallmark of cell signalling cascades. Alternatively, it could mitigate diversification of the signal by interaction with different downstream signalling molecules (Combadiere et al., 1996). ITAMs also occur in other non-ligand binding molecules such as the Igα and Igβ associated with the BCR. In mammals, the ITAM motifs present in CD3γ and CD3δ can stimulate T cell activation independently of CD3ζ (Wegener et al., 1992). The putative ITAMs in salmon CD3 are more diverse than those described in higher vertebrates including the spacing (x=8-10 in teleosts and 7 in higher vertebrates) which is considered critical in mammals (Paper I).

The CXC motif in the CYT domain of all CD8α and CD4 molecules is conserved in all higher vertebrates and interacts with a CXXC motif at the unique N terminal end of Lck (Shaw et al., 1990; Turner et al., 1990). In humans this interaction involves a single zinc ion and a clasp structure for both CD4 and CD8 (Kim et al., 2003). It results in the phosphorylation of tyrosine in the ITAMs of the cytoplasmic domain of CD3ζ and as such is a key molecule in T cell activation. Thus, the identification of this motif in all salmon CD4 molecules was a great help in determining homology. It is intriguingly absent in all teleost CD8α sequences published to date.

There are also many motifs repeated in nucleic acid sequences, which although not used for homology searches are useful in determining gene structures. These motifs include Kozak sequences indicating transcription start sites and polyadenylation signals marking the end of 3' untranslated regions.

4.8 Gene expression/RT-qPCR

The characterisation of genes also involves investigation of their expression in different tissues. Northern blotting and RT-PCR provide information on RNA in various tissues. However, most of this expression profiling is now studied using reverse transcribed quantiatitive PCR (RT-qPCR). Although no longer a new method, RT-qPCR has yet to be standardized with regard to how new methods are reported. The MIARE (**m**inimum **i**nformation **a**bout **R**T-PCR **e**xperiments) guidelines have been proposed in order to make the reporting of RT-qPCR more accountable and reproducible and should include information about:

- Efficiency of PCR
- Primer, probe and amplicon sequences
- Sample quality and quantity (RIN)
- RT details
- PCR details, including controls

- Analysis methods including calculations

The RIN refers to a RNA integrity number and is a value calculated by software used in conjunction with specialist RNA analysers. RINs lie between 1 and 10 where values over 5 can be considered adequate for use in RT-qPCR experiments. TaqMan assays were used as far as possible in this study. Absolute quantitation was not used although this could have proved useful. Relative results are usually sufficient, especially during infection studies when baselines and controls are included. Absolute values require cloned PCR products to calculate standard curves, recently published for interferon gene assays (Purcell et al., 2008).

RT-qPCR provides information about RNA, usually mRNA when priming the first strand reaction with an oligo-dT primer. Although the central dogma of biochemistry states that DNA is transcribed into RNA and RNA into protein there are many mRNAs that are degraded before translation. Thus, it is inappropiate to measure RNA and discuss expression with the assumption of discussing protein expression. In addition, when using RT-qPCR to quantitate leucocyte markers the “mobile” circulatory component due to leucocyte traffic might have to be considered.

RT-qPCR can also be performed as a confirmatory technique in conjunction with histological techniques and laser capture microdissection (LCM). LCM adds enormously to the discriminatory power of histology when coupled to the measurement of gene expression in dissected material. LCM can give quantitative information to supplement mRNA information gained from *in situ* hybridization. Immunohistochemistry which visualises actual protein expression using antibodies is also well complemented by the mRNA measurements provided by LCM, followed by RT-qPCR (Paper IV).

LCM material needs a very sensitive detection method or much optimisation of a pre-amplification step. A one step RT-qPCR assay using specific primers during the reverse transcription reaction has been shown to improve sensitivity especially for poorly expressed genes (De Paula et al., 2004; Wacker and Godard, 2005). Absolute quantification could also prove useful and overlap PCR could in theory be used to

make plasmid inserts, containing all the necessary sequences for a series of immune genes.

