UiT

NORGES
ARKTISKE
UNIVERSITET

Norges fiskerihøgskole

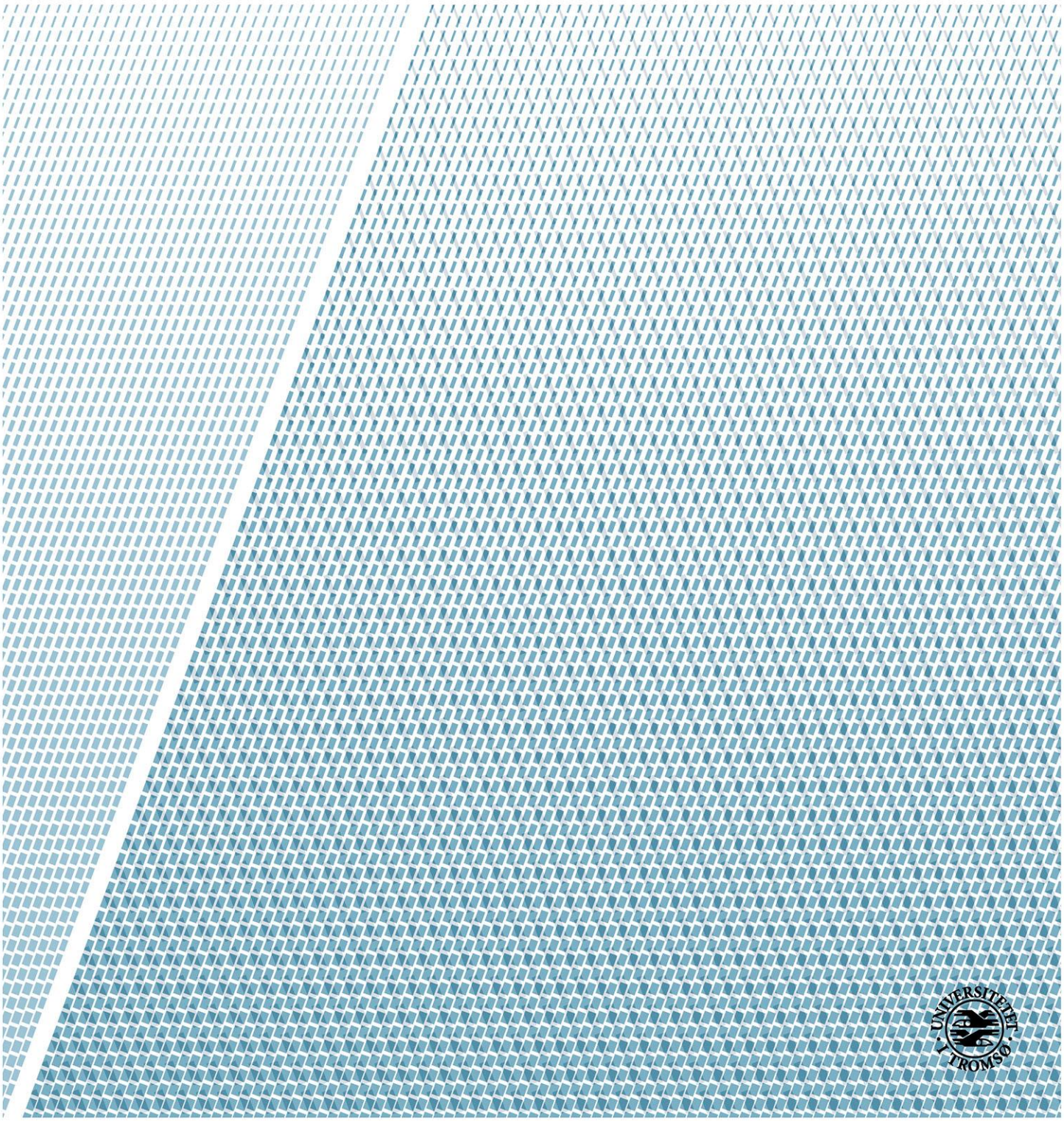
The distribution, expression pattern and effects of TNF superfamily members BAFF and APRIL in Atlantic salmon (*Salmo salar L.*) and salmon derived leukocytes

—

Mikael Fjeld Wold

Masteroppgave i Akvamedisin (60stp)

Mai 2018



Denne oppgaven ble utført ved fakultet for biologi, fiskeri og økonomi, ved Norges Fiskerihøgskole, Universitetet i Tromsø. Oppgaven ble påbegynt i februar 2017 og avsluttet i mai 2018

Først og fremst vil jeg takke min hovedveileder Jorunn B. Jørgensen for å ha gitt meg muligheten til å delta på et spennende prosjekt. Videre vil jeg takke for all oppfølgningen jeg har fått samt utsøkt veiledning i skriveprosessen. Det har aldri vært vanskelig å komme på kontoret ditt for å ta en prat. Furthermore, I wish to extend my most sincere gratitude to Michelle, who has held my hand through virtually all laboratory related work in addition to answering questions and guiding me in the process of writing; your work ethic and patience will continue to inspire me in years to come. I would like to thank Dimi and Arpita, who collaborated with me in adherent cell experiments. I wish to thank Shiferaw, who provided expert knowledge regarding ELISPOT assay procedures. Additionally, I want to thank Dennis for assistance in cDNA synthesis. Videre ønsker jeg å takke Linn og Guro som ga meg innføring på lab, dette har vært til stor hjelp. Ellers vil jeg også takke alle andre som har hjulpet meg på labben, det er mange av dere. Sist men ikke minst vil jeg rette en stor takk til alle venner og bekjente på NFH, UiT og i Tromsø for øvrig for 5 fantastiske år.

Tromsø 15. mai 2018

Mikael Fjeld Wold

Summary

Viral disease is one of the greatest challenges facing the aquaculture industry. The most serious disease at the moment is pancreas disease, caused by the salmonid alphavirus (SAV). Substantial efforts are being put into combating the disease, and fish are routinely vaccinated before being put at sea. In theory, vaccines should produce sufficient protection, but performance has been disappointing at a commercial scale. Atlantic salmon (*Salmo salar*) intrinsically possess a robust immune system. A better understanding of the mechanisms involved in host defenses upon viral infection may contribute to the development of effective therapeutic treatments. The tumor necrosis factor superfamily (TNFSF) of receptors and ligands regulate several aspects of cell functions including immune response and inflammation The B cell activating factor (BAFF) and a proliferation inducing ligand (APRIL) are TNFSF members regulating B cell function, and have in mammals shown to be important in the mounting of an efficient antibody responses upon infection. Understanding the functions of these cytokines in fish may prove useful in future vaccine development.

In this work, the distribution and expression patterns of BAFF, APRIL and their receptors are reported for the first time in Atlantic salmon. By transcriptional analyses we determined that the cytokines and their receptors were constitutively expressed in salmon systemic organs. Furthermore, we found that the transcriptional levels of BAFF and APRIL were induced in SAV-infected fish, implicating an integral role of BAFF and APRIL in viral immune responses. *In-vitro* investigations revealed that macrophage-like cells (MLCs) transcribe both BAFF and APRIL constitutively, although BAFF seems to be the dominant cytokine under normal physiological conditions. Unlike what has been reported in higher vertebrates, but consistent with what has been reported in trout, we found that resting B cells were able to transcribe both cytokines at significant levels. This mechanism may indicate unique functions of teleost B cells. Upon stimulation with cytokines and TLR-ligands, transcriptional levels of BAFF and APRIL were induced in MLCs. All receptors of BAFF and APRIL were transcribed by B cells, while MLCs transcribed one of them. The distribution of these receptors in B cell populations may provide insight into the functional roles of different B cell types in salmon. Recombinant BAFF and APRIL seemingly did not increase the amount of antibody secreting cells (ASCs) in head kidney leukocyte populations.

Contents

Term	Abbreviation
Antigen presenting cell	APC
Antibody secreting cells	ASC
A proliferation inducing ligand	APRIL
B cell activating factor	BAFF
B cell activating factor receptor	BAFF-R
B cell maturation antigen	BCMA
B cell receptor	BCR
Chum salmon heart-1	CHH-1
Cardiomyopathy syndrome	CMS
Cytosine-phosphate guanine	CpG
Cysteine rich domain	CRD
Threshold cycle	Ct
Dendritic cell	DC
Death domain	DD
Deoxyribonucleic acid	DNA
Enzyme-linked immune-spot	ELISPOT
Gut associated lymphoid tissue	GALT
Gill associated lymphoid tissue	GIALT
Heart- and skeletal muscle inflammation	HSMI
Interferon	IFN
Immunoglobulin	Ig
Intraperitoneal	i.p.
Infectious salmon anemia	ISA
Interbranchial lymphoid tissue	ILT
Interleukin	IL
Infectious pancreatic necrosis	IPN
Inter-run calibrator	IRC
Lipopolysaccharide	LPS
Macrophage colony stimulating factor	mcsfr
Mucosa associated lymphoid tissue	MALT
Major histocompability complex	MHC
Macrophage-like cell	MLC
Melanomacrophage center	MMC
Nasopharynx-associated lymphoid tissue	NALT
Nonspecific cytotoxic cells	NCC
Norges Fiskerihøgskole (Norwegian college of fishery science)	NFH
Nuclear factor kappa-light-chain-enhancer of activated B cells	NF-κB
Natural killer	NK
No reverse transcriptase	NoRT
No template control	NTC
Pathogen-associated molecular pattern	PAMP
Phosphate-buffered saline	PBS

Pancreas disease	PD
Polyinosinic: polycytidylic acid	Poly I:C
Pathogen recognition receptors	PRR
Quantitative trait locus	QTL
Recombination activing gene	RAG
Ribonucleic acid	RNA
Reverse transcriptase	RT
Quantitative polymerase chain reaction	qPCR
Skin associated lymphoid tissue	SALT
Salmonid alphavirus	SAV
Transmembrane Activator and Calcium modulator and cyclophillin ligand Interactor	TACI
T cell receptor	TCR
Toll-like receptor	TLR
Tumor necrosis factor	TNF
Tumor necrosis factor receptor	TNFR
Tumor necrosis factor super family	TNFSF
TNF receptor-associated factors	TRAF
Viral hemorrhagic septicemia virus	VHSV

1. Introduction

1.1 Background

The Norwegian aquaculture industry has seen enormous growth since its foundation in the 70s. The industry is a significant contributor to the welfare state in Norway, and continued growth is expected, as the Norwegian government hopes to increase salmon- and trout production fivefold within 2050 (St.meld. nr. 16 (2014-2015), 2015).

In the time since its beginning, the industry has encountered several problems stunting its growth. Disease has always been a limiting factor, although most bacterial diseases no longer pose a significant threat to the industry, as vaccines against these pathogens have proven effective. Effective vaccines against viral diseases are not yet commercially available, and viral diseases are thus still a major problem. The most common measure to stop the spread of viral diseases in Norwegian aquaculture is through sanitary measures by either eliminating infection routes, or stamping out infected populations.

The Jørgensen-Jensen lab at The Norwegian College of Fisheries (NFH) has an ongoing project where B-cell activation and Ig-secretion in Atlantic salmon (*Salmo salar*) is studied. In this thesis, the roles of B cell activating factor (BAFF) and a proliferation inducting ligand (APRIL) were investigated in salmon *in vivo* and *in vitro*. These are two cytokines belonging to the tumor necrosis factor (TNF) superfamily, and they are important regulators of B cell function in mammals (Bossen & Schneider, 2006). Specifically, the distribution of these cytokines were determined in healthy, pathogen-free fish, and conversely in SAV3 infected fish. Gene expression of APRIL, BAFF, and their receptors was also measured in different leukocyte-subpopulations, both constitutively and upon stimulation with TLR ligands and cytokines. Finally, the effect of BAFF and APRIL in antibody secreting cells was examined by ELISPOT assay.

1.2 Viral disease in salmon aquaculture

The viral diseases that cause problems in Norwegian aquaculture are composed of cardiomyopathy syndrome (CMS), heart- and skeletal muscle inflammation (HSMI), infectious salmon anemia (ISA) and pancreas disease (PD). The occurrences of CMS, HSMI and PD have been steadily high in the last years, with a combined number of registered outbreaks of 406, 368 and 422 in 2017, 2016 and 2015, respectively (Veterinærinstituttet, 2018). ISA has

relatively few occurrences, but each outbreak causes high morbidity and mortality in infected individuals leading to considerable economic loss. Infectious pancreas necrosis (IPN) has previously been a problem in the industry, but the disease has been almost eliminated due to the development of QTL (quantitative traits locus) fish, as well as improved hygiene in smolt facilities.

1.2.1 Pancreas disease

PD is presently a major problem in Norwegian aquaculture (Veterinærinstituttet, 2018). The virus causing this disease has been an increasing nuisance since its initial discovery in the 80s (Poppe et al., 1989) and contributes to significant economic loss for the industry (Jansen et al., 2015). The causative agent of the disease is the salmonid alphavirus (SAV), belonging to the family *togaviridae* (Nelson et al., 1995). There are six known subtypes of the virus, assigned SAV1-6 (Graham et al., 2011). SAV2 and SAV3 are endemic along the Norwegian coast (Jansen et al., 2015). The SAV 2 subtype is able to infect both rainbow trout (*Oncorhynchus mykiss)* and salmon, while SAV3 is specific to salmon. In 2016, a total of 138 new cases of PD were registered in Norway, 84 of which were of the SAV3 genotype and 53 of the SAV2 genotype (Veterinærinstituttet, 2018). In comparison, the number of SAV outbreaks in 2017 were all time high with a total of 176 outbreaks, 121 and 55 of SAV3 and SAV2, respectively (Veterinærinstituttet, 2018). The distribution of SAV outbreaks in 2017 is presented in Figure 1. SAV3 is considered to be an endemic disease in the western part of the country. The disease has not established itself north of Hustadvika, and is therefore not considered endemic north of this region. As a result, the disease is strictly regulated in the northern counties, and is controlled by "stamping out". SAV2 was first detected along the Norwegian coast in 2011. The first registered case of SAV2 infection came from north of the endemic zone, in county Nord-Trøndelag (Hjortaas et al., 2013). The route of infection of the first outbreaks have not been identified. Since the initial detection of SAV2, virtually all outbreaks of this subtype has been in counties Møre og Romsdal and Sør-Trøndelag (Veterinærinstituttet, 2018). The distribution of SAV2 and SAV3 in Norway in 2017 can be seen in Figure 1. SAV subtypes 1, 4, 5 and 6 have not been been reported in Norway. All subtypes except for SAV 3, which is unique to Norway, have been identified UK/Ireland (Graham et al., 2012), while subtype 2 have been detected in several areas of the continental Europe (Crane & Hyatt, 2011).

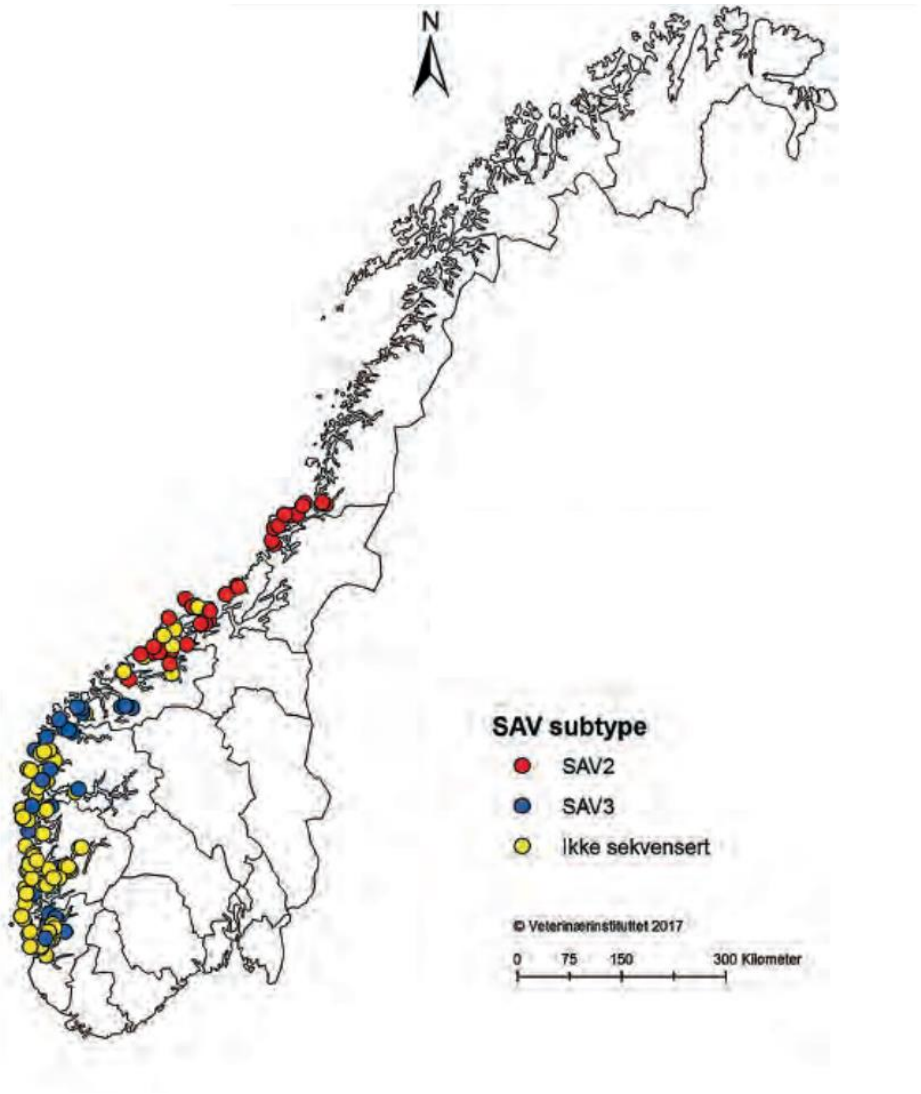


Figure 1: SAV infected localities in 2017. SAV3, SAV2 and un-sequenced SAV-outbreaks indicated with red, blue and yellow dots, respectively (Veterinærinstituttet, 2018).

PD in salmon most commonly occur in the first year in sea, but can also appear at later stages. Outbreaks commonly develop in the period between late June and November, although it can potentially occur during the entirety of the seawater phase (Bruno, 2013). The disease cause significant economic losses in the form of mortality and reduced growth (Aunsmo et al., 2010). Clinical lesions during an infection include damage to pancreas and heart- and skeletal muscle, although the severity and distribution of the lesions depend on the time after the initial infection. Symptoms include lethargy, spiraling swimming behavior, and loss of appetite (McLoughlin et al., 1996). Mortality is highly variable between incidents, suggesting a complex interaction between environment, host and pathogen (Bruno, 2013).

1.2.2 Viral vaccines

Vaccines against bacterial infections in farmed salmon are based on inactivated bacteria and are generally accepted to induce strong immunity (Sommerset et al., 2005). The present situation for viral vaccines is, however, suboptimal. The vaccine effects are limited to cause a reduction of disease severity rather than complete protection (Gomez-Casado et al., 2011). Commercial vaccines are being used to combat the diseases IPN, ISA and PD with varying results (Gomez-Casado et al., 2011). Part of the reason for the vaccines disappointing effect, is that they fail to mimic intracellular infections that are inherent to viruses (Brudeseth et al., 2013). Additionally, the shortcomings of commercial vaccines has been suggested to partly be due to insufficient concentrations of viral antigen in the vaccines (Robertsen, 2011).

Fish surviving SAV infections are resistant to re-infection, suggesting that vaccination is a viable strategy to combat the disease. Confirming this, live recombinant SAV (Moriette et al., 2006), attenuated SAV (Moriette et al., 2006) and inactivated SAV delivered by oil-adjuvanted intraperitoneal (i.p.) injection (McLoughlin & Graham, 2007) have shown protective functions against PD in salmonids. Currently, commercially available vaccines against PD are based upon inactivated whole virus antigens (Gomez-Casado et al., 2011). MSD Animal Health had the first patent to this vaccine, and was the sole supplier of the vaccine to the Norwegian aquaculture industry. Recently, MSDs patent expired, and the vaccine is now sold by both MSD and Pharmaq. Because Pharmaqs vaccine is relatively new in industrial settings, it is too early to comment on its effectiveness in the field. Vaccines based on inactivated viruses generally produce suboptimal results, in part because the antigens do not mimic a natural viral infection. Thus, live-attenuated virus may produce better protection. However, the risk of reversion to virulence and uncontrolled environmental spreading have not allowed their authorization (Gomez-Casado et al., 2011). One of the most promising preparations against viral diseases in aquaculture is currently DNA vaccines. Recently, the European Directorate for the Quality of Medicines approved the use of DNA vaccines (European commission, 2017) and this type of vaccine is also legal in Norway. Currently, the vaccine is supplied by Elanco, and has been applied in Norwegian aquaculture. The vaccine consists of naked plasmid DNA that is delivered intramuscularly, which results in the expression of viral proteins in the muscle tissue of vaccinated fish, thus mimicking an intracellular infection and triggering the adaptive immune system (Evensen & Leong, 2013). Although C. Xu et al. (2012) demonstrated poor effects of a SAV-DNA vaccine , recent studies by C. J. Chang et al. (2017) suggest that this vaccine can be

highly effective. Furthermore, IFN plasmids have shown considerable protective function when injected as an adjuvant together with DNA vaccines (Robertsen, 2018).

1.3 Immunology in teleosts

The immune system consists of several mechanisms designed to avoid and eliminate pathogens (Abbas et al., 2016). To eliminate pathogens, the immune system needs to be able to identify and expel them. All living organism have an innate immune system, while an adaptive immune system is only seen in vertebrates. Adaptive immune functions make their first appearance in jawed vertebrates, making fish the first animal phyla to possess both innate and adaptive immune systems (Magnadottir, 2010). The teleosts possess lymphocyte populations that are analogous to T cells and B cells, non-specific cytotoxic cells, macrophages and polymorphonuclear cells (Uribe et al., 2011). Fish, along with elasmobranchs are the most primitive groups to possess the Major Histocompability Complex (MHC), T cell receptors (TCR), immunoglobulins (Ig) and B cell receptors (BCR), all hallmarks of the adaptive immune system. Moreover, these animals are able to utilize recombination activating gene (RAG)-mediated recombination to diversify immune receptors (Eason et al., 2004).

1.3.1 Immunological cells and organs

The most important immunological organs in teleosts are the kidney (anterior and posterior), thymus, spleen and mucosa-associated lymphoid tissues (MALT) (Press & Evensen, 1999). Furthermore, the MALT can be divided into four distinct compartments, namely the gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT), gill associated lymphoid tissue (GIALT) and nasopharynx-associated lymphoid tissue (NALT) (Salinas, 2015). Finally, a mucosa-associated lymphoid structure has recently been identified within the GIALT of Atlantic salmon, and termed the interbranchial lymphoid tissue (ILT) (Haugarvoll et al., 2008). This tissue largely consists of a T cell-embedded meshwork of epithelial cells that has not been observed in previously described lymphoid tissues. In contrast to the mammalian immune system, fish do not possess lymph nodes nor bone marrow; organs that are otherwise considered to have important immunological functions. Teleost fish have assigned the functions of the bone marrow and lymph nodes to other organs. Instead of bone marrow, the head kidney and the thymus serve as primary lymphoid organs (Press & Evensen, 1999). In these organs, lymphocytes develop and mature. The thymus is responsible for the production of T cells, while the kidney seems to act as a bone marrow-analogue, and is the largest site of hematopoiesis in

teleost fish (Uribe et al., 2011). B cells are produced and developed in the anterior parts of the kidney, also referred to as the head kidney (Zwollo et al., 2005). In addition to having a primary lymphoid function, research also suggests that the head kidney takes part in humoral immune activation, making it a secondary lymphoid organ as well as a primary one (Press & Evensen, 1999). Mid- and posterior parts of the head kidney houses B-cell populations, although there is uncertainty regarding their developmental and functional roles, and consequently, whether these areas of the kidney act as primary- or secondary immune organs (Zwollo et al., 2005). The spleen, along with the kidney, is considered to be the most important secondary lymphoid organ. The walls of the spleen are actively involved phagocytosis of antigens by macrophages. Antigens can be stored in this organ for a long time, giving it importance in immunological memory (Uribe et al., 2011). Melanomacrophage centers (MMCs) can be found in head kidney and spleen among other tissues. The MMCs undergo chronic inflammation, and are suggested to be important in B cell development and affinity maturation (Agius & Roberts, 2003).