The ability to monitor and measure the immune response is especially important during vaccine trials and infection studies. The literature contains several infection studies where certain markers have been measured, usually by RT-qPCR (Faliex et al., 2008) and some include cellular assays (Fischer et al., 2006; Utke et al., 2007; Utke et al., 2008). Similarly microarray can produce useful information on up and down regulation of huge numbers of genes simultaneously although not quantitatively (Ferraresso et al., 2008; Majji et al., 2009).

4.9 Monoclonal antibody production

The sequence information gained during this study was used to make a collection of constructs designed to aid the production of specific monoclonal antibodies in collaboration with Dr. Bernd Köllner (Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany). An earlier collaboration using MHC class II β expressed as a thioredoxin fusion protein in pTrxFus (Invitrogen.com) had produced a successful antigen (Koppang et al., 2003). Thus, initial collaboration on this project involved the protein expression of the extracellular domains of both CD8α and CD8β as thioredoxin fusion proteins in pET102 bacterial expression vector (Invitrogen.com). This involved PCR amplification of the extracellular domain in frame with the thioredoxin sequence. As a fusion partner, thioredoxin can both chaperone and increase solubility of the expressed protein without affecting its secondary structure, adding only 11.4 kD to its molecular weight. In addition the pET102 vector included a 6 x His tag at the C terminal to simplify purification. CD8α and CD8β made in this way were used to immunize mice and produced potent antisera but fully characterised monoclonals are still under development.

In a more novel approach whole coding regions, excluding the signal sequences of CD8α, CD8β CD3γδ, CD3ε, and CD4-1 were cloned in frame with an N-terminal FLAG epitope in a mammalian expression vector (Viertlboeck et al., 2004). Expression from these vectors in transfected cells results in the whole protein with an N terminal FLAG epitope on the cell surface. These vectors were used both as antigens and as screening tools after transfection into 293T cells. The FLAG epitope was utilised to enrich positively expressing cells using anti-FLAG antibody, before the cells were used for the immunisation of mice. After several immunisations the mouse serum was tested for reactivity against cells transfected with the same vector by FACS analysis. Successful, viable clones after fusion between the mouse spleen cells and myeloma cells were screened similarly. FITC conjugated anti-mouse antibody was used to screen supernatents from surviving clones and FITC anti-FLAG was used on transfected cells as a positive control. Non-transfected cells were used as negative controls. Promising clones were purified and further characterised using western blotting and immunofluorescence using the same transfected cells as antigen and anti-FLAG as a positive control.

5. Future perspectives

The relative ease of cloning and sequencing has produced useful information on many new molecules in recent years. The challenge now is to use this information to investigate the functioning of the teleost immune system, to pave the way for rapid vaccine development and to investigate the immune response teleosts mount against pathological agents. Continued investigations are still needed on CD markers in teleosts, since there are already 339 human CD markers and it has been estimated that there could be up to 4000 different CD markers on human leucocytes (Hashimoto et al., 2003; Nicholson et al., 2005; Zola and Swart, 2005).

There is a need for more antibodies. Due to the time and expertise required for the production and not least, the characterization of monoclonal antibodies, commercially prepared polyclonal antibodies are a good choice for small research projects. An example is the CD3ε antibody presented here in the final manuscript. However, the production of monoclonal antibodies has long term possibilities. The specificity and permanent availability of monoclonal antibodies probably make them the preferred choice for use in a commercial setting, where quality control is paramount. Antibodies can help answer questions such as, how CD8 interacts with Lck without a complete binding motif. Can histidine substitute for the lack of a cysteine? What is the significance of the conserved sequences in the CYT domains of teleosts? Antibodies would also assist the further investigation of CD4-1 and CD4-2, to determine if they are co-expressed or if they identify T cell sub-populatioins.

The measurement of expressed immune genes using either microarray or RT-qPCR during infection studies will continue to be important, to provide information as to how salmon defend themselves and thus what fish health researchers can do to help them optimise this defence. An Atlantic salmon microarray containing almost 4000 relevant immune genes (Institiute for Marine Biosciences in Halifax, Canada) has been evaluated using *Aeromonas salmonicida* as a model disease (Ewart et al., 2005). Alternatively, the GRASP consortium has a massive 32,000 gene microarray for

Atlantic salmon that together with two different salmon lice arrays will be used to investigate the mechanisms of the infestation with these parasites.