1.3.2 Innate immunology

The innate immune system represents the first line of defense in any organism, and consists of a broad range of defense mechanisms, targeting an even broader range of pathogens. The innate immune system can be divided into humoral and cellular compartments, as well as mucosal/epithelial barriers. Unique for teleosts is the presence of humoral and cellular components of the immune system within the mucosal/epithelial barrier (Salinas, 2015). The humoral compartment consists of cytokines, antimicrobial peptides, the complement system and acute phase reactants among other factors (Beutler, 2004). The cellular compartment consists of phagocytic cells including neutrophils, eosinophils, monocytes and macrophages (Morel et al., 1991), nonspecific cytotoxic cells/natural killer-like cells (Evans et al., 2001), and dendritic cells (Lugo-Villarino et al., 2010). For the cellular compartment to perform its functions effectively, it needs to be able to recognize foreign molecules. This is done through a variety of germ-line encoded pattern recognition receptors (PRRs), whose function is to recognize and react to structurally conserved molecular patterns on microbes that are essential for their physiology (Medzhitov & Janeway, 2002). These structures are known as pathogen associated molecular patterns (PAMPs). Important in teleost fish immunology, and belonging to the PRRs, are toll-like receptors, considered to be the principle inducers of the innate immune system (Whyte, 2007). Typical PAMPS are microbial RNA and DNA, as either of these products are found in all microbes. These products are known to engage TLRs, and induce innate and adaptive immune responses in fish (Palti, 2011). In immunological studies, synthetic

microbial DNA and RNA can be applied to mimic infection, and thus induce immune responses in *in-vitro* assays. These types of studies have been successfully employed in salmon, using the synthetic products CpG and Poly I:C, mimicking microbial DNA and RNA, respectively (Jensen et al., 2002; Jorgensen et al., 2001; Jorgensen et al., 2003; Strandskog et al., 2008). Upon TLR-engagement, leukocytes respond by secreting cytokines, serving to enforce existing innate responses, and induce adaptive responses (Akira & Takeda, 2004; Arnemo et al., 2014). In mammals, BAFF and APRIL, which are cytokines themselves, are known to be produced and expressed/released by myeloid cells in response to other cytokines (Nardelli et al., 2001). The cytokines BAFF and APRIL are described in greater detail under heading 1.3.2.

1.3.3 Adaptive immunology

The adaptive immune system consists of a complex network of specialized cells, proteins, genes and biochemical messengers that provide the means necessary to respond specifically to antigens, antibodies and effector cells with high specificity and affinity (Uribe et al., 2011). The reaction takes longer to activate, but has a prolonged and more specific effect when compared to innate responses. The development of adaptive immunity requires several factors to ensure specific responses, where development of the thymus, B- and T-cells, RAG enzymes, and MHCs are considered the most important (Magnadottir, 2010). In the same manner as the innate immune system, the adaptive immune system can be divided in cellular and humoral components, although making clear distinctions of their functions is meaningless as they are largely connected.

The most important molecule in the humoral compartment are the immunoglobulins (Ig; antibodies), existing either as membrane bound BCRs or as secreted antibodies in plasma. Secreted antibodies function by binding specifically to foreign antigens and either neutralizing them, or activating other eliminating effector cells with antibody receptors, such as phagocytic or cytotoxic cells. Pathogen-bound antibodies are also crucial in the activation of the classical pathway of the complement system, which has also been described in fish (Boshra et al., 2006). In contrast to mammals, who possess 5 Ig classes, and several sub classes, only three Ig-classes have been identified in teleosts, namely IgM, IgD and IgZ/IgT (Hansen et al., 2005; Hikima et al., 2011; Wilson et al., 1997). The most abundant Ig in systemic circulation of fish is a tetrameric form of the IgM class, containing eight antigenic combining sites (Uribe et al., 2011). This Ig is produced mainly by plasma cells and plasmablasts located in the head kidney (Bromage et al., 2004; Zwollo et al., 2005). The B cells' ability to enhance the IgM through

affinity maturation is poor (Mutoloki et al., 2014), but the antibody has nevertheless been linked to the protection against viral diseases in salmon, such as IPN (Munang'andu et al., 2013) and PD (Grove et al., 2013; Johansen et al., 2015; C. Xu et al., 2012). IgD has been discovered in rainbow trout (Ramirez-Gomez et al., 2012), and several other species. In channel catfish (*Ictalurus punctatus*), the IgD has been suggested to be a mediator of innate immunity (Edholm et al., 2010). In teleosts, IgT is mainly found in mucosal surfaces, including the skin, the gills and the gut (Z. Xu, Gomez, et al., 2013; Z. Xu, Parra, et al., 2013; Zhang et al., 2011) and the function of the immunoglobulin lies mainly within the MALT (Ye et al., 2013). In salmon, IgM+ cells are the dominating B cell population in systemic organs. The distribution of the IgM+ and IgT+ cells in systemic organs were recently reported and is presented in Figure 2 (Jenberie et al., 2018).

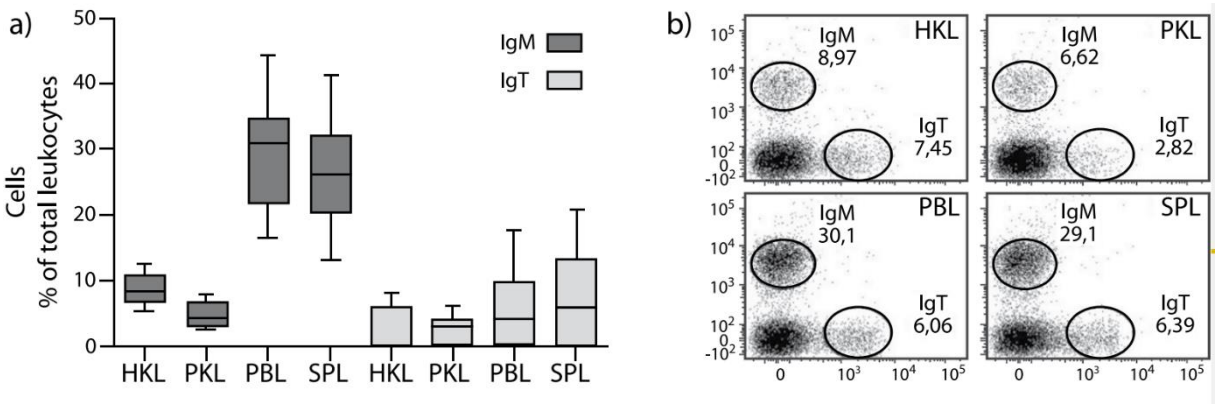


Figure 2: Distribution of IgM+ and IgT+ cells in systemic organs of Atlantic salmon. IgM+ cells are most prevalent in systemic organs. Ig expression in peripheral blood leukocytes (PBL), spleen leukocytes (SPL), posterior kidney leukocytes (PKL) and head kidney leukocytes (HKL) analyzed by flow cytometry using trout anti-IgM and IgT mAbs-stained leukocytes. Median frequencies of IgM+ and IgT+ cells of total leukocytes (a) and representative flow-cytometry dot-plots (b) (Jenberie et al., 2018).

The cellular compartment of the adaptive immune system consists of B- and T-lymphocytes, responsible for specific pathogen recognition and initiation of the adaptive immune response. These cells are able to obtain high pathogen specificity through somatic rearrangement of gene segments (Fillatreau et al., 2013). B cells are activated through engagement of the B cell receptor (BCR) and related co-receptors. When activated, the cells either proliferate or differentiate to different subsets. Rainbow trout has been demonstrated to express a variety of the of the antibody secreting cells (ASC) found in mammals; including plasmablasts, short-

lived plasma cells and long-lived plasma cells as defined by cellular (Bromage et al., 2004; Zwollo et al., 2005) and molecular characterization (Zwollo et al., 2008; Zwollo et al., 2010).

1.3.4 Antibody investigations

No doubt, the B cell and its effector molecule, the immunoglobulin, play a key role in teleost immunity. The molecule has been the subject of extensive research in all vertebrates. Several assays have been developed to better understand the underlying mechanisms for the generation of antibody responses, making it possible to observe and quantify the responsible cells. The first method developed was the plaque assay, serving to detect ASCs in response to immunization (Jerne & Nordin, 1963), which was later adapted for use in fish (Anderson et al., 1979; Georgopoulou & Vernier, 1986; Miller & Clem, 1984; Rijkers et al., 1980). Eventually the solid-phase enzyme-linked immunospot (ELISPOT) assay was developed (Sedgwick & Holt, 1983); the assay captures antibodies as they are secreted by the B cell on an antigen-coated membrane on which the cells rest. The assay was later incorporated into B cell research in salmonids (Anderson et al., 1979; Siwicki & Dunier, 1993). In this work, the ELISPOT assay was applied to quantify the amount of IgM-secreting cells in salmon-derived leukocytes upon *in-vitro* stimulation with TLR-ligands and recombinant BAFF and APRIL.

1.4 The tumor necrosis factor super family

The TNF super family is comprised of an ever-growing group of ligands and receptors, soluble and membrane bound, characterized by homologous cysteine-rich domains (CRDs). The molecules are directly related to signaling pathways for cell proliferation, survival and differentiation. The TNF/TNFreceptor superfamily proteins (TNF/TNFR) play a crucial role in the immune system where they coordinate the proliferation and protective functions of pathogen-reactive cells (Locksley et al., 2001). The ligands are type II membrane proteins with an intracellular N-terminus, and an extracellular C-terminus. Most TNF molecules are membrane bound, but many of the ligands contain proteolytic cleavage sites, used to generate soluble forms (Locksley et al., 2001). The cytoplasmic domain of TNFRs function as docking sites for signaling molecules. Signaling occurs through two principal classes of cytoplasmic adaptor proteins: TRAFs (TNF receptor-associated factors) and “death domain” (DD) (Fesik, 2000; Inoue et al., 2000). TRAF6, a TLR-associated adaptor molecule, has been identified in zebrafish (*Danio rerio*) (Phelan et al., 2005), where it serves to activate the NF-κB complex, a transcriptional factor regulating a wide range of genes, and playing a central part in

immunological processes (Baldwin, 1996). Furthermore, TRAF1 encoding sequences has been identified in grass carp (*Ctenopharyngodon idella)* (Z. Y. Xu et al., 2008), where the molecule is postulated to possess negative regulatory functions on TNF signaling (Zhu et al., 2013). The death domain has been identified in several teleost fish (Wiens & Glenney, 2011).

The immune system of teleosts share many similarities with the mammalian immune system, but it also possesses important differences, as outlined earlier. Given the important role of the TNFSF in mammals, the role of teleost TNFSF orthologs and paralogs are of interest. At least 71 teleost genes encoding proteins containing TNF homology domains has been identified as of 2007 (Glenney & Wiens, 2007). Among these are BAFF, APRIL and, unique to fish, a BAFF and APRIL-like molecule (BALM). A phylogenetic tree of mammalian, avian, amphibian and teleost TNF ligand members is presented in Figure 2.

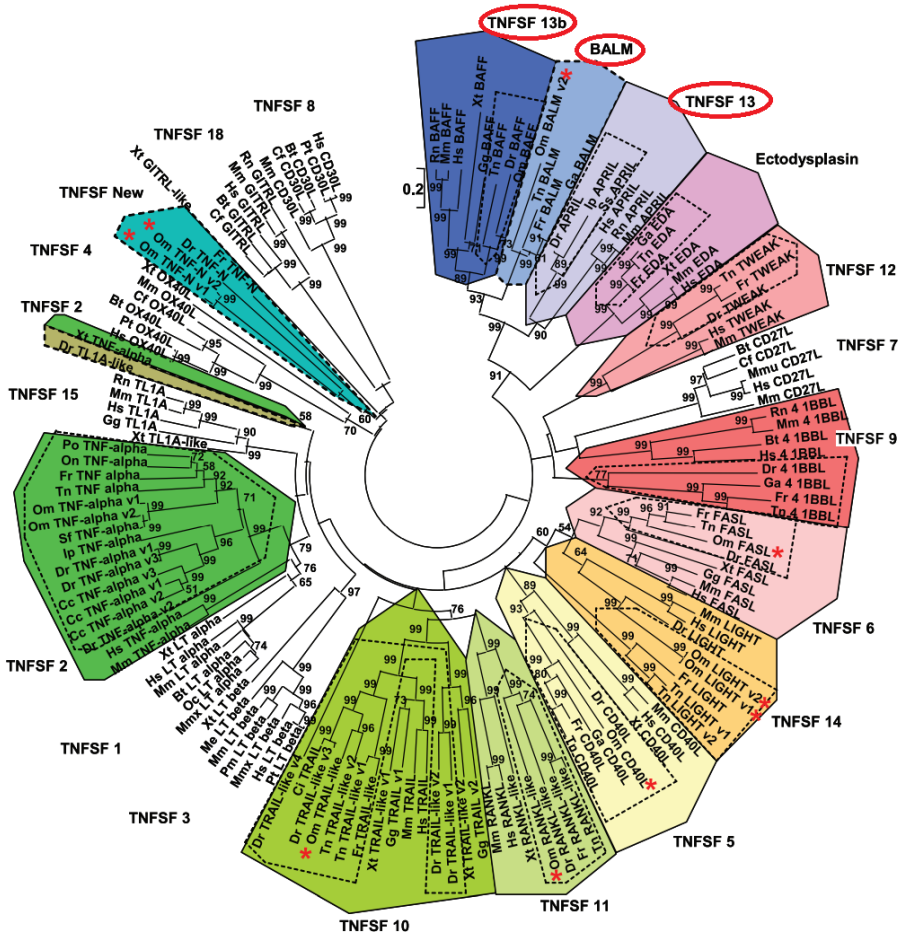


Figure 3: An unrooted phylogenetic tree of teleost, amphibian, avian and mammalian TNF ligand members. Colored boxes represent TNF super family ligands that have fish orthologues and dotted boxes represent fish-specific sequences. BAFF (TNFSF 13b), BALM and APRIL (TNFSF 13) are marked in red (Glenney & Wiens, 2007).

1.4.1 BAFF & APRIL

BAFF (also known as BLyS, TALL-1, CD257 and TNFSF13B) and APRIL (also known as CD256 and TNFSF13) are ligands belonging to the TNFSF. The two cytokines are important ligands in lymphocyte activation and survival (Hahne et al., 1998; Moore et al., 1999).

BAFF and APRIL are type II transmembrane proteins that can be released in soluble forms by proteolytic processing at a furin protease site (Bossen & Schneider, 2006). Active forms of BAFF can exist both as membrane bound- and soluble proteins (Nardelli et al., 2001). APRIL can only perform its function in soluble form, and is cleaved intracellularly in the Golgi site prior to release (Lopez-Fraga et al., 2001)

1.4.2 Mammalian BAFF & APRIL

In mammals, BAFF and APRIL are produced mainly by innate immune cells to co-stimulate B cells, however, they also have T cell co-stimulatory functions (Huard et al., 2004). BAFF expression is mainly seen in peripheral blood, spleen and lymph nodes, and to a lesser extent in other organs (Moore et al., 1999; Mukhopadhyay et al., 1999; Schneider et al., 1999; Shu et al., 1999). BAFF is predominantly produced in myeloid cells including monocytes, macrophages, dendritic cells, but is also found in T-cells (Moore et al., 1999; Schneider et al., 1999; Shu et al., 1999). APRIL is similarly expressed in monocytes, macrophages, dendritic cells and T cells (Hahne et al., 1998). APRIL transcripts are especially found in large quantities in activated T cells and monocytes (Pradet-Balade et al., 2002). The expression of BAFF and APRIL is increased in the presence type I interferons, (IFNs), IFNγ, IL-10 and granulocyte colony-stimulating factor, as well as by the activation of Toll-like receptors (TLRs) such as TLR4 or TLR9 (Mackay & Schneider, 2009). Microbial TLR-agonists and their synthetic analogs typically induce the expression of co-stimulatory molecules and the secretion of cytokines, including BAFF and APRIL, by innate immune cells (Katsenelson et al., 2007). TLR receptors provide a link between the innate and the adaptive immune system and can upon activation lead to amplified immune responses against microbial pathogens by activating B and T lymphocytes (Iwasaki & Medzhitov, 2004). Activation of TLR receptors has been shown to augment BAFF- and APRIL-mediated immunoglobulin secretion, by augmenting receptor expression in ASCs (Katsenelson et al., 2007). Additionally, TLR activation have been shown to induce production of BAFF in dendritic cells (Chung et al., 2012).

BAFF and APRILs corresponding receptors are BCMA (B Cell Maturation Antigen also known as CD269, TNFRSF13C), TACI (Transmembrane Activator and Calcium modulator and

cyclophillin ligand Interactor also known as CD267, TNFRSF13B) and BAFF-R (B cell activating factor receptor, also known as BR3, CD268 or TNFRSF17). These are type III transmembrane proteins (Bossen & Schneider, 2006), which makes them unusual, as TNF receptor proteins are generally type I transmembrane proteins (Bodmer et al., 2002). The receptors are characterized by an extracellular N-terminus, a single transmembrane domain, absence of a signal peptide and a COOH terminal region responsible for intracellular signaling. Common for all receptors are extracellular cysteine rich domains (CRDs), often formed as β-hairpin structures that are structurally conserved amongst the receptors (Magis et al., 2012). This structure binds BAFF and APRIL, and is the core of the interaction between receptor and ligand. (Hymowitz et al., 2005; Y. F. Liu et al., 2003). The receptors are expressed on the surface of B-cells (Novak et al., 2004).

BAFF is able to bind to BAFF-R, and with lower affinity, TACI and BCMA, while APRIL is only capable of binding to BCMA and TACI. BAFF binds with greatest affinity to BAFF-R, while APRIL binds with greatest affinity to BCMA, TACI has an equal affinity to both ligands (Bossen & Schneider, 2006). Affinity properties of BAFF, APRIL, BAFF-R, BCMA and TACI are presented in table 1. Interestingly, APRIL is also able to interact with the polysaccharide side chains of heparan sulfate proteoglycans, structurally unrelated to TNFRs (Ingold et al., 2005). The β-hairpin structures in the receptors are followed by a helix-loop-helix structure that is highly variable between the different receptors. This area of the receptor is determinative to the affinity characteristics of the receptor (Bossen & Schneider, 2006). An overview of the ligands and their receptors can be seen in Figure 4.

Table 1: Affinity characteristics of BAFF, APRIL, BAFF-R, BCMA and TACI in mammals. BAFF binds with greatest affinity to BAFF-R and is also able to bind BCMA and TACI. APRIL is unable to bind BAFF-R, but is able to bind TACI, and with higher affinity, BCMA. (+++), (++), (+) and (-) annotate strong, intermediate, weak and no affinity, respectively.

	BAFF-R	BCMA	TACI
BAFF	(+++)	(+)	(++)
APRIL	(-)	(+++)	(++)

The BAFF-R is important for B cell maturation and survival (Sasaki et al., 2004; Shulga-Morskaya et al., 2004). At the immature T1 stage, B cells enter the spleen and differentiate through the T2 stage to either mature B cells or marginal zone B cells. During this process, the B cells are dependent on successful expression of the BCR. The crucial role of the BCR is underscored by the fact that B cell development is arrested at the immature stage in mice carrying a mutation in the cytoplasmic domain of the BCR (Reichlin et al., 2001). In addition to signaling from the BCR, the B cells also rely on survival signals provided by BAFF during differentiation. Failure to receive the BAFF survival signals results in a 90% loss of mature B cells in mice, originating from reduced survival in all B cells beyond the immature T1 stage (Schiemann et al., 2001). During differentiation, BAFF binds to BAFF-R to provide the necessary survival signals. This is shown through loss of mature B cells in BAFF-R mutant mice (Thompson et al., 2001), and further confirmed through deletion of BAFF-R (Sasaki et al., 2004; Shulga-Morskaya et al., 2004). TACI serves as a negative regulator in this stage of B cell maturation, demonstrated by a fold increase in amount of mature B cells in TACI $^{-/-}$ mice (Yan et al., 2001). BCMA is not thought to play a part at this stage of B cell differentiation, as BCMA$^{-/-}$ mice do not show any alterations to B cell properties (Schiemann et al., 2001). At later stages of B cell differentiation, BCMA is thought to have important roles, and is shown to be crucial for the survival of long-lived bone-marrow plasma cells (O'Connor et al., 2004) and plasmablasts (Avery et al., 2004). As discussed earlier, APRIL's high affinity for BCMA suggest an importance of the BCMA-APRIL axis in B cells at later developmental stages.

All BAFF and APRIL receptors have implicated functions on the NF-κB protein complex, known to regulate DNA transcription, cytokine production and cell survival. BAFF-Rs importance in NF-κB activation has been demonstrated *in-vivo* in mice, although the exact mechanics are not yet understood (Claudio et al., 2002). BCMA similarly is a potent stimulator

of the NF-κB signaling pathway by engaging TNF receptor-associated factor (TRAF) 1, TRAF2, and TRAF3 (Hatzoglou et al., 2000). TACI seem to have an opposite effect than that of BAFF-R and BCMA, where agonistic binding triggers apoptotic pathways, although the pathway still has a connection to the NF-κB signaling pathway (Seshasayee et al., 2003).

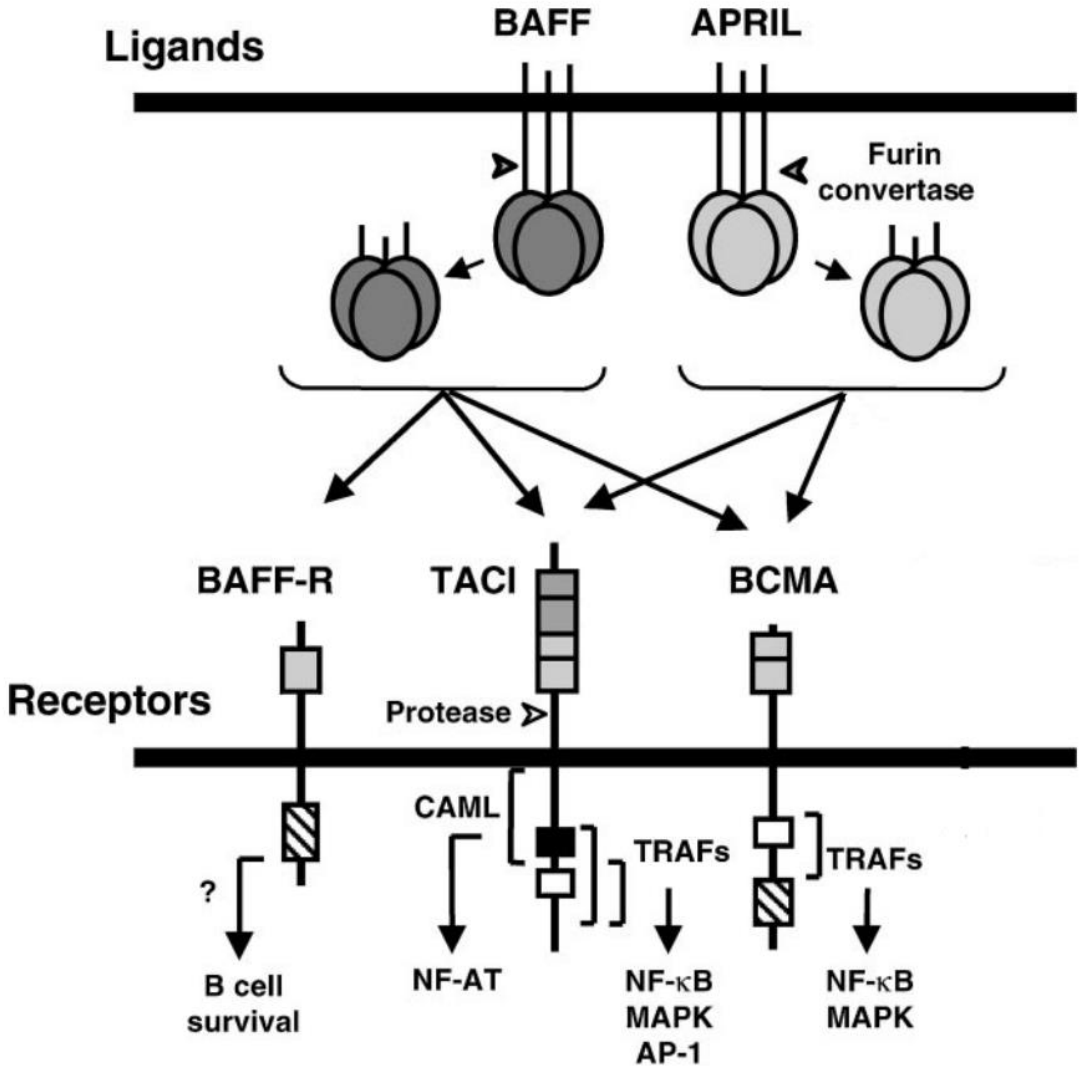


Figure 4: Affinity- and signaling properties of BAFF, APRIL, BAFF-R, TACI and BCMA. BAFF and APRIL are cleaved by furin convertase, and released in soluble form. BAFF is able to engage all receptors, while APRIL can only engage TACI and BCMA. Engagement of the different receptors trigger different signaling pathways within the target cell. Each box in the extra cellular domain represent a distinct module (Mackay et al., 2003).

1.4.3 Teleost BAFF & APRIL

Recently genes encoding BAFF, APRIL and their receptors have been reported in in different teleosts, and the proteins have proved to have similar effects as in mammals (Granja et al., 2017). BAFF sequences have been reported in zebra fish *(Liang et al., 2010)*, mefugu (*Takifugu obscurus*) (Ai et al., 2011), Japanese sea perch (*Lateolabrax japonicus*) (Cui et al., 2012), Grass carp (Pandit et al., 2013), Yellow grouper (*Epinephelus awoara*) (Xiao et al., 2014), miiuy croaker (*Miichtys miiuy*) (Meng et al., 2015), Tongue sole (*Cynoglossus semilaevis*) (Sun & Sun, 2015), Nile tilapia (*Oreochromis niloticus*) (H. Z. Liu et al., 2016), rock bream (*Oplegnathus fasciatus*) (Godahewa et al., 2016), rainbow trout (*Oncorhynchus mykiss*) (Glenney & Wiens, 2007), rohu (*Labeo rohita*) (Basu et al., 2016), as well as several cartilaginous fish (Li et al., 2012; Li et al., 2015; Ren et al., 2011). APRIL sequences have, however, only been reported in channel catfish, zebrafish (Min et al., 2012), salmon and rainbow trout (Glenney & Wiens, 2007). Additionally, a third ligand designated BALM, unique for teleosts and elasmobranchs, has been identified (Glenney & Wiens, 2007). This ligand is postulated to be an ancestral homologue to BAFF and APRIL (Das et al., 2016), and possess lymphoproliferative effects, not shared by its descendants (Granja et al., 2017).

BAFF-R-, BCMA- and TACI homologues have, to our knowledge, only been characterized in rainbow trout, although homologous sequences have been reported in several species, including salmon (Granja et al., 2017). A phylogenetic analysis of rainbow trout TNF receptors is presented in Figure 5. TNF receptors usually possess multiple CRDs (Magis et al., 2012), while in rainbow trout, BCMA only has one, TACI two and only a partial one has been reported in BAFF-R (Granja et al., 2017). The differences in rainbow trout CRDs suggest that affinity characteristics of the receptors in fish may be different compared to mammals. Granja, Holland *et.al* suggested that APRIL might be able to engage BAFF-R, and that BAFF might be able to potently engage BCMA in rainbow trout. In the same work, Granja, Holland *et al.* also postulated an altered intracellular signaling pathway upon TACI activation in fish, on account of a missing TRAF binding consensus in the COOH terminal region of the receptor. BAFF has been shown to have variable effects on different B cell subsets; the mechanisms responsible for this are related to the receptors displayed on the surface of the B cells. BAFF is able to engage all receptors, while engagement of the different receptors trigger different signaling pathways within the cell. The composition of the receptors expressed on the membrane of the B cells is in this way determinative of the cells reaction to the stimulant (Granja & Tafalla, 2017), and

gives different B cell subsets the ability to react differently upon BAFF and APRIL exposure. It should be noted that BCMA in mice is APRIL-specific (Dillon et al., 2006)

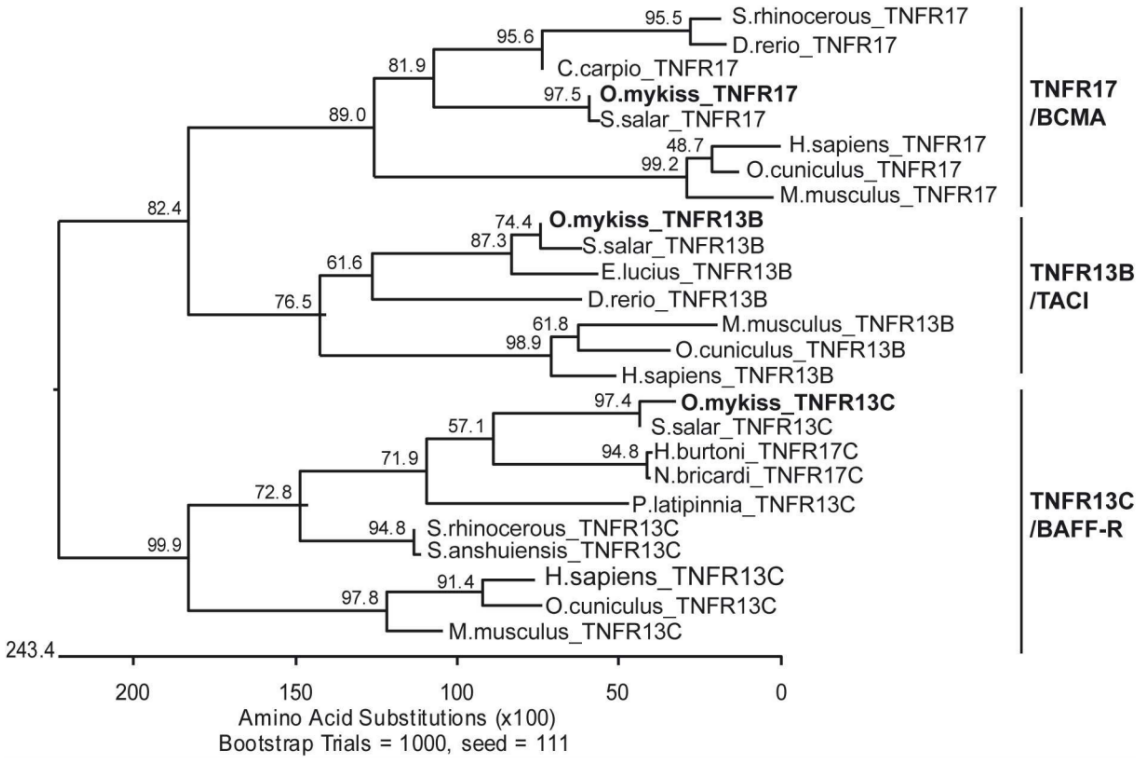


Figure 5: Phylogenetic analysis of rainbow trout BAFF-R, BCMA and TACI. Phylogenetic tree created by Granja et al. (2017) with emphasis on rainbow trout receptors.

In teleosts, the spleen and kidney are the most important immune organs, and are considered the primary source of B cells (Salinas et al., 2011). Basal expression of BAFF in bony fish has been shown to be highest in head kidney and spleen (Basu et al., 2016; Tafalla et al., 2017), while their receptors in rainbow trout show the highest basal expression in spleen, peripheral blood leukocytes and kidney (Granja et al., 2017). The basal distribution of this cytokine implicates important functions of BAFF in the normal function of B cells in fish. Similarly to mammals, BAFF-gene expression is induced in response to TLR-activation in teleosts. Poly I:C has been shown to elicit significant upregulation of BAFF transcripts in both *in-vivo*- and *in-vitro* stimulated fish (Basu et al., 2016). In this thesis, salmon derived leukocytes were stimulated with several TLR-ligands to investigate this interaction further. Functional studies using recombinant BAFF has been carried out in several bony fish (Basu et al., 2016; Cui et al., 2012; Godahewa et al., 2016; Liang et al., 2010; H. Z. Liu et al., 2016; Sun & Sun, 2015; Xiao

et al., 2014), as well as a study using both recombinant BAFF and APRIL in rainbow trout (Granja et al., 2017).

As in mammals (Mackay et al., 2003), salmonid BAFF promotes survival, but not proliferation of IgM+ B cells (Granja et al., 2016). In mammals, treatment with BAFF in absence of pathogens results in increased levels production of IgM and IgD (Do & Chen-Kiang, 2002) as a result of augmented survival of plasma cells (Avery et al., 2004). The same mechanism is observed in rainbow trout spleen IgM+ B cells treated with rBAFF (Tafalla et al., 2017). Additionally, another paper investigating the role of BAFF, APRIL and BALM on rainbow trout peritoneal resident IgM+ B cell survival found that rBAFF did not promote survival in these cells, however, rAPRIL and rBALM had significant effects on the survival of the leukocytes (Soleto et al., 2017); thus, splenic and peritoneal IgM+ B cells react differently upon engagement to BAFF, further underscoring the fact that different B cell subsets react differently to the cytokine. Upon introduction of viral hemorrhagic septicemia virus (VHSV) to the peritoneum of rainbow trout, upregulation of BAFF-, but not APRIL and BALM, transcripts in peritoneal leukocytes was observed (Soleto et al., 2017). Furthermore, i.p injection of VHSV triggered upregulation of BAFF-R, but not BCMA and TACI mRNA levels, in peritoneal leukocytes (Soleto et al., 2017). The BAFF homologue (CsBAFF) found in tongue sole was found to be most abundant in immune organs such as spleen and head kidney. Upon *in vivo* bacterial challenge CsBAFF expression was shown to be upregulated. Furthermore, elevated levels of CsBAFF was shown to enhance macrophage activation and reduce bacterial infection in tissues. CsBAFF plasmids used as adjuvants in DNA vaccines have shown considerable protective functions, implicating BAFF as a prospect in future therapeutic treatments of fish disease (Sun & Sun, 2015).

Indeed, BAFF, APRIL and their receptors play important roles in the immune system under normal physiological conditions, as well as in response to invading pathogens. The mechanisms of the cytokines, especially APRIL, are poorly investigated in teleost fish. In this work, we set out to investigate the role of BAFF, APRIL and their receptors in salmon, both *in-vivo* and *in-vitro.*

1.5 Objectives

The main objective of this work is to investigate the gene expression of BAFF and APRIL in Atlantic salmon. The distribution of the cytokines and related receptors are to be investigated *in-vitro*, both constitutively and upon stimulation and *in-vivo* under normal physiological conditions and upon infection, gene transcripts are to be measured by qPCR methods. Finally, leukocytes will be stimulated with recombinant BAFF and APRIL to determine their effect on antibody secretion in ELISPOT assays.

Sub goals:

- Measure the basal expression of Atlantic salmon BAFF and APRIL in different organs (head kidney, spleen, gills heart, liver and pancreas).

- Measure the expression of BAFF and APRIL in in different organs (head kidney, spleen, gills heart, liver and pancreas) from salmon alphavirus challenged fish relative to uninfected controls. Samplings are performed at 3, 8, and 15 days post infection. The load of SAV RNA in the heart will be estimated using SAV specific primers.

- Measure the basal expression of Atlantic salmon BAFF and APRIL (and corresponding receptors) in adherent head kidney leucocytes and peripheral blood- head kidney- and spleen-derived B cells.

- Measure the expression of salmon BAFF and APRIL in stimulated adherent head kidney leukocytes. Cells are to be stimulated with CpG, poly I:C, LPS, IFNγ and IFNα To verify that the stimulations have worked, the expression of different immune genes known to respond to the different stimulants will be measured as well.

- Investigate the effects recombinant BAFF and APRIL have for the secretion of salmon IgM. HK spleen and peripheral blood leucocytes will be treated with these recombinant proteins, alone, or I combination with TLR ligands and the number of cells secreting IgM will be quantified by an ELISPOT assay. This assay is established in the lab.

2. Materials and methods

2.1 Experimental fish and organ extraction

2.1.1 Experimental fish

All fish used were healthy naïve Atlantic salmon (*Salmo salar L.)* QTL fish from Aquagen kept at Havbruksstasjonen i Tromsø, Kårvika. All fish were fed Nutura Olympic (Skretting) in accordance to feeding regimes from manufacturer.

In vivo experiments were carried out using fish kept at freshwater throughout their lives. Seven weeks prior to the experiments the mean weight of the fish was 33 gram, at this point the temperature was brought to 10 °C to achieve the desired weight for the experiment. The fish were kept in continuous dark before initial feeding, and continuous light after initial feeding. The fish were fed with a feed factor of 0,75 from initial feeding to 3-5 gram and a feed factor of 1 after initial feeding. At sacrifice, the mean weight of the fish was 61.4 gram.

Fish used for quantitative analyses of gene expression in cells were hatched and smoltified at Tromsø aquaculture station and kept at Norges fiskerihøgskole (NFH). The fish were kept at natural temperatures (minimum 4 °C) under 12 hours light/day. At sacrifice, the mean weight of the fish was ~500 gram.

Fish used for ELISPOT assays were kept in Kårvika throughout their lives. They were smoltified and put on sea water (10 °C) roughly 3 months before the first sampling. The fish were kept on a natural light regime (December-February; Tromsø, Norway). At sacrifice, the mean weight of the fish was ~250 gram.

2.1.2 Organ extraction

Before extraction of organs, fish were killed by over-exposure to benzocaine before being given a blow to the cranium to ensure that death had occurred. To make the extraction process cleaner and to exclude peripheral blood leukocytes, blood was drained through *Vena caudalis* using a syringe. Organs were aseptically extracted using a scalpel and tweezers. Equipment was dipped in ethanol and burned between extractions to ensure sterile working conditions. Tissues to be used in qPCR were cut into appropriately sized pieces and put on RNA later (Life Technologies), while tissues for cell isolation were suspended in transport medium (described

in section 2.3) and placed on ice while brought to the laboratory at NFH. An overview of materials and corresponding treatment methods and investigations is presented in table 2 and 3.

Table 2 Summary of *in-vivo* experiment.

Experiment	Number of fish	Organs	Days post infection	Investigation
Constitutive expression of BAFF and APRIL in organs	6	HK, liver, heart, spleen, gills and pancreas	0	**RT-qPCR**
Distribution of BAFF and APRIL in SAV-infected fish	12 per time point (8 infected, 4 control)	HK, liver, heart, spleen, gills and pancreas	3, 8 and 14	**RT-qPCR**

Table 3 Summary of *in-vitro* experiments.

Experiment	Number of fish	Cells derived from	Stimulation	Incubation	Investigation
Constitutive expression of BAFF-R, BCMA and TACI in B cells (provided material)	5	HK, PB, spleen	n/a	n/a	**RT-qPCR**
Constitutive expression of BAFF, APRIL, BAFF-R, BCMA and TACI in HK MLCs	3	HK	n/a	n/a	**RT-qPCR**
Induction of BAFF- and APRIL expression in stimulated HK MLCs	3	HK	Control, Non-CpG, CpG, Poly I:C, INFg, IFNa	6-, 24- and 48 hours	**RT-qPCR (**EF1aB, TNF, Mx, BAFF, APRIL**)**
Antibody secretion in LPS-stimulated IgM+ cells (pilot 1)	3	HK	Control, LPS	3 days	**ELISPOT**
Antibody secretion in LPS-stimulated IgM+ cells (pilot 2)	3	HK, spleen	Control, LPS	3 and 5 days	**ELISPOT**
Antibody secretion in BAFF- and APRIL stimulated IgM+ cells	4	HK	Control, LPS, CpG, BAFF, APRIL	3 days	**ELISPOT**

2.2 In-vivo SAV3 experiment

2.2.1 Virus

Salmonid alphavirus subtype 3 (SAV3) (PDV-H10-PA3) was provided by professor Øystein Evensen (Faculty of Veterinary Medicine, Norwegian University of Life Sciences). The virus was originally isolated from the heart of Atlantic salmon with clinical outbreak of PD and was identified as SAV3 by sequencing (Cheng Xu et al., 2012; C. Xu et al., 2010). The virus was replicated in CHH-1 cells from heart tissue in Chum salmon (*Oncorhyncus keta*) in L-15 medium w/ 100 U/ml penicillin, 10 µl/ml streptomycin and 5% FBS at 15 °C. Virus titer was determined using $TCID_{50}$, the method was done using a monoclonal mouse anti-SAV3 antibody (Moriette et al., 2005) provided by Dr. M. Bremont to quantify SAV-positive cells (Thim et al., 2012). Virus was cultivated and quantified by Guro Strandskog.

2.2.2 Virus challenge

Before the start of the experiment, the fish were distributed randomly in 2 tanks. Forty eight fish were injected i.p. with 100 µl 10^5 $TCID_{50}$ SAV3, while 58 fish were injected with 100 µl PBS as mock-infected control group. After injection, the fish were kept at 10 °C on continuous light. Fish were starved for a minimum of 1 day prior to sampling or relocation. The experiment was concluded 2 weeks after SAV was administered. The experiment was approved by the Norwegian Animal Research Authority (ID 11258).

Tissues (Table 2) were aseptically collected from 4 mock-infected and 8 SAV3-infected fish at 3, 8, and 14 days post-SAV3 infection. Additionally, tissues from 6 fish were harvested before the challenge for constitutive expression analyses. Tissues were subsequently used for gene expression profiling, described in further details in section 2.4

2.3 Samples for *in-vitro* studies in Atlantic salmon

In-vitro studies were conducted using adherent cells and B cells, an overview of materials and corresponding investigations is presented in table 2.

2.3.1 Leukocyte isolation

Leucocytes from head kidney and spleen were isolated on Percoll gradients (GE Healthcare) as described in earlier works (Jorgensen et al., 2001). The isolation was done by forcing a single cell suspension through a discontinuous Percoll gradient by centrifugation. The Percoll gradient

used had a density of 25% and 54 % Percoll. Percoll consists of colloid silica-particles that form the density gradient described. When centrifuged, the leucocytes will sediment in the interface between the 25% and 54% Percoll solutions.

The starting solution of Percoll is prepared by adding 10 ml 1.5M NaCl to 90 ml Percoll, before adding 400 µl heparin. Twentyfive % and 54% Percoll solutions were then prepared from the starting solution in accordance with the description presented in table 3. Transport medium was made with 98 ml L-15 medium added penicillin (60 µg ml^{-1}), streptomycin (100 µg ml^{-1}) (L-15+) (Gibco®, Life Technologies), 2 ml FBS and 400 µl heparin (Leo pharma). The gradients were layered in a 50 ml centrifuge tube. The 54 % gradient was added first and the 25 % gradient was then carefully pipetted on top, making sure to not mix the gradients.

Table 4: Preparation of 90 %-, 54 %- and 25 %-Percoll gradients

Starting solution (90% percoll)	90 ml percoll 10 ml 9% NaCl 0.4 ml heparin
54% percoll	59 ml starting solution 41 ml L-15 w/heparin, antibiotics and FBS
25% percoll	28 ml starting solution 72 ml PBS 288 µl heparin

Head kidney and spleen were aseptically sampled and kept on ice-cold transport medium during transportation. Organs were homogenized by crushing in a 100 µm cell strainer (Falcon), using the plunger of a 2 mL syringe (Falcon). Transport medium was cautiously poured through the strainer, into a centrifuge tube, while crushing the organ. This leaves debris and connective tissue in the strainer, while cells pass through the strainer along with the transport medium. The solution was topped off with additional L-15+ adjusting the final volume to 30 ml. The cell suspension was carefully pipetted on top of the gradients, making sure that it does not mix with the Percoll gradients. The gradients were centrifuged at 400x g (acceleration- and deceleration speed set to 4) in Multifuge 1S-R (Heraeus) for 40 minutes at 4 °C. During centrifugation, the cells will accumulate in the interface between the 25 % and 54 % Percoll solutions, the gradient

is demonstrated in Figure 6. Layers above the cell layer was removed by suction and the cells were harvested using a sterile pipette and transferred to a 50 ml centrifugation tube. The tube was topped up with ice-cold L-15+, adjusting the final volume to 30 ml. The tubes were centrifuged at 400x g for 10 minutes at 4 °C. The pellet was resuspended in 5 ml L-15+.

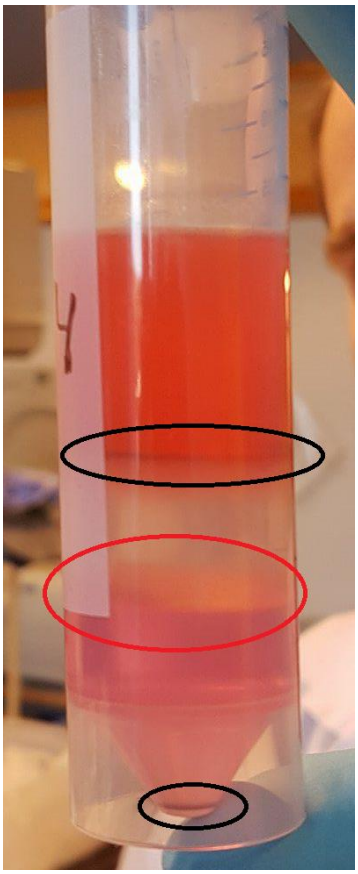


Figure 6: Percoll gradient with leukocyte band encircled in red. Layers consist of, in descending order, superfluous cell medium, cell band (encircled in black), 25 % Percoll, leukocyte band (encircled in red), 54 % Percoll and red blood cells (encircled in black).

The cell concentration was then determined using Nucleocounter™ NC-100 (ChemoMetec). This device detects signals from Propium iodide bound to DNA, so that total cell concentration can be determined based on the amount of DNA in the sample (ChemoMetec, 2015). One hundred µl cell suspension, 100 µl lysis buffer and 100 µl stabilizing buffer (ChemoMetec) were mixed in Eppendorf tubes. The suspension was sampled using a NucleoCassette™, and placed in Nucleocounter™ NC-100. Cell count was then determined. The cell density was adjusted by adding L-15+ w/ 5% FBS.