Histology, the backbone of morphological studies has made a quantum leap as a method in conjunction with LCM and RT-qPCR. Further analysis of the form and function of the immune system, including the recently identified lymphoid gill tissue, is warranted.

Model species such as the zebrafish whose virtues are well recognized (Meeker and Trede, 2008; Sullivan and Kim, 2008) are not useful for *in vitro* cellular studies due to their small size. Cell lines would abrogate the cell number problem for small model species such as zebrafish. There are cell lines for some species, but well characterised lymphocyte cell lines are needed. Cell lines in Atlantic salmon are being characterized (Pettersen et al., 2008). The numerous leucocyte cell lines from catfish are already useful and lend an extra dimension to all cloning and sequencing work in this species while further characterizing the actual cell line (Miller et al., 1998; Miller et al., 1994; Stuge et al., 2000).

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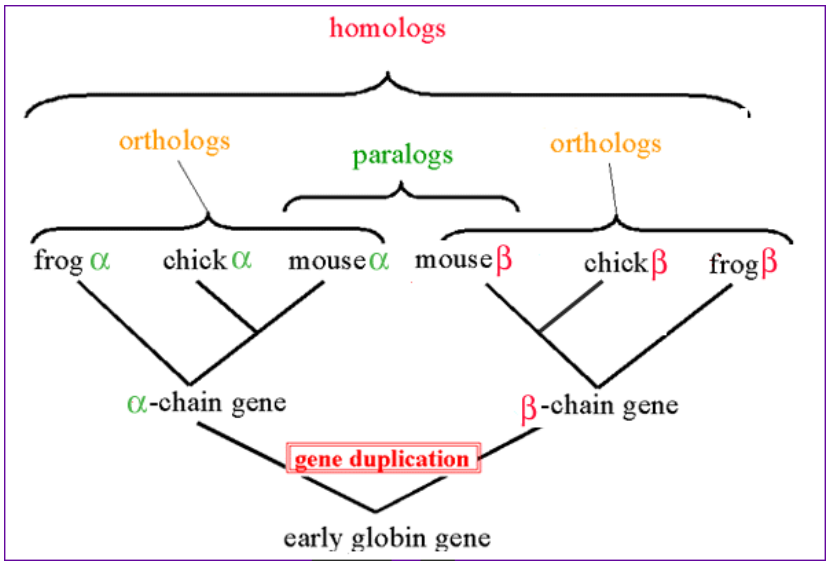
Appendix A: GenBank accession numbers

CD3			CD4			CD8		
EF421412	CD3ζ-1	cDNA	EU409792	CD4-2a	cDNA	AY693391	CD8α	gDNA
EF421413	CD3ζ-1	gDNA	EU409793	CD4-2b	cDNA	AY693392	CD8β	gDNA
EF421414	CD3ζ-2	cDNA	EU409794	CD4-1	cDNA	AY693393	CD8α	cDNA
EF421415	CD3ζ-2	gDNA	EU585750	CD4-1	gDNA	AY693394	CD8β	cDNA
EF421416	CD3γδ-A	cDNA	EU585751	CD4-2a	gDNA	AY701521	CD8α alt.spl.	cDNA
EF421417	CD3γδ-A	gDNA	EU585752	CD4-2b	gDNA	AY701522	CD8β trunc.	cDNA
EF421418	CD3γδ-B	cDNA				AY701523	br. trout CD8α	cDNA
EF421419	CD3γδ-B	gDNA				AY701524	br. trout CD8β	cDNA
EF421420	CD3ε	cDNA				AY563420	r. trout CD8β	cDNA
EF421421	CD3ε	gDNA						
EF421422	ψCD3ε	cDNA						
EF421423	ψCD3ε	gDNA						

Appendix B. The concepts of homology, orthology and paralogy.

From the explainatory figure below the term homologous can be applied to both orthologs and paralogs. Orthology is defined as the same gene in a different species derived from a common ancestor. Other definitions of homology stipulate related function as well as common descent which in the present work has not been confirmed. However, the initial characterisation of the genes and transcripts presented in this thesis suggest that they perform common functions. Therefore, the term homology has been used throughout this work.