2.3.2 Sorted Atlantic salmon B cells (provided material)

RNA samples were obtained from a previous study in Jørgensen-Jensen Laboratory. Briefly, salmon IgM+ B cells were sorted by magnetically activated cell sorting (MACS) from isolated peripheral blood, head kidney, and spleen leukocytes following a modified protocol from Miltenyi Biotec (Jenberie et al., 2018). cDNAs were prepared from provided B cell RNA samples and used for qRT-PCR assay to determine the constitutive expression of BAFF, APRIL and their receptors in salmon B cells (table 3). Refer to subsection 2.5 for details of RNA isolation, cDNA synthesis, and qPCR assays.

2.3.3 Stimulation of adherent cell leukocytes

For stimulation of adherent cells, $7x10^6$ cells were seeded in Thermo Fisher™ Nunc™ Delta cell culture plates and incubated overnight at 15 °C. Adherent leukocytes will adhere to the bottom of the well. Cell medium was removed by suction thus removing non-adherent cells and debris. Fresh L-15+ with 5% FBS was added to the wells, and the plates were incubated overnight at 15 °C. Cell medium was again removed, and fresh medium with or without stimulants was added to the wells. Head kidney-derived leukocytes were stimulated with 2 µM/ml CpG (Integrated DNA technologies), 10 µg/ml Polyinosinic-polycytidylic acid (Poly I:C) (Pharmacia Biotech), 100 ng/µl recombinant interferon gamma (IFNγ) (Skjaeveland et al., 2009) or 500 units/µl recombinant interferon a1 (IFNα) (Robertsen et al., 2003). Control cells were incubated with medium alone or non-CpG, in which the CpG dinucleotides are inverted(Integrated DNA technologies).

At 6, 24 and 48 hours post-stimulation, cells were harvested by sucking out cell media in wells before adding 350 µl lysis buffer, included in RNeasy Mini kit (Qiagen), to each well. The lysis buffer was pipetted up and down extensively before being transferred to Eppendorf tubes. The tubes were then stored at -80 °C until further use in total RNA isolation, cDNA synthesis, and qPCR assays (described in details in section 2.4)

2.4 Gene expression profiling in *in-vivo* and *in-vitro* Atlantic salmon samples

Quantitative real time polymerase chain reaction (qPCR) was used to measure expression of selected gene transcripts in tissues and cells. The method is used to detect and quantify DNA or RNA sequences (Life-Technologies, 2012). qPCR is highly sensitive, enabling detection and quantification of rare transcripts and subtle changes in expression levels (Pfaffl, 2001). Gene

expression can be measured in cells, blood and tissue (Heid et al., 1996; Higuchi et al., 1993). Materials from *in-vivo* and *in-vitro* experiments were used in this assay, an overview of the materials, stimulations and incubation periods are presented in table 3.

The method is based on a reaction where, using specific primers and shifts in temperature, gene sequences are amplified. Added to the PCR reaction is a fluorescent molecule that binds double stranded DNA. The molecules' fluorescence increases when in bound form, making the amount of fluorescence in the sample proportional to the amount of PCR product. Fluorescence is measured after each amplification cycle. A qPCR run can be divided into 3 phases: Initiation, exponential and plateau. The initiation phase occurs during the first PCR cycles where emitted fluorescence cannot be distinguished from background noise. In the exponential phase, there is an exponential increase in emitted fluorescence. In the plateau phase, the reagents in the reaction are exhausted, and no increase in fluorescence can be observed. In the exponential phase, the fluorescence in the sample will eventually distinguish itself from background noise. The amplification cycle where this happens is termed the threshold cycle (Ct). The Ct value is used to estimate the concentration of the specific gene transcript in the sample. The Ct value is inversely proportional to the amount of cDNA in the sample, thus, a low Ct-value indicates a high concentration, while a high Ct-value indicates a low concentration (Life-Technologies, 2012). Relative quantification was used to determine change in expression of target genes. Expression of target genes are normalized in relation to a reference gene, usually a housekeeping gene, which is expressed at virtually the same level in all samples; this method is proved to be effective in examining differences in expression of gene transcripts (Pfaffl, 2001). In our data analysis, we used elongation factor 1 α/ β (EF1aB) for this purpose. EF1aB has been shown to be expressed at virtually constant levels in different organs and life stages in Atlantic salmon (Olsvik et al., 2005), as well as in SAV infected Atlantic salmon (Lovoll et al., 2011).

SYBR®Green (Applied biosystems) was used to detect increases in gene expression. SYBR®Green binds to minor grooves in dsDNA. The fluorescence of the molecule is stronger when bound to DNA than when in unbound form. Knowing this, we can use SYBR®Green to determine the amount of dsDNA in a sample, and determine how much dsDNA has been replicated. SYBR®Green can bind to all dsDNA, this will lead to some degree of unspecific binding if the samples are contaminated. To be able to determine to what extent unspecific binding has occurred, a melt curve stage is incorporated in the method. The melt curve stage is

an extension of the PCR method, where the temperature is brought up to 95 °C over a longer period of time (temperature cycles presented in table 8 and 9). The melt curve depicts the changes in fluorescence as dsDNA attached to SYBR®Green melts into two ssDNA when temperature rises and subsequently loses its fluorescence. Different gene sequences will dissociate at different temperatures and time points, yielding different melt curves, thus implicating unspecific binding.

To measure the amount of mRNA in cells and tissues, we used a two-step qPCR method. In this method, mRNA is transcribed to cDNA by reverse transcription. The cDNA product is then used in qPCR method. cDNA synthesis is described in further detail in section 2.5.2. A qPCR reaction typically consists of 40 amplification cycles. In these cycles, cDNA is replicated. Each step in the process consists of three stages: Denaturing, amplification and elongation. The steps are controlled by shifts in temperature. In the denaturing stage, the samples are exposed to a high temperature, usually 95 °C. This splits the dsDNA to ssDNA. In the amplification stage, the temperature is lowered, typically to 60°C. Complimentary primers are attached to the ssDNA. The elongation stage also occurs at 60°C, in this stage the DNA-polymerase synthesizes a new strand of DNA, staring at the primers. Under optimal conditions, the amount of the DNA in the sample will be doubled after each cycle, resulting in an exponential increase of the DNA sequences (Life-Technologies, 2012). A summary of details regarding RNA extraction, cDNA synthesis and qPCR assays is presented in table 5.

Table 5: Summary of qPCR assays performed

In vivo Experiments	Organs (Gills, head kidney, heart, liver, pancreas, and spleen)				
	Number of samples	RNA input	cDNA dilution	cDNA input per qPCR reaction	Target genes
Basal/Constitutive expression (Day 0)	6 naïve fish	600 ng	1:10	15 ng	BAFF, APRIL, EF1ab
Gene expression changes during SAV3 infection (Day 3, 8, and 14)	8 SAV3-infected, 4 mock-infected control	600 ng	1:10	15 ng	BAFF, APRIL, EF1ab

In vitro Experiments	Leukocyte Subpopulations (IgM+ B cells, Adherent HK MLCs)				
	Number of samples	RNA input	cDNA dilution	cDNA input per qPCR reaction	Target genes
Constitutive expression in IgM+ B cells (provided materials)	5 (RNA) per B cell type	300 ng	1:5	15 ng	BAFF, APRIL
	5 (cDNA) per B cell type	150 ng	1:5	7,5 ng	BAFF-R, BCMA, TACIvar1, EF-1ab
Constitutive expression in adherent HK MLCs	3	300 ng	1:5	15 ng	BAFF, APRIL, EF1ab
	3	300 ng	1:10	7,5 ng	BAFF-R, BCMA, TACIvar1 EF-1ab
Gene expression changes in stimulated adherent HK MLCs	3	300 ng	1:5	15 ng	BAFF, APRIL, Mx-1, TNF-1, EF1ab

2.5.2 Isolation of total RNA from cells and tissues

Gene transcripts from cells and tissues were extracted and transcribed to cDNA to allow for use in qPCR runs. Isolation of RNA is based on a method originally described in 1987 (Chomczynski & Sacchi, 1987), where RNA from cells and tissues are isolated by a single extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture. The solution effectively lyses cells and isolates RNA, while maintaining the integrity of the RNA. Ethanol is added to the solutions to give RNA optimal binding conditions. The solution is then run through special silicate membranes that specifically binds RNA, while contaminants are washed out (Qiagen, 2012). Finally, RNA is eluted from the membranes using ultrapure water. RNA is easily degraded by ribonucleases, found both endogenous and in the environment. To minimize the effects of ribonucleases RNase-free equipment was used throughout the procedure. Centrifugation of samples were done using Eppendorf centrifuge 5417R at 4 °C.

RNA extraction from tissues and adherent cells was done using RNeasy Mini Kit (Qiagen) following the manufacturer's recommendations (Qiagen, 2012). When isolating RNA from tissues, roughly 30 mg tissue sample was transferred to 600 µl homogenizing buffer in a 2 ml homogenizing tubes, containing steel pellets. The tubes were placed in a homogenizing machine (TissueLyser II Qiagen). The machine was run for 15 seconds at 5900 rpm; in some cases, tissues were not sufficiently homogenized, and the tubes were again placed in the homogenizing machine, and run again. Total RNA was isolated from tissue homogenates in accordance to the protocol developed by the manufacturer of the kit (Qiagen, 2012) When extracting RNA from heart and gills, tissues that are hard to digest, we included a step where proteinase K was added (also described in the manufacturer's protocol), for all other tissues and cells, this step was not included. Columns were elated twice to maximize RNA yield. Isolation

of total RNA from cells were done using the same protocol as for tissues, excluding homogenization steps.

After isolation, RNA was quantified using NanoDrop spectrophotometer (ND-1000, Thermo Fischer Scientific). The system displays values for A_{260} and A_{280}, indicating RNA- and protein concentration, respectively. A ratio of $A_{260}/ A_{280} \sim 2.0$ is generally accepted as «pure» for RNA (Thermo-Scientific, 2010b). Extracted RNA was stored at -80 °C until further use use.

2.5.3 Reverse transcription of RNA sequences

Isolated and purified RNA was transcribed to complementary DNA strands (cDNA) using the enzyme reverse transcriptase. The enzyme attaches short oligo nucleotides to the RNA strand, a complementary DNA-strand is synthesized and the RNA strand is degraded. cDNA synthesis consists of three steps: Primer annealing, reverse transcription and RNA degradation. The steps are controlled through shifts in temperature. The resulting product is a single stranded cDNA transcript that can later be used in an qPCR method. Under optimal conditions, the amount of resulting PCR product is equal to original amount of mRNA in samples. gDNA wipeout buffer was used to remove any potential genomic DNA contaminations in the samples.

In this procedure, Quantitect® reverse transcriptase kit (Qiagen) with the accompanying protocol (Qiagen, 2009) was used. Reagents used are demonstrated in table 6. Samples were kept on ice throughout the procedure to prevent degradation of RNA. All incubations were done in a thermocycler (2720 Thermal cycler, Applied Biosystems). RNA input was adjusted by addition of RNase free water in accordance to measured RNA concentration.

Table 6. Reverse transcription reagents.

Reagent	Volume
Sample + H2O	12 µL
gDNA wipeout buffer	2 µL
Quantiscript RT buffer	4 µL
RT primer mix	1 µL
Quantiscript reverse transcriptase	1 µL
Total	20 µL

The wanted input (Table 5) of RNA was added to all tubes before RNase free water was added to make the total volume 12 µL. gDNA wipeout buffer was added to samples, the samples were spun and incubated for 2 minutes at 42 °C. Quantiscript RT buffer, RT primer mix and

Quantiscript reverse transcriptase had beforehand been mixed together in the correct concentration in a master mix and was dispensed to the tubes (6 µL/tube). The reverse transcriptase reaction was done in a thermocycler under the following conditions: 25 °C for 10 min, 48 °C for 30 min and 95 °C for 5 min. cDNA was diluted to the desired concentrations (Dilutions presented in table 5) and stored at -20 °C until use.

2.5.4 Quantitative PCR

mRNA from *in-vivo* and *in-vitro* experiments were analyzed in an qPCR method. Table 7 illustrates the components in each reaction.

Table 7. Template for preparation of master mix (per reaction).

Component	Volume	Final concentration
SYBR®Green (2x)	10µl	1x
Forward-reverse primer mix (4µm)	1µl	200 nm
RNAse-free water	4µl	
cDNA (7,5-15µl)	5µl	

Quantitative qPCR was done in accordance to the producer's protocol (Life-Technologies, 2012). All qPCR analyses were done in 7500 Fast Real-Time PCR system software (Applied Biosystems).

Fifteen µl mastermix was added to each well in a 96-well plate (MicroAmp® Fast Optical 96-well Reaction Plate). 5ul cDNA was added to each well in duplicates. All steps were done on ice. Additionally, NoRT (no reverse transcriptase), NTC (no template control) and IRC (inter-run calibrator) was included in each plate. IRCs are included to make different qPCR runs comparable. In NTCs, cDNA was replaced with ultra pure water. This is done to detect nonspecific binding, which is an indicator of contamination in water, primers, cDNA or SYBR®Green reagents. NoRT controls consist of master mix, and cDNA that has been synthesized using ultra-pure water in place of reverse transcriptase, and are included to specifically detect genomic DNA contamination in cDNA samples. Primer efficiency was determined using a standard curve made from two-fold dilution series using cDNA from different samples, table 10 presents an overview of primer sequences used and their efficiency.

The plates were centrifuged in an MPS mini plate spinner 1000 (Labnet), and loaded in an Applied Biosystems® 7500 Real-Time PCR System. An overview of run methods are presented in table 2 and 3. All samples were analyzed using 7500 Fast Real-time PCR software (Applied Biosystems). Threshold values were adjusted according to IRCs for each plate to make qPCR runs comparable.

Two variants of the SYBR®Green Master Mix were used, the SYBR™ Green PCR Master Mix and Fast SYBR™ Green Master Mix. The master mixes uses different variants of the same polymerases, the reaction speed of the Fast SYBR™ Green Master Mix is faster than that of the standard SYBR®Green Master Mix. Because of this, the two master mixes require different run methods. The run methods for the respective reactions are listed in table 8 and 9.

Table 8. qPCR run method SYBR®Green reagents

Stage	Temperature °C	Duration (seconds)	Cycles
Denaturing	95	600	Hold
Denaturing	95	15	40
Amplification and elongation	60	60	40
Melt curve	95	15	Continuous
	60	60	
	95	30	
	60	15	

Table 9. qPCR run method FAST SYBR®Green reagents

Stage	Temperature °C	Duration (seconds)	Cycles
Denaturing	95	20	Hold
Denaturing	95	3	40
Amplification and elongation	60	30	40
Melt Curve	95	15	Continuous
	60	60	
	95	15	
	60	15	

2.5.5 Data analyses

Data from the qPCR analyses were analyzed in Microsoft Excel. To calculate the relative fold change in gene expression, the Pfaffl-method (Pfaffl, 2001) was applied.

$$Ratio = \frac{(E_{Target})^{\Delta Cttarget(control-sample)}}{(E_{ref})^{\Delta Ctref(control-sample)}} \quad (1)$$

Calculations were done in accordance to equation 1. By using this equation, we find the fold change of gene expression in a stimulated group compared to a control group, and normalized to a reference gene (EF1aB). E_{Target} and E_{Ref} represent the efficiency of target- and reference-gene primers, respectively. ΔCt represents the difference between the means of Ct values of stimulated and unstimulated or infected and uninfected tissues or cells.

Relative expression of genes sequences in cells and tissues was calculated using $2^{-\Delta Ct}$ method (Schmittgen & Livak, 2008). Each Ct value is normalized to the reference gene, but is not compared to a control sample. The method assumes 100% primer efficiency.

$$Relative\ expression = 2^{-(Mean\ CtTarget - Mean\ CtRef)} \quad (2)$$

Equation 2 demonstrates how relative expression is calculated using $2^{-\Delta Ct}$.

Relative expression- and fold change-figures are presented as columns while Ct values are presented as dot plots on an inverse y-axis.

Table 10. Primer sequences for qPCR and PCR efficiency

Samples	Gene		Master mix	Sequence (5'-3')	PCR eff.
Tissues	**EF1aB**	**F**	FAST SYBR™ Green	TGCCCCTCCAGGATGTCTAC	1,99
	EF1aB	**R**	FAST SYBR™ Green	CACGGGCCCACAGGTACTG	
	BAFF	**F**	FAST SYBR™ Green	GAGATGAAGCTCAGCATGTCAC	2,03
	BAFF	**R**	FAST SYBR™ Green	CGACCTCCAGCTTCACTATACC	
	APRIL-var-all	**F**	FAST SYBR™ Green	CTGCGGGATATCTGACTCTCA	1,99
	APRIL-var-all	**R**	FAST SYBR™ Green	CCGTCTCCCATGTCTTGAAATC	
Cells	**EF1aB**	**F**	Standard SYBR™ Green	TGCCCCTCCAGGATGTCTAC	1,97
	EF1aB	**R**	Standard SYBR™ Green	CACGGGCCCACAGGTACTG	
	BMCA	**F**	Standard SYBR™ Green	CTCAATCATCAGAGAGCAAAGC	1,94
	BCMA	**R**	Standard SYBR™ Green	CAGCAGCAGGAGAGTAGTAAA	
	TACI	**F**	Standard SYBR™ Green	AAAGGGCCAAGAGCCTAAC	1,99
	TACI	**R**	Standard SYBR™ Green	CACACTGTTAACCTGGAGCA	
	BAFF-R	**F**	Standard SYBR™ Green	TCCATCCTGGCAATGTTTCTAT	1,94
	BAFF-R	**R**	Standard SYBR™ Green	GCTTTAGCTGGAGGGTTAAGT	
	mcsfr	**F**	Standard SYBR™ Green	CACCAGTAACCCTAACCACTTC	2,00
	mcsfr	**R**	Standard SYBR™ Green	GACCTGCTTGTCCTGCATTA	
	TNF	**F**	Standard SYBR™ Green	TGCTGGCAATGCAAAAGTAG	1,89
	TNF	**R**	Standard SYBR™ Green	AGCCTGGCTGTAAACGAAGA	
	MX	**F**	Standard SYBR™ Green	TGCAACCACAGAGGCTTTGAA	2,01
	MX	**R**	Standard SYBR™ Green	GGCTTGGTCAGGATGCCTAA	
	BAFF	**F**	Standard SYBR™ Green	GAGATGAAGCTCAGCATGTCAC	1,90
	BAFF	**R**	Standard SYBR™ Green	CGACCTCCAGCTTCACTATACC	
	APRIL-var-all	**F**	Standard SYBR™ Green	CTGCGGGATATCTGACTCTCA	1,96
	APRIL-var-all	**R**	Standard SYBR™ Green	CCGTCTCCCATGTCTTGAAATC	

2.4 Quantification of ASCs by ELISPOT assays

Enzyme linked immunosorbent spot (ELISPOT) assays are used to investigate cellular immune responses in living organisms. The assay was originally developed to detect and quantify the amount of specific ASCs (Czerkinsky et al., 1983), and has since then been developed to identify and quantify cytokine producing cells. When applied for B cells the assay can be used to detect and quantify the total number of ASCs in a sample or B cells secreting antibodies specific for a given antigen.

A general outline of the ELISPOT method is presented in Figure 7. In our experimental set-up 96-well ELISPOT PVDF (Polyvinylidene fluoride) plates were used. These plates were used because of their high antibody binding capacity, and because the white background in the wells provide a suitable backdrop for ELISPOT enumeration (Kalyuzhny, 2012). Monoclonal antibodies (mAb), specific to IgM, were coated on the membranes. When leukocytes are introduced to the wells, IgM secreted by cells will immediately bind to the membrane-coated antibodies. The wells are subsequently washed and a biotinylated detection anti-IgM antibody is added, which binds the coating-antibody-IgM complex. Next, a streptavidin-enzyme conjugate, which binds the biotinylated antibody, is added. Finally, a substrate producing color by catalyzing the enzyme is added. This process produces a visible spot on the membrane for each area where enzyme has been catalyzed.

The biotinylated antibody needs to be prepared before use in an ELISPOT assay. This was done by Shiferaw Jenberie as per the recommendations from the manufacturer (Thermo-Scientific, 2010a).

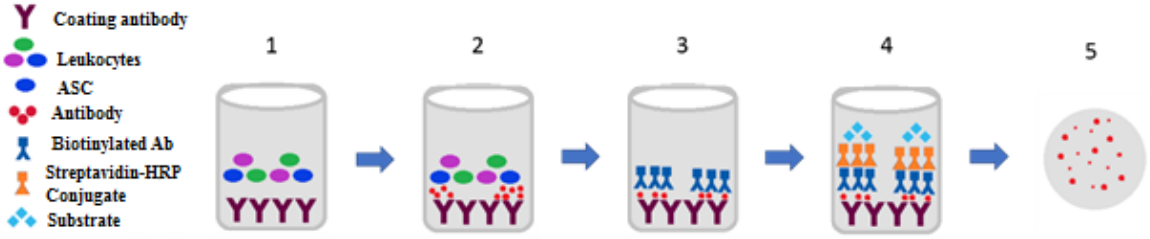


Figure 7: Execution of ELISPOT assay. (1) Membrane is coated with antibodies before leukocytes are added. **(2)** ASCs secretions bind nearby coating antibodies. **(3)** Cells are removed by washing and an IgM-specific biotinylated detection antibody is added and binds to IgM-coating antibody complex. **(4).** A streptavidin-enzyme conjugate and substrate is added, the substrate catalyzes the enzyme, producing color. **(5).** The reaction produces visible spots on the membrane, each representing an ASC (EMD-Millipore, 2012).

For this study, a total of 3 ELISPOT assays were carried out, the assays are summarized in Table 3.

2.4.1 Preliminary Experiments to Optimize ELISPOT Assay Protocol

Two pilot assays were performed to optimize the execution of method including establishing an optimal concentration for LPS as a positive control and optimal incubation periods. Head kidney- and spleen-derived leukocytes were isolated as described in section 2.3.1. In the first pilot experiment, head kidney leukocytes (n=3 fish) were seeded onto 96-well plates ($2{,}0x10^6$ cells/well) and stimulated with different concentrations of lipopolysaccharides (LPS) (10 µg/ml, 20 µg/ml, 50 µg/ml and 100 µg/ml). At 24h post-stimulation, cells were collected into sterile Eppendorf tubes after mixing the suspension by extensive pipetting. To ensure complete harvesting of cells, additional 100 µl L-15+ w/ 5% FBS was added to the wells, and the remaining cell suspension were pipetted up and down extensively, before being transferred to the same Eppendorf tubes. The Eppendorf tubes were centrifuged at 400g for 10 minutes at 4 °C. The supernatant was carefully removed, and the pellet was re-suspended in 200 µl L-15+ w/ 5 % FBS. Cells were enumerated in Nucleocounter™ NC-100 in the same manner as described in section 2.3.1. Cells were then seeded out on ELISPOT plates for quantitation of IgM^+ ASCs.

In the second pilot experiment, HK- and spleen leukocytes from 3 fish were stimulated with LPS (10ug/ml and 100ug/ml) and incubated for 24- and 72 hours, before being transferred to

ELISPOT plates (as described above). In both pilot experiments, leukocytes were seeded onto ELISPOT plates in a concentration of 50.000 cells/well in triplicates. Negative controls were included, containing cells incubated with medium alone and medium with no cells. ELISPOT plates were incubated for 48 hours, before further processing and counting.

2.4.2 Processing and Counting of ELISPOTs

The ELISPOT protocol used was originally described in 2009 (Buisman et al., 2009), but has been optimized for our purposes. MSIPS4510-plates (Merck Millipore) were activated by wetting the membranes with 15µL 35% ethanol (99,8 % ethanol (Sigma-Aldrich) diluted in ultrapure H_2O) for 30-60 seconds. The wells were then washed 2 times with ultrapure water and 2 times with PBS. The wells were then coated with 1.5µg/well mAb anti-trout IgM F1-18 (Hedfors et al., 2012) in PBS (100 µl/well), and incubated overnight at 4 °C. After incubation, the wells were washed 4 times with PBS. To block non-specific binding to the membrane, plates were incubated with (100 µl/well) L-15 w/ 10U/ml penicillin, 10 µl/ml streptomycin and 2 % bovine serum albumin (BSA) (Sigma-Aldrich) for 1 hour and 30 minutes at room temperature. Head kidney and spleen leukocytes from individual fish diluted in L-15 w/ 10U/ml penicillin, 10 µl/ml streptomycin and 5 % FBS, were added to the wells in triplicates in the desired concentration (100 µl total medium/well). After incubating wells for 48 hours at 15 °C, residual cells and unbound IgM were washed away (2 washes with PBS w/ 0.2% tween 20, 2 washes with PBS). Wells were added 1.5µg/well biotinylated detection antibody (mAb anti-trout IgM F1-18, EZ-Link NHS-PEG Solid Phase Biotinylation kit, Thermo Scientific) diluted in PBS w/ 0.2% tween 20 and 1% BSA, and incubated for 1 hour and 30 minutes at room temperature. After additional washing steps (2 washes with PBS w/ 0.2% tween 20, 2 washes with PBS), the plates were developed by addition of streptavidin-HRP conjugate (Mabtech) diluted 1:5000 in PBS for 1 hour at room temperature. Unbound complex was discarded by washing (2 washes with PBS w/ 0.2% tween 20, 2 washes with PBS). Tetramethylbenzidine (TMB) (Mabtech) was filtered through a 0,45 µm filter (Falcon) and added to the wells (100 µl/well). The plates were incubated for 10 minutes in the dark. The reaction was stopped by washing extensively in tap water. The plastic cover under the plates were removed to allow washing the membranes from underneath. Plates were incubated overnight in the dark at 4°C. Images of the wells were acquired using ImmunoSpot® S6 Core Analyzer, running ImmunoSpot image acquisition

software (ImmunoSpot). Spots were enumerated using C.T.L. cell counting software (ImmunoSpot).

2.4.3 Effect of rBAFF or rAPRIL in IgM+ ASCs

Following optimization of ELISPOT assay, a final ELISPOT assay was conducted to determine the effect of recombinant BAFF (rBAFF) or recombinant APRIL (rAPRIL) pre-treatment on ASCs in leukocyte culture. Custom-made rBAFF and rAPRIL were commercially produced by MRC PPU Reagents and Services (University of Dundee, Scotland).

HKLs (1.5x106 cells/well) were incubated with rBAFF (2.5ug/mL), rAPRIL (2.5ug/mL), LPS (100ug/mL), or CpG (5 µM) for 72h in cell culture plates. HKLs treated with L-15 culture medium was used as negative control. Cells were then collected and split into two groups labeled “counted” and “uncounted” groups. Viable cells in the “counted” group was determined using Nucleocounter as described in section 2.3.1, and 75.000 viable cells from 4 fish were seeded out in ELISPOT well. For our “uncounted” group, we seeded out what was estimated to be 75.000 cells from 4 fish in ELISPOT wells. All cells were seeded in triplicates per treatment group. ELISPOT plates were processed and quantified after 48h as described above (2.4.2).

2.5 Statistical analyses

Statistical analyses were performed using GraphPad Prism. All analyses were done using Mann-Whitney tests. P-values under 0,05 were considered significant.

3. Results

3.1 The distribution of BAFF and APRIL in naïve and SAV-infected Atlantic salmon

BAFF and APRIL are molecules important in B cell function and homeostasis (Mackay et al., 2003). Little is known of the cytokines in teleosts (Soleto et al., 2017; Sun & Sun, 2015), while even less is known in Atlantic salmon. Many microbes, including viruses, are known to influence the expression of these molecules (Pandit et al., 2013; Soleto et al., 2017). In this study, we set out to examine the expression of BAFF and APRIL in salmon i.p. injected with SAV. Prior to this, we determined the steady-state levels of these factors in the immune tissues head kidney, spleen, and gills, and additionally in the liver, pancreas and heart derived from healthy naive salmon. All investigations were done using qPCR and expression levels were normalized using reference gene EF1aB. Mean Ct values are presented in appendix II

3.1.1 Normal distribution of BAFF & APRIL mRNA transcripts in tissues

Figure 8 demonstrates the constitutive/basal expression levels of BAFF and APRIL in salmon tissues presented as relative expression (A) and as Ct values (B).

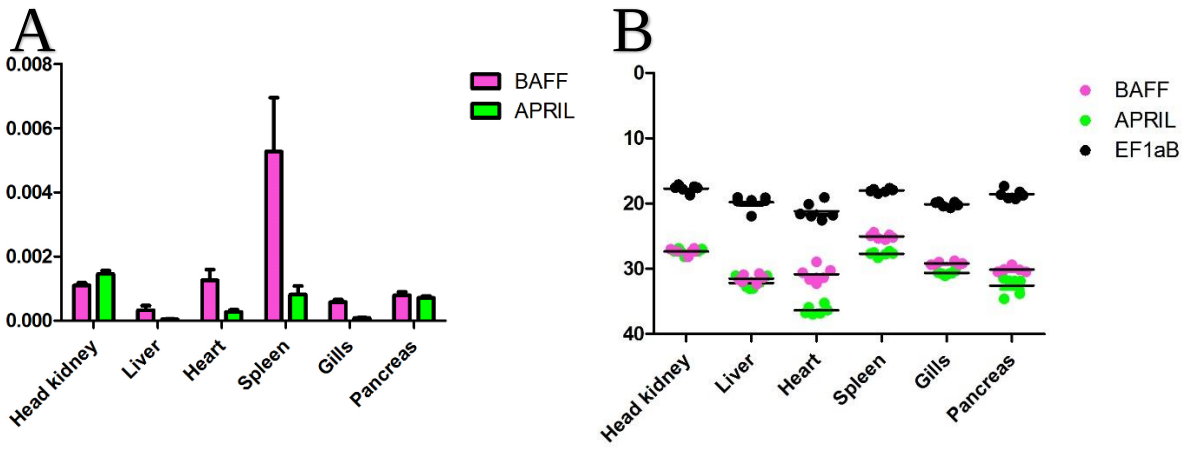


Figure 8: BAFF and APRIL expression levels in healthy Atlantic salmon. The columns (A) represent relative gene expression of BAFF and APRIL in head kidney, liver, heart, spleen, gills and pancreas. Transcription level for each sample was measured by qPCR and normalized with the housekeeping gene EF1aB. The columns show mean values ±standard error (n=6). The dot plots (B) illustrate Ct values of BAFF, APRIL and EF1ab in HK, liver, heart, spleen, gills and pancreas from 6 individual fish; lines represent mean values (inverted scale).

The highest BAFF transcript levels were detected in the spleen, while intermediate levels were observed in head kidney, heart and pancreas and the lowest levels were found in gills and liver. For APRIL, the immunological organs head kidney and spleen had the highest relative expression, while lower levels were detected in pancreas and heart. In organs liver and gills, expression of APIRL was very low. BAFF is expressed to a higher magnitude than APRIL in spleen, heart, gills and liver (6.5, 4.6, 7.2 and 6.8 times greater, respectively). In organs head kidney and pancreas, the expression of the ligands were comparable.

3.1.2 Induction of BAFF & APRIL in SAV3-infected Atlantic salmon

BAFF and APRIL are two of the main cytokines produced by innate immune cells to co stimulate B cells (Huard et al., 2004). Both cytokines regulate B cells during innate immune responses (Mackay & Schneider, 2009). Studies have shown that $BAFF^{-/-}$ mice are unable to sustain effective immune responses (Rahman et al., 2003). Knockout studies in mice reveal that APRIL augments Ig dynamics (Castigli et al., 2004). Because of BAFF and APRILs function in the immune system of vertebrates, we expect them to play a part in the pathogenesis in fish. To examine the role of these cytokines in PD, induction of BAFF and APRIL were examined in Atlantic salmon after SAV3 infection. Organs were extracted from infected and mock-infected fish at 3, 8, and 14 days post infection. Fold change values were calculated in accordance to equation 1 (section 2.4.3) using Ct values of BAFF, APRIL and Ef1aB in infected (n=8) and mock-infected (n=4) fish. Results are presented in Figure 3 A and B.

To confirm that the i.p. injection of the SAV-virus had caused a systemic infection, viral RNA in the heart was investigated. SAV-RNA can be detected in multiple tissues in SAV3 infected salmon, including cardiac muscle, skeletal muscle, pancreas, gills, kidney and pseudobranchia (Andersen et al., 2007). The nsP1 gene codes for a non-structural protein in the virion that plays a part in the replication of the virus (Andersen et al., 2007; Hodneland & Endresen, 2006). The amount of nsP1 transcripts in the heart of SAV3-injected fish was measured (Figure 9).

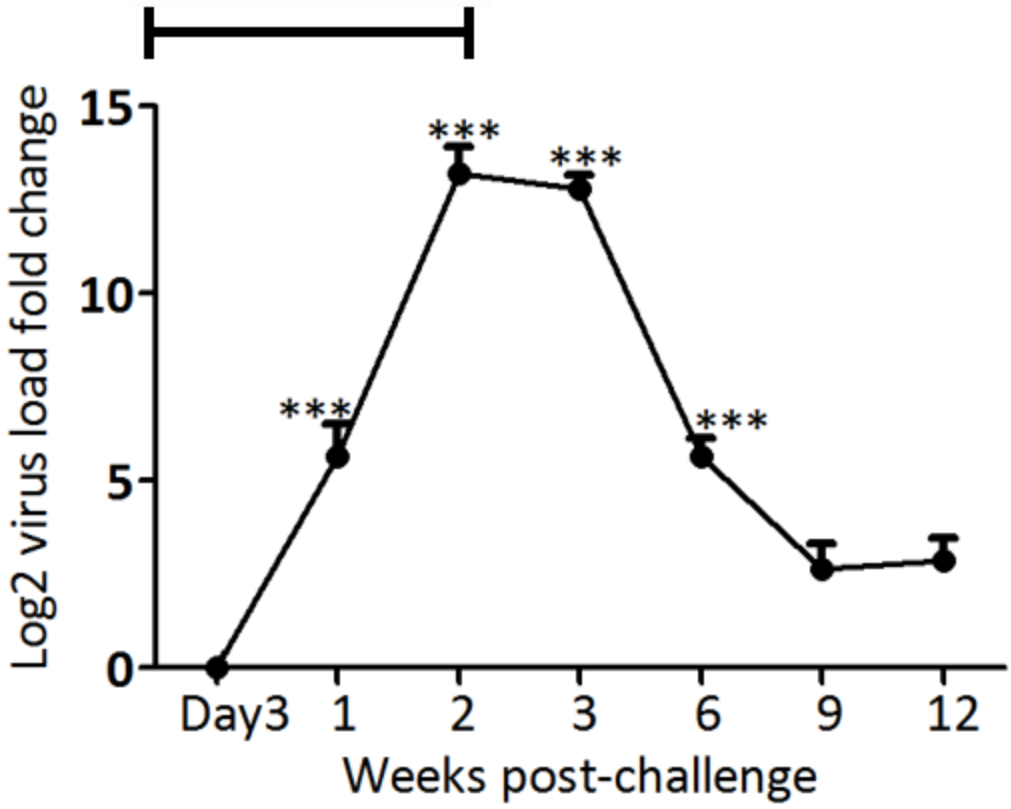


Figure 9. Relative increase of viral transcripts in heart after i.p injection of SAV3 in Atlantic salmon. Viral RNA (nsP1) detection in heart analyzed with qPCR presented as log2 relative values of host gene compared to viral gene. RNA was extracted from 30-50 mg heart tissue, 600 ng RNA was used in a cDNA synthesis method. PCR was done in a 20 µL reaction with 5 µL cDNA (diluted 1:10). The samples were analyzed in duplicates with an internal control. Ct threshold value was set to 0.2 for target gene. Ct cutoff was set to 38.5. Changes in gene expression was calculated using control group. Columns represent mean values ± standard error (n= 6-10). Significant fold changes compared to 3 d.p.i. are annotated by "*". Bracket indicates sampling time points relevant for this thesis.

No viral transcripts were detected in control fish at any time point. Viral transcripts were first detected in the heart at 8 days post challenge and increased considerably by 14 days post infection. At this point, the viral load in the heart was 192 times higher than it was at 8 days post-infection. Our results demonstrate that i.p injection of SAV3 leads to viral colonization of the heart and likely other organs through the circulatory system.

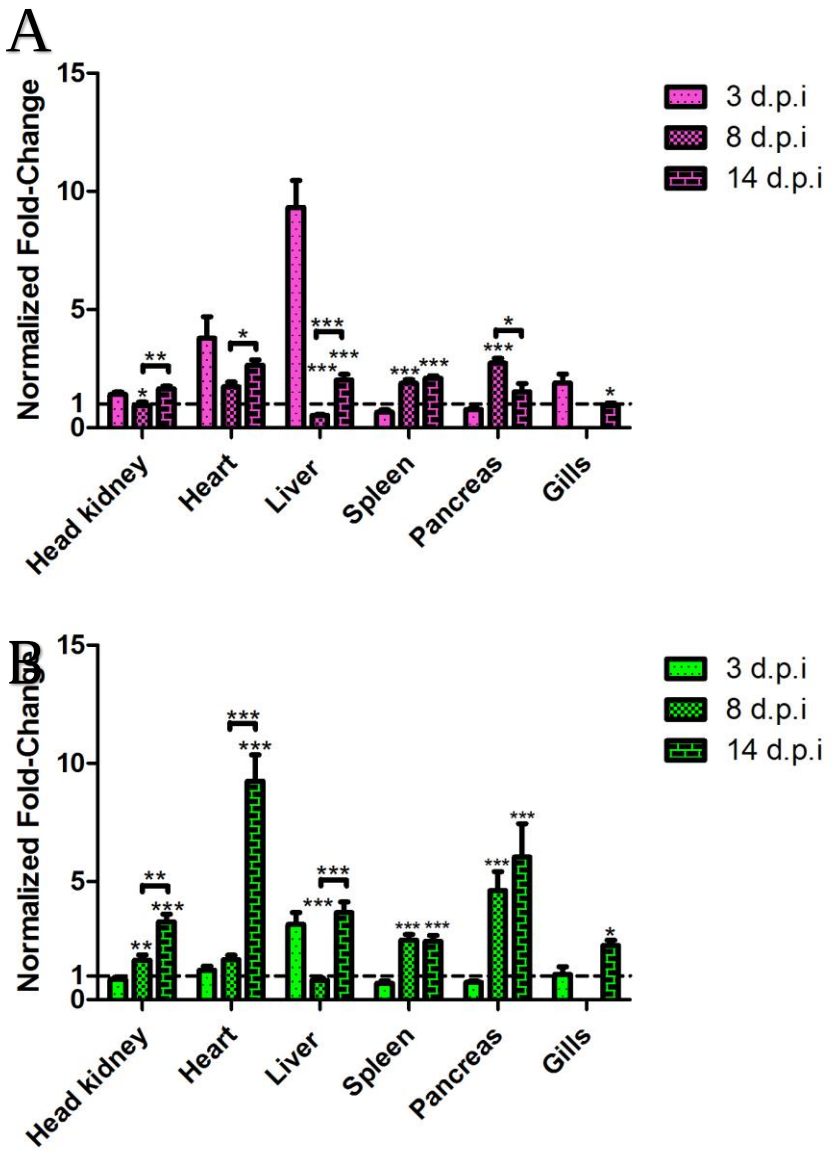


Figure 10: Fold induction of BAFF and APRIL in SAV3-infected Atlantic salmon. The columns represent normalized fold change in expression of BAFF (A) and APRIL (B) in pancreas, spleen, head kidney, liver, heart and gills at 3, 8 and 14 days post-infection. Broken line at Y=1 represents point of no induction. Gene expression was measured using qPCR and normalized with reference gene EF1aB. Fold change in tissues of SAV3-infected fish was calculated using the mean relative expression in control PBS-injected fish (n=4) of the same tissue and time point. Columns display mean values ±standard error (n=8). Fold-change data for gills at day 8 were excluded, as Ct values are unreliable. Statistical significance between day 8 and day 14 are indicated by brackets and asterisks, statistical significance in relation to day 3 are indicated asterisks: *p <0.05 **p <0.01 ***p <0.001.

BAFF was induced in all organs upon infection (Figure 10 A), and was most highly upregulated in the liver and heart. In these organs, the highest fold induction occurred at 3 days post infection, and declined to an approximately two-fold induction at 14 days post infection. BAFF transcripts were induced approximately two-fold in all other tissues, and the peak induction

occurred at different time points. In the head kidney and spleen, peak induction occurred at 14 days post infection while in the pancreas, the greatest induction was seen at 8 days post infection. APRIL transcripts were induced in all organs upon infection (Figure 10 B). The level of APRIL expression was upregulated the most in the heart and pancreas, both known target organs of the SAV3 virus. A 9- and 6-fold induction was seen in heart and liver, respectively. At 3 days post infection, the liver was the only organ where a considerable upregulation of APRIL transcripts was seen. At 8 days post infection all organs had induced levels of APRIL. The level of APRIL transcripts were most highly induced 14 days post infection in all organs except the spleen, where peak induction occurred at 8 days post infection, and was maintained at 14 days post infection. Expression of APRIL was induced to a comparatively higher extent than BAFF in all organs except the liver.

3.2 *In vitro* expression of BAFF, APRIL and receptors

In this work, we examined the steady-state levels of BAFF, APRIL and related receptors in IgM+ B cells and adherent cells; we also examined fold induction of BAFF and APRIL in adherent cells upon stimulation with various cytokines and synthetic microbial products. Binding of bacterial- and viral products to PRRs in a variety of cells have shown to induce the secretion of BAFF (Ittah et al., 2008) and APRIL (Hardenberg et al., 2007). In trout, BAFF has been shown to be produced by myeloid cells as well as splenic and peritoneal B cells (Tafalla et al., 2017). The binding of these cytokines to TLRs and BCRs on the membrane of B cells regulate the expression of BAFF-R, BCMA and TACI (Katsenelson et al., 2007). The different receptors trigger different signaling pathways within the fish, thus, IgM+ B cells with differing expression of the receptors will be differently regulated by BAFF and APRIL (Granja & Tafalla, 2017). All investigations were done using qPCR and expression levels were normalized using reference gene EF1aB. Mean Ct values are presented in appendix III

3.2.1 Description of the leucocyte populations utilized in the study

Head kidney derived macrophage-like cells were isolated as described in section 2.3, and their macrophage-like phenotype was confirmed by measuring their expression of macrophage colony stimulating factor (mcsfr), a marker of monocyte-macrophage linages in mammals (Murphy et al., 2005) and fish (Roca et al., 2006). B cell RNA samples from a previous experiment in Jørgensen-Jensen Laboratory were used in the preparation of cDNA for

subsequent B cell gene expression analyses in this study. Sorted IgM+ B cells, originating from peripheral blood, head kidney and spleen, had been purified using MACS (magnetic-activated cell sorting) and confirmed by flow-cytometry to have a purity >95% for peripheral blood and spleen, and >92% for head kidney (Jenberie et al., 2018).

Figure 11 presents the relative expression of mcsfr for the adherent cells used in this study (pink columns). The levels were compared with mcsfr expression data previously obtained from MLC and IgM^+ B cells (Jenberie et al., 2018) (black columns). As shown, mcsfr expression in adherent head kidney cells was comparable between these two studies. Furthermore, the relative expression of mcsfr in MLCs from Jenberies' publication were 2690, 217 and 560 times greater than that of peripheral blood, spleen and head kidney IgM^+ B cell populations, respectively.

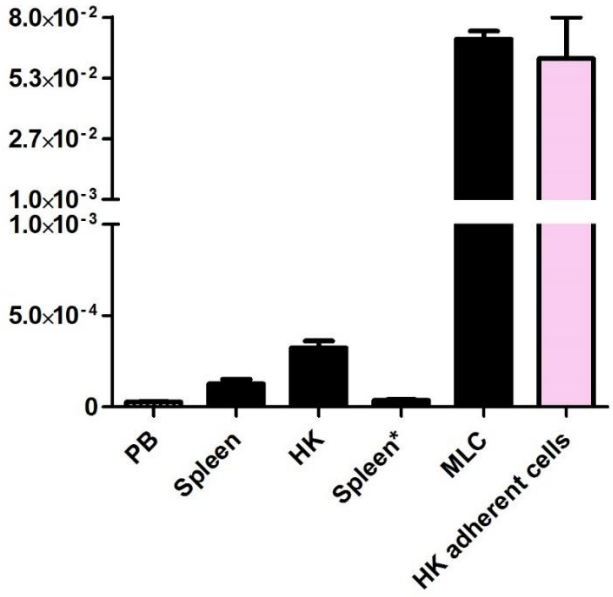


Figure 11: Relative expression of mcsfr in cell populations: Relative expression in MACS sorted IgM^+ cells and in head kidney MLCs from previous experiment (Jenberie et al., 2018) and head kidney adherent cells from this present study (pink). Ct values measure in qPCR and normalized with reference gene EF1aB. The columns show mean values ±standard error (n=3).

Based on their morphology, ability to adhere during cultivation and the high expression levels of the macrophage marker mcsfr we conclude that the head kidney adherent cells have a macrophage phenotype. These cells will therefore be referred to as head kidney MLCs from this point on.

3.2.2 Steady-state levels BAFF & APRIL transcripts in leukocyte populations

BAFF have previously been shown to be produced by myeloid cells and a subset of splenic B cells (Tafalla et al., 2017) and peritoneal B cells (Soleto et al., 2017) in rainbow trout, while the little is known regarding the origin of APRIL in teleost fish. To gain insight to where these cytokines are produced in Atlantic salmon, the steady-state levels of BAFF and APRIL in peripheral blood, head kidney and spleen IgM+ B cells, as well as in head kidney MLCs of Atlantic salmon was examined.