Homologous sequences



Orthologs and paralogs are two types of homologous sequences. Orthology describes genes in different species that derive from a common ancestor. Orthologous genes may or may not have the same function. Paralogy describes homologous genes within a single species that diverged by gene duplication.

http://www.ncbi.nlm.nih.gov/Education/BLASTinfo/Orthology.html

Appendix C: Table of Key Homologous T cell markers in teleosts

CD	Species	Accession № mRNA(s)	Accession № Gene(s)	Refs
TCRα	Alantic salmon *(Salmo salar)*	AY552002		[1]
	catfish *(Ictalurus punctatus)*	U58505		[2]
	Cod *(Gadus morhua)*	AJ133845		[3]
	flounder *(Paralichthys olivaceus)*	AB054227		[4]
	pufferfish *(Sphaeroides nephelus)*	U22676		
	rainbow trout *(Oncorhynckus mykiss)*	U50991.		[5]
TCRβ	Atlantic salmon *(Salmo salar)*	X97435		[6]
	flounder *(Paralichthys olivaceus)*	AB053228		[4]
	Cod *(Gadus morhua)*	AJ143849		[3]
	rainbow trout *(Oncorhynckus mykiss)*	U18122		
	catfish *(Ictalurus punctatus)*	U39193		[2]
TCRγ	Atlantic salmon *(Salmo salar)*			[7]
	flounder *(Paralichthys olivaceus)*	AB07671		[4]
TCRδ	Atlantic salmon *(Salmo salar)*			[8]
	flounder *(Paralichthys olivaceus)*	AB076073		[4]
CD3γδ	Atlantic salmon *(Salmo salar)*	EF421416/17	EF421418/19	[9]
	fugu *(Takifugu rubripes)*	AB166800		[10]
	flounder *(Paralichthys olivaceus)*	AB044572/	AB054068	[11]
CD3ε	Atlantic salmon *(Salmo salar)*	EF421420	EF421421	[9]
	flounder *(Paralichthys olivaceus)*	AB081751		[12]
	fugu *(Takifugu rubripes)*	AB166798/99		[10]
	sterlet *(Acipenser ruthenus)*	AJ242941-45		[13]
CD3ζ	Atlantic salmon *(Salmo salar)*	EF421412/13	EF421414/15	[9]
	zebrafish *(Danio rerio)*	EF601086		[14]
CD3ζL	zebrafish *(Danio rerio)*	EF158448		[14]
CD4-1	Atlantic salmon *(Salmo salar)*	EU409792	EU585750	[15]
	carp *(Cyprinus carpio L.)*	DQ400124		[16]
	catfish *(Ictalurus punctatus)*	DQ435301		[17]
	fugu *(Takifugu rubripes)*	AB164054/55		[18]
	rainbow trout *(Oncorhynckus mykiss)*	AY973028	AY973030	[19]
	sea bass *(Dicentrarchus labrax)*	AM849811		[20]
CD4-2	Atlantic salmon *(Salmo salar)*	EU409793/4	EU585751/2	[15]
	catfish *(Ictalurus punctatus)*	DQ4354302		[17]
	rainbow trout *(Oncorhynckus mykiss)*	AY772711(a) AY899932 (b) AY973029(REL)	AY899933(a) AY973031(REL)	[21], [19]
CD8α	Atlantic salmon *(Salmo salar)*	AF693391/93	AY 701521	[22]
	flounder *(Paralichthys olivaceus)*	AB082957		
	fugu *(Takifugu rubripes)*	AB232548		[23]
	rainbow trout *(Oncorhynckus mykiss)*	AF178053	AF178055	[24]
	sea bass *(Dicentrarchus labrax)*	AJ846849		[25]
CD8β	Atlantic salmon *(Salmo salar)*	AY693392/94	AY701522	[22]
	carp *(Cyprinus carpio L.)*	DQ324046		[16]
	fugu *(Takifugu rubripes)*	AB281056		[23]
	rainbow trout *(Oncorhynckus mykiss)*	AF178053		[22]
	zebrafish *(Danio rerio)*	BX005379		

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