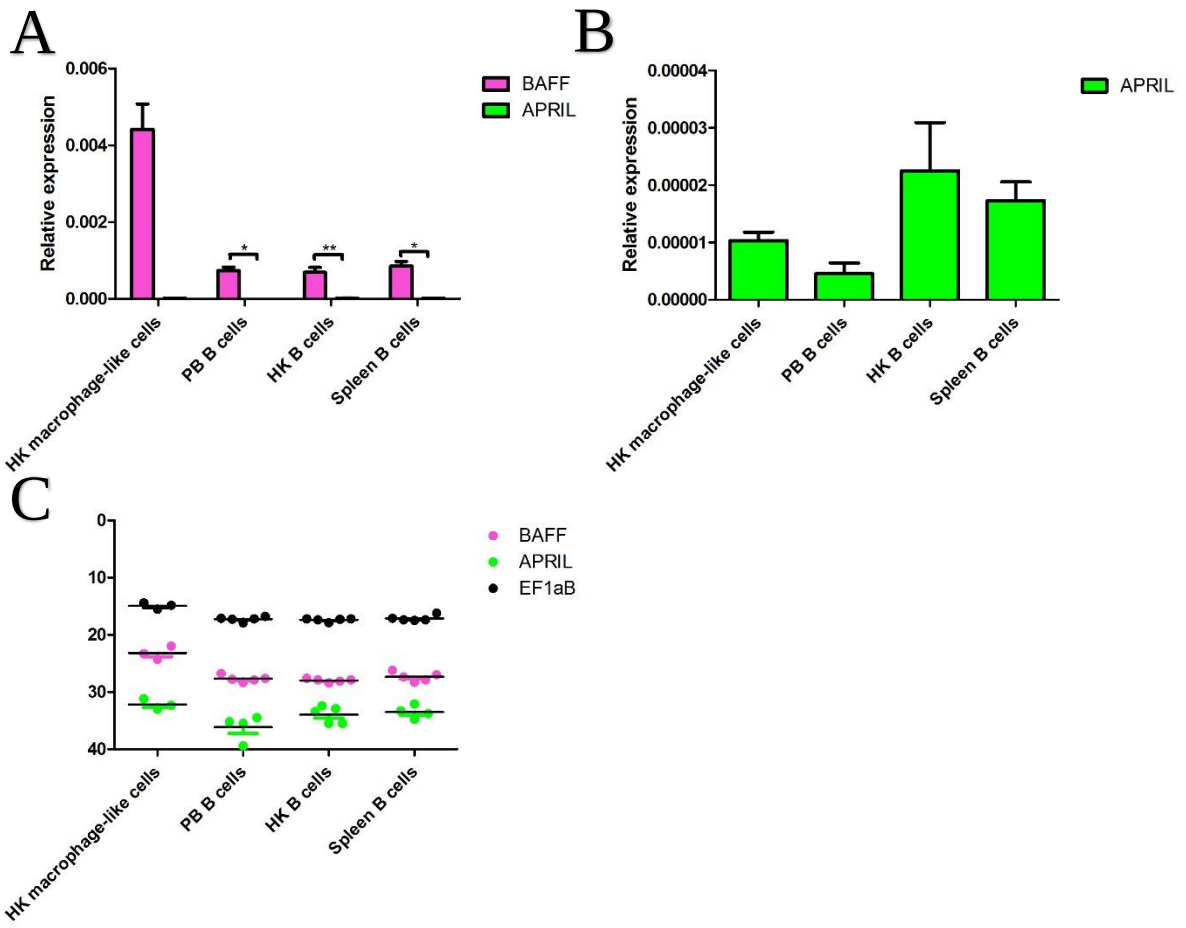


Figure 12: Constitutive expression of BAFF and APRIL in healthy, naïve Atlantic salmon cell populations. Columns representing constitutive expression of BAFF (A) and APRIL (B). Notice different scales in A and B. Dot plots (C) representing Ct values of unstimulated cell populations, lines represent mean values (inverted scale). Gene expression was measured using qPCR, and normalized using reference gene EF1aB. The columns show mean values ±standard error (n=4-5 for B cells; n=3 for MLCs). Statistical significance between BAFF and APRIL expression levels are indicated by brackets, asterisks indicate the strength of significance: *p <0.05 **p <0.01 ***p <0.001.

Expression of BAFF was highest in head kidney MLCs, while the expression of the ligand was comparable between samples in the B cell subpopulations. The relative expression of BAFF in the cell types were considerably higher than that of APRIL (4288, 2333, 418 and 686 times higher, respectively) (Figure 12 A). For BAFF, the highest levels of mRNA transcripts were found in head kidney MLCs, where its levels were 6, 6.4 and 5.2 times higher than in the peripheral blood, head kidney and spleen IgM+ B cells, respectively. APRIL expression levels were comparably low throughout all cell types (Figure 12 B and C). Peripheral blood B cells and Spleen B cells had 1 sample each where APRIL transcripts were undetectable.

3.2.3 Normal distribution of BAFF-R, BCMA and TACI transcripts in salmon-derived leukocytes

In mammals BAFF-R, BCMA and TACI are B cell receptors known to mediate the functional activity of their ligands BAFF and APRIL. In mammals, both BAFF and APRIL engage the receptors BCMA and TACI, while only BAFF is able to engage BAFF-R (Schneider, 2005). Affinity properties are presented in table 2. Examining the expression of these receptors may give insight to the functions of the different leukocyte subpopulations and B cell types, as well as to where these signaling molecules perform their function.

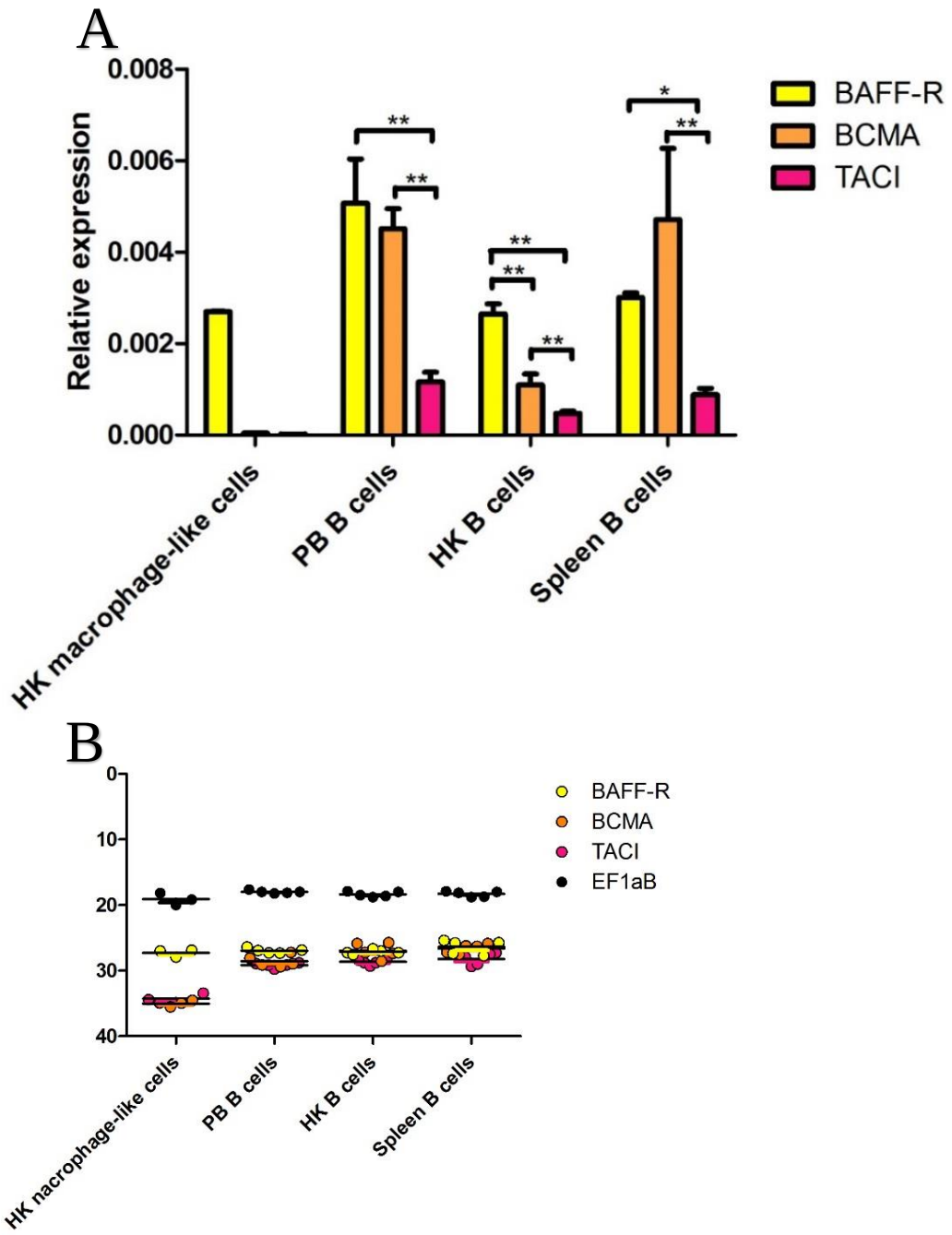


Figure 13: Constitutive expression of BAFF-R, BCMA and TACI in leukocyte subpopulations of healthy, naïve Atlantic salmon. Dot plots (A) representing Ct values of unstimulated cell populations (inverted scale). The columns (B) represent constitutive expression of BAFF-R, BCMA and TACI. Gene expression was measured using qPCR and normalized with reference gene EF1aB. Columns show mean values ±standard error (n=5 for B cells; n=3 for MLCs). Statistical significance between BAFF-R-, BCMA and TACI expression levels are indicated by brackets, asterisks indicate the strength of significance: *p <0.05 **p <0.01 ***p <0.001.

All BAFF and APRIL receptors were expressed in significant levels in all B cell subpopulations examined (Figure 13). We observed comparable levels of TACI mRNA transcripts in all B cell subpopulations, while variable observations were made in the expression patterns of BAFF-R

and BCMA. Peripheral blood B cells expressed the greatest amounts BAFF-R transcripts, while the receptor was expressed at a lower- and approximately equal level in head kidney and spleen B cells. BCMA was most highly expressed in peripheral blood and spleen B cells and a modest expression in head kidney B cells. BCMA and TACI transcripts were expressed in negligible levels in MLCs while BAFF-R transcripts were expressed to an approximately equal level in MLCs, head kidney and spleen B cells.

3.2.4 Effect of TLR ligands and cytokines on BAFF & APRIL transcription in adherent head kidney macrophages

Having determined the normal distribution of BAFF and APRIL mRNA transcripts in tissues and cell populations, we wanted to investigate if, and how, the distribution changes when cells are stimulated in-vitro. Head kidney MLCs of naïve, healthy Atlantic salmon were stimulated with TLR ligands CpG and poly I:C and cytokines, IFNγ and IFNα. CpG and poly I:C are synthetic nucleic acids mimicking bacterial DNA and viral dsRNA, respectively, and binds to PRRs on the surface of cells, thus triggering immune responses. CpG and Poly I:C have shown to produce antiviral activity in Atlantic salmon leukocytes (Jorgensen et al., 2001). IFNγ and IFNα are cytokines secreted by host cells that play important roles in the signal transduction in immune responses following viral infections (Robertsen, 2008). Non-CpG- and L-15-treated (unstimulated) groups were used as oligonucleotide- and negative controls, respectively. Fold induction values are presented in Figure 14 A and B.

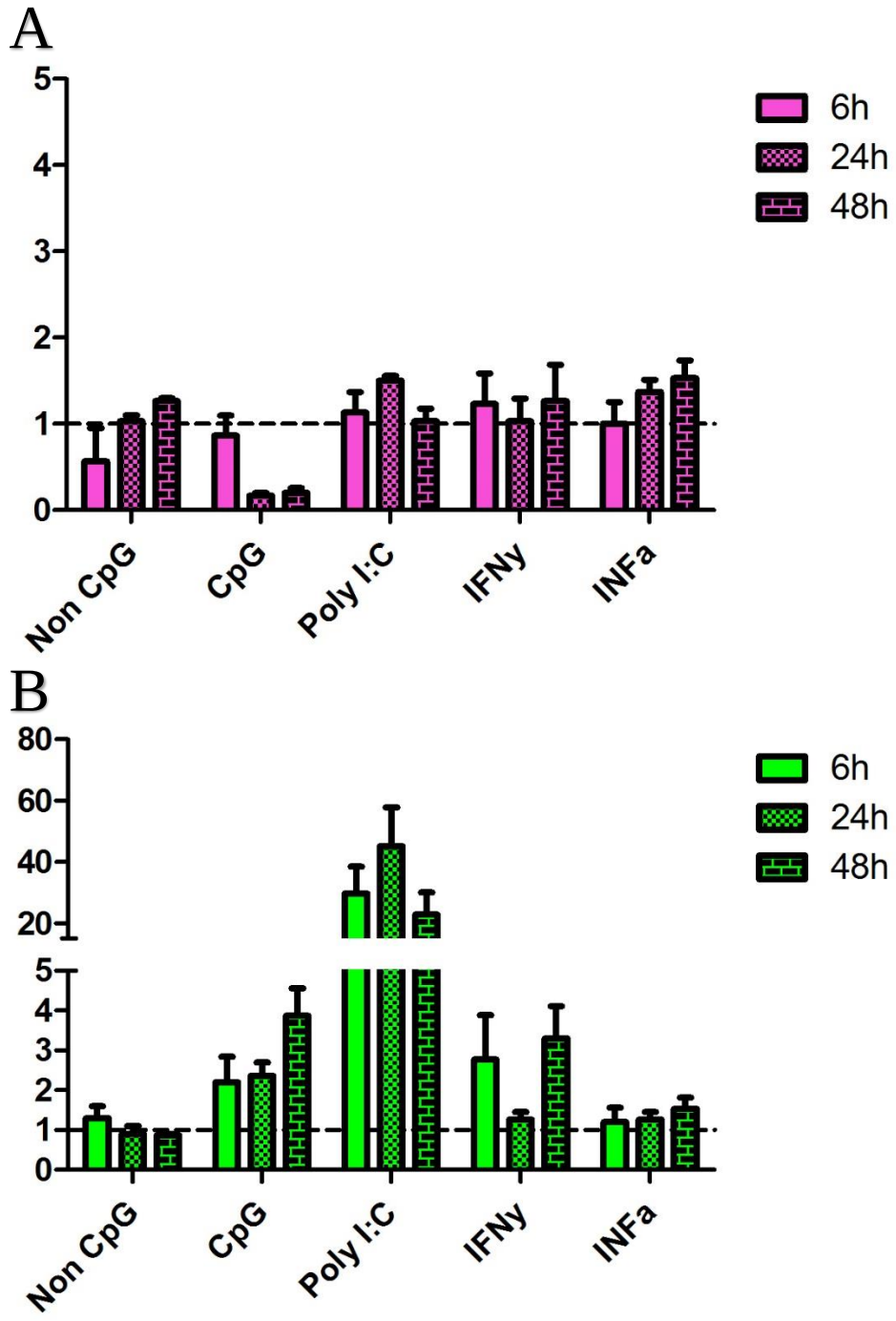


Figure 14: Expression changes in BAFF(A) and APRIL(B) in stimulated MLCs at 6, 24, and 48 hours post stimulation. MLCs stimulated with non-CpG, CpG, Poly I:C, IFNγ and IFNα. Broken line at Y=1 represents point of no induction. Gene expression was measured using qPCR and normalized with reference gene EF1aB. Columns display mean values ±standard error (n=3). No statistically significant differences in fold induction within treatment groups.

Little induction of BAFF mRNA transcripts were observed in stimulated cell, and fold induction values never exceeded 2 for any stimulation group at any time point. Down-regulation of BAFF expression was detected in cells stimulated with CpG at 24h and 48h post-stimulation.

A very modest fold induction of BAFF expression was observed in groups stimulated with non-CpG, poly I:C, IFNγ and IFNα. Expression of APRIL was induced in all stimulation groups, with the most pronounced inductions occurring in CpG-, Poly I:C- and IFNγ treated groups. We observed the highest fold induction of APRIL mRNA transcripts in Poly I:C treated groups, with an almost 50-fold mean induction at 24 h.p.i. Induction patterns of BAFF and APRIL were similar in head kidney MLCs stimulated with IFNγ and IFNα, although the severity of the induction of APRIL is considerably greater than that of BAFF.

To verify that the stimulation of the cells had worked, fold induction of Mx-1 and TNF-α mRNA transcripts were measured in stimulated cells. Because of their role in immune responses, we assume that a successful stimulation has led to the upregulation of Mx-1- and TNF-α gene transcripts.

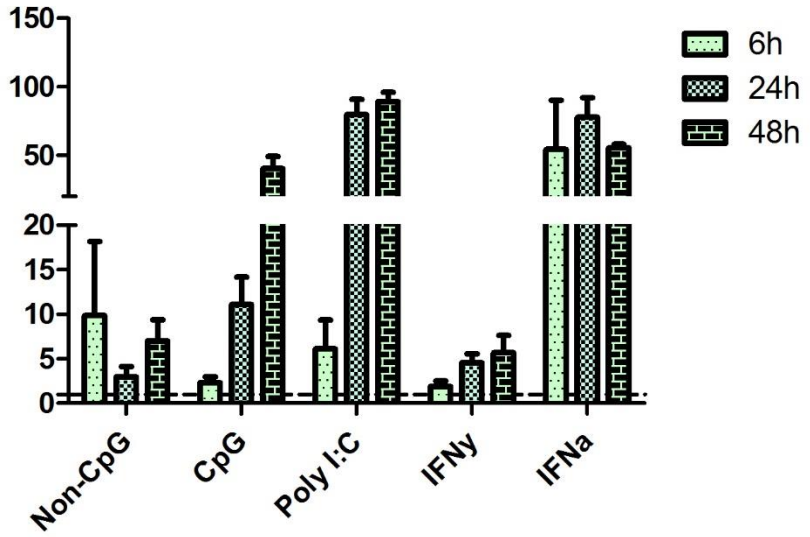


Figure 15: Fold induction of Mx-1 in stimulated MLCs at 6, 24, and 48 hours post stimulation. MLCs stimulated with non-CpG, CpG, Poly I:C, IFNγ and IFNα. Broken line at Y=1 represents point of no induction. Gene expression was measured using qPCR and normalized using reference gene EF1aB. Columns display mean values ±standard error (n=3).

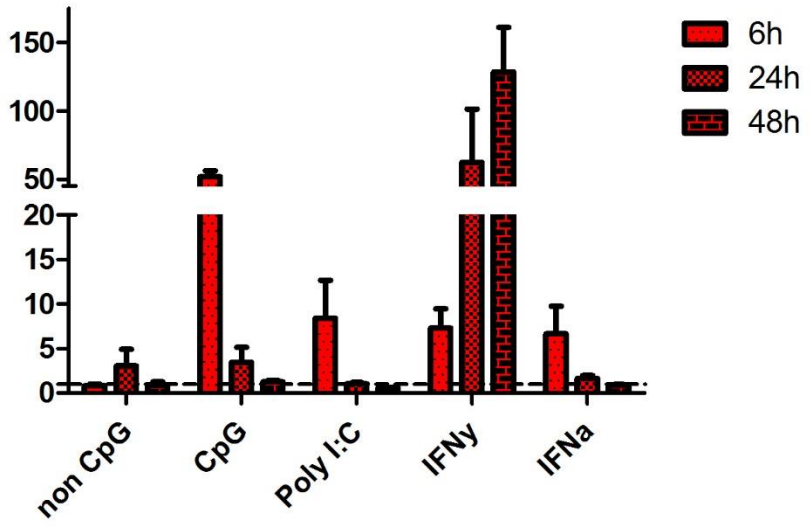


Figure 16: Fold induction of TNF-α in stimulated MLCs at 6, 24, and 48 hours post stimulation. MLCs stimulated with non-CpG, CpG, Poly I:C, IFNγ and IFNα. Broken line at Y=1 represents point of no induction. Gene expression was measured using qPCR. Columns display mean values ±standard error (n=3).

TNF-1A (Figure 15) and Mx-1 (Figure 16) and were up-regulated, albeit at different levels and time points, in all TLR ligands and cytokines used, thus confirming that the stimulation experiment in adherent MCs worked. Highest Mx-1 up-regulation was observed in CpG, poly I:C and IFNα stimulated groups, while highest induction of TNF was found in IFNγ- and CpG-stimulated groups. Non-CpG, intended to work as a control stimulation, also provoked a considerable fold induction in the gene expression of both Mx-1 and TNF-1A.

3.3 ELISPOT assays with LPS-, CpG-, APRIL-, and BAFF-stimulated MLCs

In very recent work done in our research group, it has been shown that BAFF and APRIL contribute to survival, but not proliferation of B cells in Atlantic salmon (Peñaranda, 2018). To gain further insights in the role of BAFF and APRIL in antibody secretion and survival of salmon IgM+ B cells, ELISPOT assays were carried out in salmon head kidney leukocytes (HKLs) in the presence and absence of recombinant BAFF (rBAFF) and APRIL (rAPRIL). Before performing the assays using BAFF and APRIL as stimulants, two pilot experiments were set up. The goal of the pilot experiments was to investigate the suitability of LPS and CpG as positive controls for B cell activation and IgM antibody secretion, as well as to optimize execution of the method in terms of cell densities and incubation periods used.

3.3.1 Preliminary Experiments to Optimize ELISPOT Assay Protocol

In the first pilot experiment, head kidney leucocytes were stimulated with different concentrations of LPS.

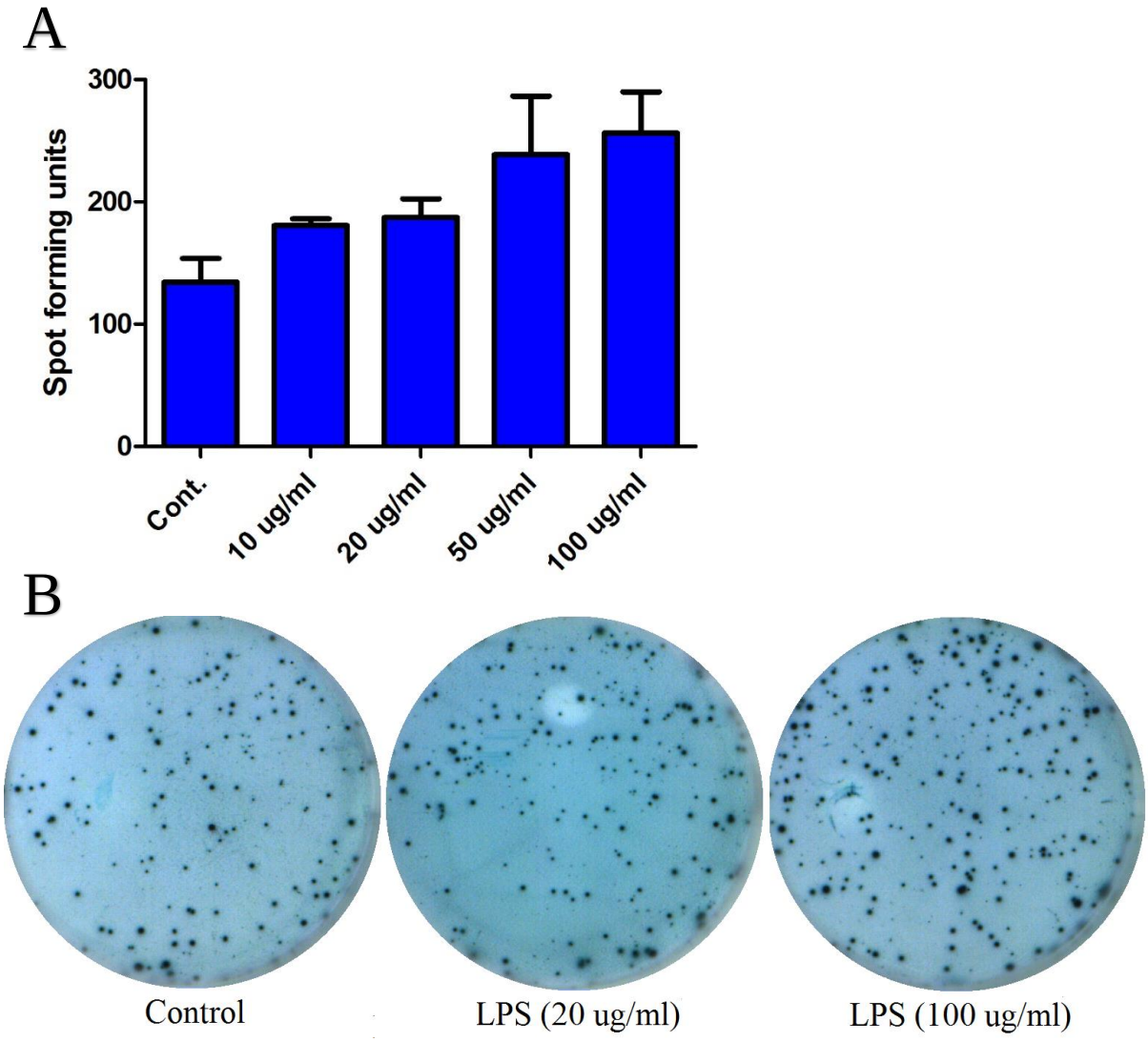


Figure 17: Spot forming units in HKLs at 5 days post stimulation. Cells stimulated with LPS (10 ug/ml, 20 ug/ml, 50 ug/ml and 100 ug/ml) for 3 days. 50.000 cells were seeded into ELISPOT wells (50000 cells/well in triplicates). Plates were developed 2 days after transfer to ELIspot plates. Columns (A) display mean spot counts ±standard error (n=3). Representative raw pictures of wells is included (B), demonstrating cells from the same fish receiving control-, LPS (20 ug/ml)- and LPS (100 ug/ml)-treatment. No statistical significance between treatment groups.

Untreated cells produced the lowest amount of spots, while cells treated with the largest concentration of LPS produced the highest amount of spots (Figure 17). Head kidney leukocytes treated with the highest concentration of LPS produced 1.9 times as many spots as untreated cells. The pilot experiment proved that the method is effective. Seeding 50.000 cells

per well yielded satisfactory spot counts. Head kidney leukocytes proved to be suitable for use as positive controls in this method.

In the second pilot experiment, head kidney leukocytes and spleen leukocytes were stimulated with different concentrations of LPS. Cells were kept in culture for 3 and 5 days before being seeded out on ELISPOT plates. This was done to investigate how incubation time affects antibody secretion. We also wanted to investigate the performance of spleen leukocytes in the assay. Results are illustrated in Figure 18.

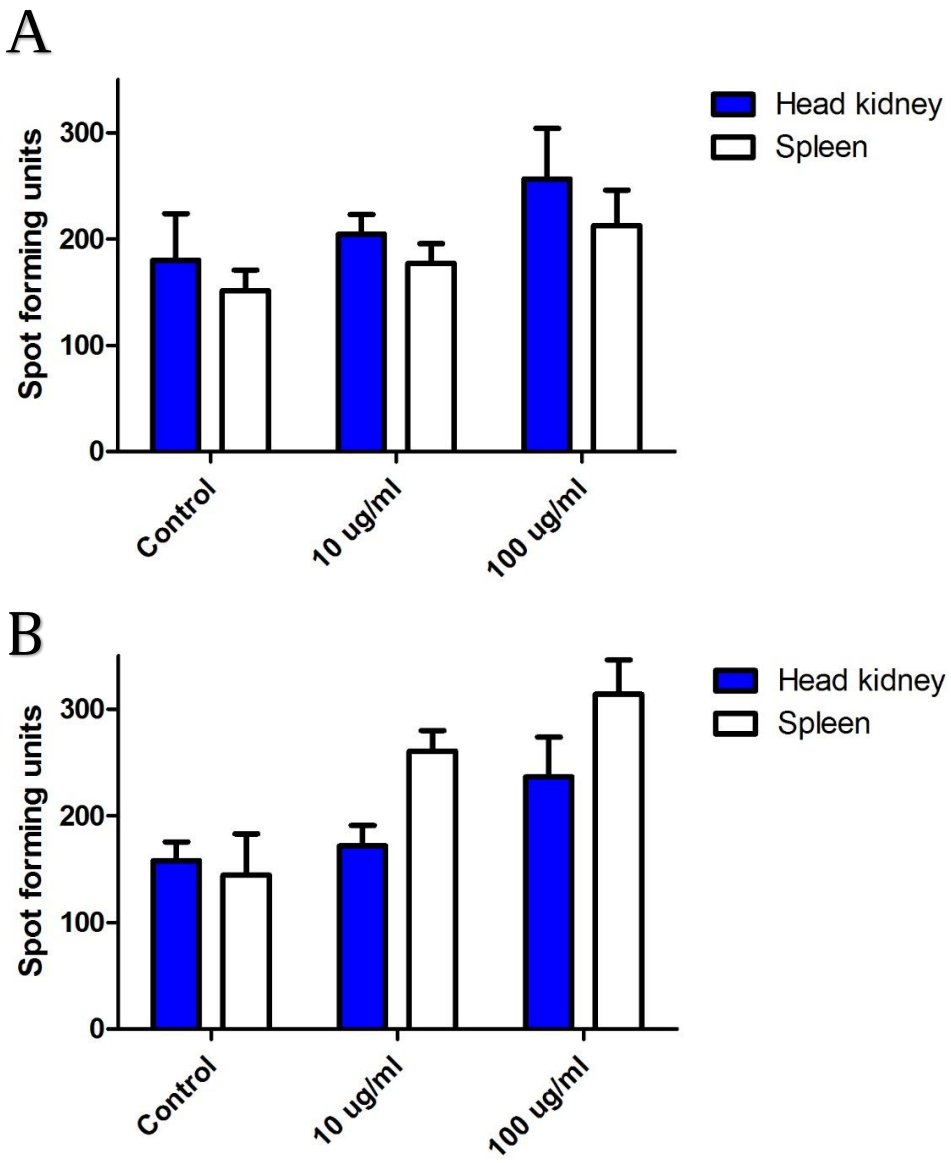


Figure 18: Spot forming units in head kidney leukocytes and spleen leukocytes at 5 (A) and 7 (B) days post stimulation. Leukocytes stimulated with LPS (10 ug/ml and 100 ug/ml) for 3- and 5 days were seeded into

ELISPOT wells (50000 cells/well in triplicates). Plates were developed 2 days after transfer to ELISPOT plates. Columns display mean spot counts ±standard error (n=3). No statistical significance between treatment groups.

Five days post stimulation, head kidney leukocytes produced the highest amount of spots in all stimulation groups. The cells stimulated with the highest concentration of LPS produced 1.4 times more spots than non-stimulated cells in both cell types. Head kidney leukocytes produced 1.2 times more spots than spleen leukocytes in all stimulation groups. Seven days post stimulation, unstimulated head kidney leukocytes and spleen leukocytes produced an approximately equal amount of spots. Stimulated spleen leukocytes produced a higher amount of spots than head kidney leukocytes in cells treated with both concentrations of LPS. In LPS-treated groups, spleen leukocytes produced 1.4 times more spots than head kidney leukocytes at both concentrations. At both time points and in both organs, cells treated with the highest concentration of LPS yielded the highest amount of spots.

From the pilot experiments, we were able to optimize the method and prove that it worked. The pilot experiments made us able to select a control (LPS) and a concentration (100 µg/mL) that confirms stimulation of cell populations. We also found that a total incubation period of 5 days (3 days in culture and two days in ELISPOT wells) was most suited for our purposes. During the establishing of this method, we included two negative controls, one where the biotinylated antibody was excluded and one where cells were excluded. This was done to confirm that the spots detected by the method represents antibody secreting cells. We observed no spots in either controls, confirming that our method is specific to antibody secreting cells.

3.3.2 Effect of rBAFF or rAPRIL IgM+ antibody secreting cell populations in head kindey leukocytes

Lastly, an ELISPOT assay was set up to investigate the effect of BAFF and APRIL on head kidney leukocytes. In rainbow trout, the cytokines have shown to augment survival and antibody secretion of B cells (Tafalla et al., 2017). The cells were stimulated with LPS, CpG, BAFF and APRIL at concentrations listed earlier, or left unstimulated. The cells were further split into two groups labeled “counted” and “uncounted”. Our “uncounted” group was assumed to have had 100% viability since the start of the incubation period, while our “counted” group was counted before being seeded on the ELISPOT membranes. This was done to gain insight in the role of BAFF and APRIL on the survival of Atlantic salmon ASCs. Moreover, the assays were set up to provide information on the role of BAFF and APRIL in augmenting the antibody secretion in Atlantic salmon leukocytes. Results are presented in Figure 19.

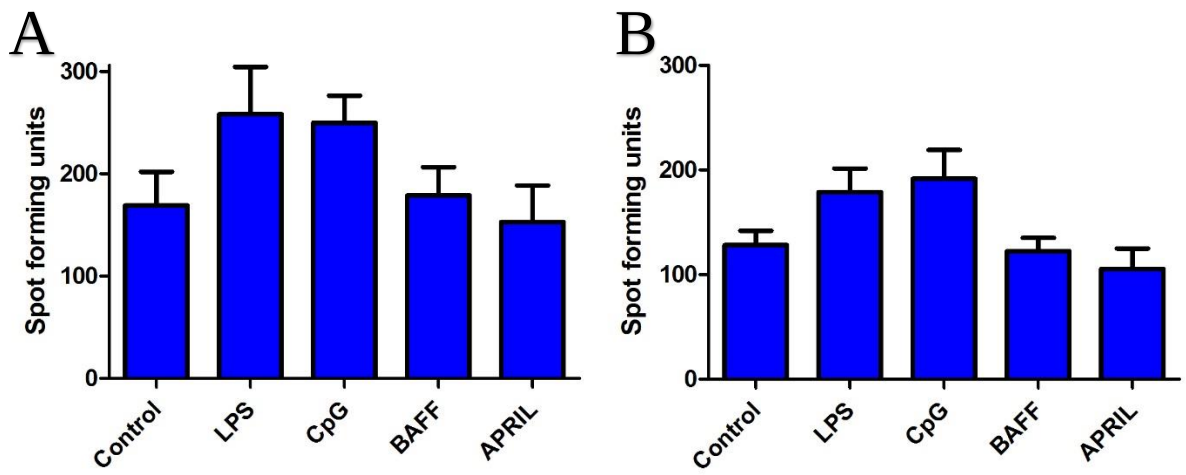


Figure 19: Spot forming units in counted (A) and uncounted (B) head kidney leukocytes at 5 days post stimulation. Cells stimulated with LPS (100ug/ml), CpG (5 µM), BAFF (2,5ug/ml), or APRIL (2,5ug/ml) for 3 days were seeded into ELISPOT wells (75.000 cells/well in triplicates). Plates were developed 2 days after transfer to ELISPOT plates. Columns display mean spot counts ±standard error (n=4). No statistical significance between treatment groups.

Little difference was observed between cells treated with CpG and LPS. CpG- and LPS treated cells produced approximately 1.5- and 1.4 times more spots in the "counted and "uncounted" groups, respectively. Treatment with BAFF and APRIL produced no notable differences in spot counts in any of the groups. Cells in the "counted" group produced higher amounts of spots than cells from the uncounted group. Interestingly, several small spots, that were not included in the automatic counts,were observed in higher numbers in the CpG-, BAFF- and APRIL treated wells compared to the control (results not shown). We were unable to attain accurate manual counts of these wells.Images of the control-, LPS-, BAFF-, and APRIL treated wells are presented in appendix V.

4. Discussion

Future growth in the Norwegian aquaculture sector is wholly dependent on solving todays viral- and environmental challenges. In 2017 a total of 407 reports of viral disease outbreaks were reported in Norway (Veterinærinstituttet, 2018). Viral diseases not only account for huge economic loss in the form of mortality and reduced growth, but also poses ethical obstacles in terms of fish welfare. In spite of nationwide employment of viral vaccines, outbreaks are on the rise and the development of effective vaccines is a prerequisite for the continued growth of the industry (Robertsen, 2011). To achieve control of viral diseases, knowledge of fish immunology needs to be enhanced. More specifically, a better understanding of the interactions between the immune system and the virus is required. Cytokines are signaling molecules of the cellular communications, and essential for the functioning of innate and adaptive immune responses (Magnadottir, 2010). BAFF, APRIL, BAFF-R, BCMA and TACI are cytokines and receptors belonging to the TNF superfamily and are especially important in the B cell arm of the immune system (Bossen & Schneider, 2006). B cells are the effector cells of humoral immunity, providing protection from pathogens through antibody production. Teleost BAFF has been shown to significantly contribute to innate and adaptive immune responses, and it has protective functions when applied as an adjuvant in DNA vaccines (Sun & Sun, 2015). On the other hand, APRIL has not been studied to the same extent as BAFF, but has similarly been shown to enhance teleost B cell functions (Soleto et al., 2017).

4.1 *In-vitro* studies of BAFF, APRIL and receptors

4.1.1 Constitutive expression of BAFF, APRIL and receptors

B cells are known targets of BAFF and APRIL which are secreted primarily by myeloid cells (Moore et al., 1999; Nardelli et al., 2001). Binding of BAFF and APRIL to their respective receptors trigger an array of functions within B cells, including survival, proliferation and differentiation (Mackay & Schneider, 2009) and, in healthy individuals, BAFF and APRIL play important roles in B lymphocyte homeostasis (Schneider, 2005). We investigated the constitutive expression of BAFF, APRIL and their receptors in different immune cell types to gain an understanding of BAFF and APRILs function in normal physiological states of salmon.

4.1.1.1 The distribution of BAFF & APRIL

BAFF- and APRIL transcripts were detected in all cell types examined. BAFF and APRIL are both synthesized as membrane-bound proteins that can be released in soluble form by proteolytic processing (Moore et al., 1999; Schneider et al., 1999).

BAFF transcripts were expressed in a hundred- to thousand-fold greater magnitude than APRIL across all cell types examined. Highest transcriptional levels of BAFF were observed in head kidney macrophage-like cells, while levels were comparable and low between B cell subtypes. These results confirm that head kidney MLCs transcribe BAFF under normal physiological conditions, but also demonstrate that B cells are capable of, at least, transcribing the cytokine themselves. Similar to our results, constitutive expression of BAFF transcripts has been observed in rainbow trout splenic, head kidney and peripheral blood B cells. In trout, the presence of BAFF protein in splenic B cells was further confirmed through flow cytometry and confocal imaging (Tafalla et al., 2017). Since trout BAFF transcripts were detected at protein levels in splenic IgM+ B cells, it is reasonable to believe that protein expression also occurs in salmon peripheral blood, head kidney and spleen B cells. BAFF is of great importance in normal function and development of B cells (Mackay et al., 2003), furthermore, the head kidney of fish houses a variety of B cell populations (Zwollo et al., 2005), thus, the presence of BAFF in head kidney MLCs suggests that salmon BAFF is involved in B cell development and function under normal physiological conditions. In humans and mice, BAFF is produced by dendritic cells, follicular dendritic cells, macrophages, T cells and stimulated neutrophils, but never by resting B cells (Craxton et al., 2003; El Shikh et al., 2010; El Shikh et al., 2009; Moore et al., 1999; Nardelli et al., 2001; Scapini et al., 2003). This suggests that the function of BAFF is different in teleosts than in mammals. APRIL was barely detectable in all cell types examined, suggesting that APRIL is produced in low amounts in these cells. Studies in mammals have shown APRIL to be produced constitutively and in abundance in bone marrow and function as a long-term survival signal to plasma cells (PCs) (Matthes et al., 2011). Teleost head kidney is suggested to function as an analogue to mammalian bone marrow and houses plasma cells (Zwollo et al., 2005), and we would therefore expect to find high levels of APRIL transcripts in head kidney MLCs.

4.1.1.2 The distribution of BAFF and APRIL Receptors

We were able to detect BAFF-R, BCMA and TACI transcripts in all cell types examined. BAFF-R, BCMA and TACI are classified as transmembrane III receptors, characterized by an

extracellular N-terminus, a COOH region that is responsible for intracellular signaling, absence of a signaling peptide and a single transmembrane domain (Magis et al., 2012).

Head kidney MLCs expressed negligible amounts of both BCMA- and TACI transcripts but considerable amounts of BAFF-R transcripts. This is in contrast to what is observed in humans, where BAFF-R is mainly expressed by B cells, and to a lesser extent by T cells (Ng et al., 2004). TACI has been shown to be expressed constitutively and intracellularly in low amounts in human monocytes. When the monocytes are exposed to BAFF, transcription of TACI is upregulated and the receptor is translocated to the cell membrane. BAFF is able to stimulate monocytes through engagement to TACI, thus activating the NFκB pathway (S. K. Chang et al., 2006). This further promotes activation and differentiation of monocytes and, by doing so, increases production of IL-6, TNFα and IL-1β within the monocytes and further amplifies the immune response (S. K. Chang et al., 2006). Since the BAFF-R in B cells is shown to be connected to the NFκB pathway (Hildebrand et al., 2010), it is reasonable to believe that the receptors are connected to this pathway in head kidney MLCs as well. Thus, engagement of BAFF to BAFF-R on head kidney MLCs might exert the same functions as has been observed in human monocytes expressing TACI, and can possibly be a mechanism to provide survival signals to MLCs and also function to further amplify ongoing immune responses. In support of this theory is the fact that rBAFF has been shown to induce macrophage activation in tongue sole (Sun & Sun, 2015). However, our results only demonstrate the transcriptional expression of the receptor; we do not know whether it is expressed as a functional protein or not. Additionally, if transcripts are indeed translated to protein, we do not know if the protein is expressed as a membrane bound receptor, or if it is contained intracellularly. Moreover, because of structural differences in mammalian and fish BAFF-R, it is questionable whether the receptor is functional and whether it trigger the same signaling pathways as in mammals. Further investigations on the production, expression and activation of BAFF-R in salmon MLCs are warranted.

Within B cell populations, notable differences in the expression of receptor-transcripts were observed. Because the different receptors trigger different signaling pathways within the cell (Figure 2), the observed differences may imply that the cells react differently upon engagement to BAFF and APRIL, and may represent different maturation stages and functional groups of B cells. This further builds on the notion that the outcome of BAFF- and APRIL-engagement to B cells is highly dependent on context.

In mammals, signals received from BAFF-R is of great importance in developing B cells (Schiemann et al., 2001), and with the exception of plasma cells, the receptor is expressed in the majority of mature B cell types, , where it delivers continuous survival signals to the cells (Darce, Arendt, Wu, et al., 2007). What is known of BAFF-R function in mammals seem to be true in salmon, as BAFF-R expression was prevalent in all B cell types examined, suggesting that BAFF-R has a global function in B cell maturation and survival. BAFF-R was expressed to the highest extent in peripheral blood B cells, while comparable levels were seen in head kidney and spleen B cells. What is known from mice, is that BAFF-R is expressed in a small scale in immature B cells, and that the expression of the receptor gradually increases during B cell maturation, and is finally expressed in considerable amount on all mature B cells (Darce, Arendt, Chang, et al., 2007). Our results might suggest that the peripheral blood of salmon contain a higher proportion of mature B cells, while spleen and head kidney houses a bigger proportion of developing B cells types; alternatively, a higher proportion of the B cells in head kidney and spleen are plasma cells/plasmablasts.

In this investigation, we have shown that B cells derived from different immune tissues transcribe both BAFF and BAFF-R constitutively. If both transcripts are indeed expressed as proteins, this may suggest that salmon B cell survival and development is supported by an autocrine pathway. Notably, several autoimmune disorders and cancer types, such as lymphoma (He et al., 2004) and systemic lupus erythematosus (Chu et al., 2009), are caused by B cells producing BAFF, thus resulting in an autocrine loop. Human B1 cells have been shown to express levels of BAFF transcripts but do not express the protein (Chu et al., 2007). This may suggest that the expression of BAFF in B lymphocytes is an ancient mechanism that is not conserved in higher vertebrates.

BCMA signaling is closely related to the survival of antibody secreting cells and crucial for the survival of bone marrow plasma cells in mammals (O'Connor et al., 2004). In humans, BCMA is only expressed by tonsillar memory B cells and plasma cells (Darce, Arendt, Wu, et al., 2007). BCMA transcripts were found in all B cell types examined and was most highly expressed in spleen and peripheral blood B cells, while the lowest expression was found in head kidney B cells. B cells used in this work was isolated using MACS, a technique that sorts cells based on membrane IgM expression. Although it has been speculated that human plasma cells retain IgM (D. Pinto et al., 2013), there is general uncertainty whether this is true, and the situation is even more uncertain in fish. If we assume that the B cell types examined do not

contain plasma cells, then the expression of BCMA indicates that BCMA is expressed by other cells than plasma cells. This may suggest that salmon BCMA signaling is different from what is observed in higher vertebrates. Further supporting an alternative function of BCMA in fish is the fact that the structure of BCMA is different in trout and mammals. A limiting amino acid residue, known to inhibit the engagement of BAFF to BCMA is not found in fish (Granja et al., 2017). This might imply that BAFF is able to bind BCMA with high affinity, and further suggesting a different function of BCMA altogether. If we are to assume that B cells in salmon retain membrane-bound IgM when differentiating to plasma cells, then this suggests that plasma cells reside in peripheral blood, spleen and head kidney. This is indeed possible, as plasma cells have been suggested to circulate between these tissues, however, the head kidney of trout is thought to house the vast majority of plasma cells (Bromage et al., 2004).

In mammals, TACI is shown to have important functions in the differentiation and survival of plasmablasts, particularly those derived from innate B cells (von Bulow et al., 2001). Additionally, TACI is able to negatively regulate B cells (Mackay & Schneider, 2008); this is demonstrated in $TACI^{-/-}$ mice where a significant increase in B lymphocytes is observed (von Bulow et al., 2001). In mammals, TACI is expressed mainly in mature B lymphocytes (Groom et al., 2007). In rainbow trout, TACI has been found in high quantities in mucosal surfaces and a role of TACI in immunological tolerance in these sites are suggested by the authors Granja et al. (2017). Rainbow trout TACI has shown to have significant structural differences when compared to its mammalian counterpart. Trout TACI does not possess a TRAF binding consensus site, implicating key differences in intracellular signaling upon TACI engagement (Granja et al., 2017). Based on the salmon genome, TACI seems to have two variants. In our studies, we only used primers for one variant, and as such, our studies may only represent part of the receptor expression. As in mammals (Treml et al., 2007), the constitutive expression of TACI in salmon was, in general, modest when compared to that of BAFF-R and BCMA. In our investigations, TACI was detected in negligible amounts in head kidney MLCs and in low but detectable levels in all B cell populations examined, this is in contrast to what has been observed in trout by Granja et al. (2017), who did not detect TACI in B cell populations. Because modest and comparable levels of TACI was found between B cell types, our results may suggest that, like in mammals, salmon TACI possess regulatory functions of B cells.

In conclusion, we have demonstrated that head kidney, spleen, and peripheral blood IgM+ B cells, as well as head kidney MLCs, were able to constitutively transcribe BAFF, APRIL and

their receptors. Considerable transcription of BAFF and BAFF-R was observed in head kidney MLCs, while considerable transcription of BAFF and all the receptors were observed in B cells derived from different tissues. If the transcripts are translated at a protein level, the ability of the cells to produce both ligand and receptor suggest that these cells are regulated partly by autocrine pathways. Additionally, the ability of B cells to produce BAFF, thus being able to further activate adaptive immune cells, suggests that salmon B cells possess innate functions. Furthermore, BCMA and TACI is expressed differently within B cell types, suggesting that B cells from different compartments within the fish have distinct functions. BAFF-R, BCMA and TACI possess key structural differences compared to their mammalian counterparts, making it possible that the function of the receptors is vastly different in fish than in mammals. The distribution of BAFF and APRIL receptor transcripts were mostly in line with what has been observed in rainbow trout by Granja et al. (2017), however, we were able to detect TACI transcripts in all our samples, while Granja *et. al.* were not. BAFF-R transcripts were detected in head kidney MLCs, suggesting that these cells react upon exposure to BAFF. Because these cells are seemingly able to produce both BAFF and BAFF-R, autocrine signaling pathways might be present in these cells; however, head kidney MLCs are likely a heterogeneous group of cells, and expression of receptor and ligand might not be limited to one cell type. BAFF was expressed to a significantly greater magnitude than APRIL in all cell types, suggesting that most signaling through BAFF/APRIL receptors under normal physiological conditions occur through BAFF. This is likely possible because BAFF can bind the same receptors as APRIL, in addition to being able to bind BAFF-R. This may implicate that APRIL signaling is not commonly used under normal physiological conditions. In response to infection, we might expect to see an upregulated expression of APRIL and receptors BCMA and TACI, as APRIL binds more readily to these receptors signaling through these receptors can exert regulatory functions on B cell populations.

4.1.2 Induction of BAFF and APRIL in myeloid cells upon stimulation

Similar to most other members of the TNF superfamily, the expression of BAFF and APRIL is dependent on the activation state of the cells producing them. In mammals, transcriptional levels in BAFF- and APRIL producing cells are usually low or undetectable in resting cells, but is upregulated in response to cytokines, in particular interferons (Nardelli et al., 2001) and TLR-ligands (Vincent et al., 2014). Evidence suggest that BAFF is an important factor in B cell expansion and antibody responses upon infection (Nardelli et al., 2001). Additionally, upon stimulation with TLR-ligands and cytokines, B cells are known to augment the expression

patterns of BAFF/APRIL receptors on their surface, thus priming them to react specifically upon stimulation (Katsenelson et al., 2007). Cells of the innate immune system are capable of stimulating B cells (Cerutti et al., 2011), and considerable cross-talk between innate and adaptive compartments has been observed (Striz et al., 2014). In Atlantic salmon, several TLRs have been identified (Arnemo et al., 2014; Skjaeveland et al., 2009; Skjaeveland et al., 2008), and engagement of TLRs to their respective ligands have shown to promote the production of various cytokines (Strandskog et al., 2008). We therefore hypothesized that an increased production of BAFF and APRIL upon TLR-engagement to their respective ligands in Atlantic salmon head kidney MLCs would occur. An experiment was conducted where adherent head kidney MLCs were stimulated with the cytokines IFNγ and IFNα and TLR-ligands CpG and Poly I:C.

The stimulations generally provoked an up-regulation of BAFF- and APRIL transcripts in the cells. APRIL transcripts were upregulated by all stimulants, with the exception of IFNα. APRIL was most highly induced by Poly I:C, a molecule mimicking double stranded RNA, which is present is some viruses, and is known to stimulate antiviral responses. In all stimulation groups, with the exception of IFNα, APRIL was upregulated to a considerably higher extent than BAFF. This is not surprising, as steady-state levels of the cytokine were particularly low, such that even a modest quantitative increase might easily result in a high fold-induction. Transcriptional levels of BAFF were very modestly up-regulated upon stimulation with Poly I:C, IFNγ and IFNα, while CpG-stimulation resulted in the down-regulation of BAFF 24- and 48 hours post stimulation. One would expect that secretion of BAFF would be upregulated upon stimulation with CpG, as CpG is able to bind TLRs, and is shown to induce immune responses. CpG can independently and directly stimulate B lymphocytes (Buchanan et al., 2011), and has in mouse splenic B cells shown to upregulate the expression of both BAFF-R and TACI, thus priming them to react to BAFF and APRIL (Katsenelson et al., 2007). It is therefore unexpected that our cells should downregulate the expression of the cytokines upon CpG-exposure. Our results warrant further investigations on the effect of CpG on BAFF and APRIL production in salmon myeloid cells.

The engagement of Poly I:C and CpG to innate cells of the immune system induces the production of cytokines in teleosts (Robertsen, 2006). In our experiment, it seems likely that the production of BAFF and APRIL is induced by the stimulants directly, by engaging

BAFF/APRIL producers, as well as indirectly, by stimulating the production and release of cytokines from innate cells, thus stimulating BAFF- and APRIL producing cells.

Our results confirm that stimulations with microbial products and cytokines trigger the transcription of BAFF and APRIL in head kidney MLCs in salmon. This further underscores the role of BAFF and APRIL in the recruitment and stimulation of B cells and the mounting of an adaptive immune response following an infection. The very modest induction of BAFF in *in vitro* stimulation compared to *in vivo* stimulation might indicate that MLCs are not the primary producers of the cytokine. We know that B cells constitutively produce BAFF, and thus, we would expect transcription of the cytokines to be induced in these cells as well. Indeed, a considerable amount of the BAFF transcripts observed in our *in vivo* investigations may be product of B cells. Although not investigated in this work, the ability of BAFF and APRIL to augment BAFF/APRIL receptor expression on the surface of B cells, thus priming them for activation, may have implications in future vaccine development, and should be investigated further in Atlantic salmon.

4.1.3 Effect of BAFF and APRIL on IgM production and survival in head kidney IgM+ B cells

To date, there are only a few functional studies of fish BAFF and APRIL. The cytokines have been investigated in rainbow trout (Tafalla et al., 2017) and tongue sole (Sun & Sun, 2015). So far, there have been contradictory reports of the lymphoproliferative effects of BAFF in fish, as Tafalla et al. (2017) postulates that lymphoproliferative effects do not exist, while Sun and Sun (2015) claim that they do. This may be due to inherent differences between fish species examined in the two investigations, but is more likely due to the fact that Sun andSun did not discriminate between survival and proliferation in their investigation. As for BAFF and APRIL as B cell survival factors, it has been shown that presence of rBAFF and rAPRIL do not promote survival in trout head kidney B cells, although survival effects of rBAFF has been demonstrated in trout splenic B cells (Tafalla et al., 2017).

In this work, we aimed to determine whether recombinant BAFF and APRIL would increase the number of IgM secreting cells and/or promote survival of ASCs in head kidney leukocyte culture. To do this, ELISPOT assays were carried out. Unpublished data from our lab (Peñaranda, 2018) demonstrate that head kidney B cell survival is increased in the presence either rBAFF or rAPRIL. To further investigate the survival effects, an ELISPOT assay was set up using a “counted” and an “uncounted” group. Both groups were seeded in culture plates in known cell numbers. After incubation, the “counted” group was recounted for the presence of

viable cells before being reseeded on the ELISPOT membranes, while our "uncounted" group was assumed to represent 100% survival. The aim with these procedures was to estimate whether any observed increase in spot counts were due to increased survival of ASCs or due to increased production of secreted IgM itself. LPS was included as a positive control because it has previously been shown to stimulate survival and antibody secretion in teleost IgM+ B cells (Abos et al., 2016). Similarly, the TLR9-agonist CpG was included, also as a positive control of lymphocyte activation and Ig-secretion (Jenberie et al., 2018).

Previous ELISPOT assays done using rainbow trout splenocytes showed an increase in IgM secreting cells in the presence of rBAFF (Tafalla et al., 2017). In the present ELISPOT assays, using salmon head kidney leukocytes, we did not observe any increase in IgM secreting cell numbers in the presence of rBAFF or rAPRIL. However, in mainly BAFF-, but also APRIL- and CpG-treated leukocytes, we observed an evident increase of smaller spots that were not detectable by our counting software, and are not represented in the results. Raw pictures of representative ELISPOT wells are presented in appendix V. Our contrasting results to rainbow trout is likely stemming from different compositions of ASCs in leukocytes isolated from the two different organs, additionally, there could be differences between trout and salmon B cells. From our previous results, we know that B cell types from these two tissues have different expression patterns of BAFF and APRIL receptors, and thus will react differently upon exposure to the cytokines. In salmon, head kidney- and spleen B cells express BAFF-R and TACI in comparable amounts, but splenic B cells transcribe BCMA to a considerably higher extent than head kidney B cells; this might suggest that splenic B cells are able to react more readily upon exposure to BAFF. To our knowledge, there are no other reports of BAFF- and APRIL treated leukocytes producing small spots in ELISPOT assays. A probable explanation of the small size of these spots is that the B cells producing them are naïve and has not yet been activated by antigens and, as such, they inherently do not produce large quantities of immunoglobulins. Naïve B cells produce small amounts of antibodies, and it is only when they bind an antigen that they proliferate and differentiate to antibody-secreting effector cells (Lamond, 2002). LPS and CpG are intrinsically antigens, so it is reasonable that we observe activated leukocytes in these treatment groups. If the majority of head kidney B cells are naïve, then we would not expect them to significantly increase production of IgM in response to BAFF or APRIL. Plasma cells, however, inherently express BCMA in considerable amounts and the activation of the receptor results in increased survival of the cells (O'Connor et al., 2004). Increased survival in plasma cell can likely be observed as an increased number of spots in an

ELISPOT assay. In our investigations of B cell receptors, we observed that splenic B cells express BCMA to a greater extent than head kidney B cells. The ability of spleen B cells to produce more spots upon exposure to BAFF may be due to different maturation stages of plasma cells. Splenic plasma cells may represent a more immature stage of plasma cell development that react more readily upon exposure to cytokines by expressing more receptors than head kidney plasma cells. Additional analyses should include both LPS+BAFF/APRIL and LPS treated groups determine the effect of BAFF and APRIL on activated B cells. Additionally, ELISPOT assays should be done using cells from both head kidney and spleen to determine the effects of rBAFF and rAPRIL on different B cell types.

4.2 *In-vivo* investigations of BAFF and APRIL

4.2.1 The distribution of BAFF and APRIL in tissues

As stated earlier, several cells have the ability to produce BAFF and APRIL, in humans these cells primarily consist of myeloid lineage cells (Nardelli et al., 2001). From our *in-vitro* experiments, we observed that both head kidney MLCs and a variety of B cell types were able to produce BAFF and APRIL. Additionally, we observed that production of BAFF and APRIL was induced in stimulated head kidney MLCs. Taking into consideration the amount of cells that are capable of producing the cytokines, it is reasonable to believe that the cells we examined are not the sole producers of BAFF and APRIL. As such, examining transcriptional levels in whole organs will provide us with a global understanding of where the cytokines are produced.

We analyzed levels of constitutive BAFF- and APRIL expression in tissues obtained from naïve salmon. We found that expression of BAFF was highest in spleen; this is in line with observations done in other fish species (Ai et al., 2011; Cui et al., 2012; H. Z. Liu et al., 2016; Tafalla et al., 2017). The exceptions are miiuy croaker and grass carp, where the highest levels of BAFF mRNA was found in the skin (Meng et al., 2015; Pandit et al., 2013). Intermediate levels of BAFF was found in head kidney heart and pancreas, while the lowest levels of the cytokine were found in gills and liver. The distribution of BAFF is similar to what has been reported in rainbow trout by Tafalla et al. (2017) and tongue sole by Sun and Sun (2015). APRIL was expressed to the highest extent in organs head kidney, spleen and pancreas, and was modestly expressed in liver, heart and gills. We expected to find high levels of BAFF in spleen and head kidney as these organs are considered to be the primary source of developing- and mature B cells (Salinas et al., 2011), both reliant of BAFF signaling to survive (Rolink et

al., 2002). We know that APRIL serves as a regulator of mature B cells, especially plasma cells, by signaling through TACI and BCMA (O'Connor et al., 2004; Yan et al., 2001), and as such, we expected to find levels of APRIL in these organs as well. The distribution of APRIL might serve as an indication that the function of APRIL is more prevalent in plasma cells. We did not expect the relatively high constitutive expression of BAFF in the heart. The heart is not typically associated with immunological functions, although in Atlantic cod (*gadus morhua*), the organ has been shown to be involved in antigen trapping (Arnesen et al., 2002; Dalmo et al., 1996; Dalmo et al., 1998; Smedsrod et al., 1995). Thus, BAFF and APRIL might be secreted by APCs within the heart. Intermediate levels of both BAFF and APRIL were observed in the pancreas, this was also unexpected, as teleost pancreas is not closely associated with B cell populations. Levels of BAFF and APRIL in the pancreas could present artefacts of the organ harvesting procedure, as pancreas tissue is interspersed in the pyloric caeca of the gastrointestinal tract, and pancreas samples will inevitably include intestinal tissues. Cells from both the innate- and adaptive immune systems are present in teleost gut (Rombout et al., 2011), and immunoglobulins have important functions in these areas (Rombout et al., 2014). Both BAFF and APRIL are involved in immunological functions of the gut in mammals (Fagarasan & Honjo, 2004), so it is not surprising that we should find the cytokines is salmon gut. Further investigations in the function of BAFF and APRIL in the salmon gut might be of interest.

4.2.2 Expression patterns of BAFF and APRIL in SAV-infected tissues

To determine the effect of an infection on the distribution of BAFF and APRIL in salmon, we infected salmon by i.p. injection of the virus. By measuring SAV3 transcripts, we confirmed that the virus had been taken up into the blood stream of the fish. Through the cardiovascular system, the virus is able to reach its target tissues, the pancreas and the heart (Bruno, 2013).

Expression of both BAFF and APRIL was upregulated in all tissues upon infection, confirming that the cytokines take part in the mounting of an immune reaction against pathogens. Notably, BAFF and APRIL were most highly upregulated in the target organs of the virus. In target tissues, virus particles and virus-infected cells are endocytosed by resident myeloid cells. In fish, these cells alert other cells of the immune system by secreting, among others, the cytokines IFNγ (Robertsen, 2006) and IL-10 (R. D. Pinto et al., 2007; Zou et al., 2003). We know that in mammals, these cytokines enhance the production of BAFF (Nardelli et al., 2001), and as such, it seems reasonable to assume that similar dynamics are taking place in the present infection

study. Synthesis and release of APRIL from myeloid cells is poorly mapped in both mammals and fish, however, we observed an induction of APRIL transcripts, so it is reasonable to infer that signaling pathways are similar. BAFF and APRIL can then augment B cell function by promoting survival and increasing IgM secretion, as demonstrated in rainbow trout (Granja et al., 2017; Tafalla et al., 2017). Additionally, we know that in mammals, BAFF serves to activate monocytes (S. K. Chang et al., 2006), and we have observed that salmon MLCs express BAFF-R, thus, BAFF secretion likely aid in innate responses during the infection.

We observed a modest and delayed upregulation of both BAFF and APRIL in the spleen and head kidney following infection; the upregulation appeared 8 days post infection, and was maintained at 14 days post infection. In fish, affinity maturation of immunoglobulins is believed to occur (Kaattari et al., 2002), and likely occurs within melano-macrophage centers located in the spleen and head kidney (Agius & Roberts, 2003). BAFF is shown to have an important role related to affinity maturation in mammals, as demonstrated in mice (Rahman et al., 2003). The increased levels of BAFF and APRIL in the systemic tissues from virus infected fish suggest their involvement in the mounting of an adaptive immune response.

Significant induction of BAFF transcripts was observed as early as 3 days post infection in heart and liver samples, while APRIL induction peaked at 14 days post infection in all organs tested. The liver is closely related to the production of acute phase proteins, participating in the acute phase reaction in fish upon infection (Bayne & Gerwick, 2001). This reaction is an innate mechanism that rapidly responds to tissue trauma and infection. Under an acute phase reaction, hepatocytes secrete large amounts of acute phase proteins (APPs) that act to limit the dispersal of- and eliminate pathogens, repair tissue damage and restore homeostasis in the individual (Bayne & Gerwick, 2001). We know that in humans, BAFF plays a role in the activation of innate immune cells by binding TACI on these cells (S. K. Chang et al., 2006), and we have demonstrated the capability of innate salmon cells to express BAFF-R; it therefore seems reasonable that BAFF might be involved in the activation and stimulation of hepatocytes secreting APPs. Further supporting this hypothesis is the fact that BAFF was extensively upregulated 3 days post infection, but returned to sub-constitutive levels at 8 days post infection, this is in line with the dynamics of APPs observed in fish (Bayne & Gerwick, 2001). Interestingly, in regard to kinetics, BAFF has been shown to behave like an APP in humans (Pongratz et al., 2013). APRIL transcripts in the liver were similarly upregulated at three and 14 days post infection, albeit not to the same extent as BAFF. APRIL transcripts reach their

peak induction at 14 days post infection, while BAFF transcripts reach their peak induction at 4 days post infection. Since APRIL is upregulated in the same pattern as BAFF, it seems likely that APRIL might also be involved in the acute phase reaction. Elevated levels of both BAFF and APRIL in liver at 14 days post infection may point toward the fact that our hypothesis does not hold true, as the acute phase does not persist until this time point, on the other hand, the cytokines may serve other functions in this organ at that time point.

4.3 Conclusions

- BAFF and APRIL mRNA was detected in all examined organs in naïve, healthy Atlantic salmon, suggesting that the cytokines have regulatory functions in all organs during normal physiological conditions. The highest expression of BAFF was found in the spleen, while the highest expression of APRIL was found in the head kidney.
- *In-vivo* investigations of SAV3-infected fish revealed up-regulation of BAFF- and APRIL transcripts in all organs, suggesting that the cytokines have important functions in response to infection. Considerable inductions of the cytokines were seen in target organs of the virus, suggesting that the cytokines are produced by local effector cells during an infection.
- Steady-state BAFF and APRIL transcripts were observed *in-vitro* in B lymphocytes and macrophage-like cells. In all cells examined, APRIL transcripts were relatively low, while BAFF transcripts were expressed at considerable levels, suggesting that these cells mainly produce BAFF.
- Steady-state expression of BAFF- and APRIL receptor transcripts were observed in cultivated B lymphocytes and macrophage-like cells. In B cells derived from different tissues, all transcripts were found in considerable quantities. In macrophage-like cells, BCMA and TACI transcripts were close to undetectable, while BAFF-R transcripts were found at higher levels. Within B cell populations, expression patterns of the receptors differed; different expression patterns of the receptors indicate different response types upon engagement to either BAFF or APRIL in these cell.
- Head kidney macrophage-like cells stimulated *in-vitro* with CpG, Poly I:C, IFNγ and IFNα resulted in the upregulation of APRIL mRNA levels, while all stimulants, except CpG, increased BAFF transcription. These results imply that BAFF and APRIL take part in the immune responses following both viral and bacterial infection.
- No apparent increase in IgM+ secreting cells was observed by ELISPOT in head kidney leukocytes cultured in the presence of BAFF or APRIL alone. This could suggest that BAFF and APRIL functions are geared toward activated ASCs.

5. References

Abbas, A. K., Lichtman, A. H., & Pillai, S. (2016). *Basic immunology : functions and disorders of the immune system* (Fifth edition. ed.). St. Louis, Missouri: Elsevier.

Abos, B., Wang, T. H., Castro, R., Granja, A. G., Leal, E., Havixbeck, J., . . . Tafalla, C. (2016). Distinct Differentiation Programs Triggered by IL-6 and LPS in Teleost IgM(+) B Cells in The Absence of Germinal Centers. *Scientific Reports, 6*. doi:ARTN 30004

10.1038/srep30004

Agius, C., & Roberts, R. J. (2003). Melano-macrophage centres and their role in fish pathology. *Journal of Fish Diseases, 26*(9), 499-509. doi:DOI 10.1046/j.1365-2761.2003.00485.x

Ai, H. X., Shen, Y. F., Min, C., Pang, S. Y., Zhang, J. X., Zhang, S. Q., & Zhao, Z. H. (2011). Molecular structure, expression and bioactivity characterization of TNF13B (BAFF) gene in mefugu, Takifugu obscurus. *Fish & Shellfish Immunology, 30*(6), 1265-1274. doi:10.1016/j.fsi.2011.03.020

Akira, S., & Takeda, K. (2004). Toll-like receptor signalling. *Nature Reviews Immunology, 4*(7), 499-511. doi:10.1038/nri1391

Andersen, L., Bratland, A., Hodneland, K., & Nylund, A. (2007). Tissue tropism of salmonid alphaviruses (subtypes SAV1 and SAV3) in experimentally challenged Atlantic salmon (Salmo salar L.). *Archives of Virology, 152*(10), 1871-1883.

Anderson, D. P., Robertson, B. S., & Dixon, O. W. (1979). Cellular Immune-Response in Rainbow-Trout Salmo-Gairdneri Richardson to Yersinia-Ruckeri O-Antigen Monitored by the Passive Hemolytic Plaque-Assay Test. *Journal of Fish Diseases, 2*(3), 169-178. doi:DOI 10.1111/j.1365-2761.1979.tb00156.x

Arnemo, M., Kavaliauskis, A., & Gjoen, T. (2014). Effects of TLR agonists and viral infection on cytokine and TLR expression in Atlantic salmon (Salmo salar). *Developmental and Comparative Immunology, 46*(2), 139-145. doi:10.1016/j.dci.2014.03.023

Arnesen, S. M., Schroder, M. B., Dalmo, R. A., & Bogwald, J. (2002). Antigen uptake and immunoglobulin production in Atlantic cod (Gadus morhua L.) after intraperitoneal injection of Vibrio anguillarum. *Fish & Shellfish Immunology, 13*(2), 159-170. doi:10.1006/fsim.2001.0390

Aunsmo, A., Valle, P. S., Sandberg, M., Midtlyng, P. J., & Bruheim, T. (2010). Stochastic modelling of direct costs of pancreas disease (PD) in Norwegian farmed Atlantic salmon (Salmo salar L.). *Preventive Veterinary Medicine, 93*(2-3), 233-241. doi:10.1016/j.prevetmed.2009.10.001

Avery, D. T., Kalled, S. L., Ellyard, J. I., Ambrose, C., Bixler, S. A., Thien, M., . . . Tangye, S. G. (2004). BAFF selectively enhances the survival of plasmablasts generated from human memory B cells (vol 112, pg 286, 2003). *Journal of Clinical Investigation, 113*(7), 1069-1069. doi:10.1172/Jci200318025c

Baldwin, A. S. (1996). The NF-kappa B and I kappa B proteins: New discoveries and insights. *Annual Review of Immunology, 14*, 649-683. doi:DOI 10.1146/annurev.immunol.14.1.649

Basu, M., Lenka, S. S., Paichha, M., Patel, B., Banerjee, R., Das, S., . . . Samanta, M. (2016). B cell activating factor is induced by toll-like receptor and NOD-like receptor-

ligands and plays critical role in IgM synthesis in Labeo rohita. *Molecular Immunology, 78*, 9-26. doi:10.1016/j.molimm.2016.08.010

Bayne, C. J., & Gerwick, L. (2001). The acute phase response and innate immunity of fish. *Developmental and Comparative Immunology, 25*(8-9), 725-743. doi:Doi 10.1016/S0145-305x(01)00033-7

Beutler, B. (2004). Innate immunity: an overview. *Mol Immunol, 40*(12), 845-859.

Bodmer, J. L., Schneider, P., & Tschopp, J. (2002). The molecular architecture of the TNF superfamily. *Trends in Biochemical Sciences, 27*(1), 19-26. doi:Doi 10.1016/S0968-0004(01)01995-8

Boshra, H., Li, J., & Sunyer, J. O. (2006). Recent advances on the complement system of teleost fish. *Fish & Shellfish Immunology, 20*(2), 239-262. doi:10.1016/j.fsi.2005.04.004

Bossen, C., & Schneider, P. (2006). BAFF, APRIL and their receptors: structure, function and signaling. *Semin Immunol, 18*(5), 263-275. doi:10.1016/j.smim.2006.04.006

Bromage, E. S., Kaattari, I. M., Zwollo, P., & Kaattari, S. L. (2004). Plasmablast and plasma cell production and distribution in trout immune tissues. *Journal of Immunology, 173*(12), 7317-7323. doi:DOI 10.4049/jimmunol.173.12.7317

Brudeseth, B. E., Wiulsrod, R., Fredriksen, B. N., Lindmo, K., Lokling, K. E., Bordevik, M., . . . Gravningen, K. (2013). Status and future perspectives of vaccines for industrialised fin-fish farming. *Fish & Shellfish Immunology, 35*(6), 1759-1768. doi:10.1016/j.fsi.2013.05.029

Bruno, D. N. (2013). *A colour atlas of salmonid diseases. second edition*. New York: Springer.

Buchanan, B. M., Popowych, Y., Arsic, N., Townsend, H. G. G., Mutwiri, G. K., Potter, A. A., . . . Wilson, H. L. (2011). B-cell activating factor (BAFF) promotes CpG ODN-induced B cell activation and proliferation. *Cellular Immunology, 271*(1), 16-28. doi:10.1016/j.cellimm.2011.05.016

Buisman, A. M., de Rond, C. G. H., Ozturk, K., ten Huscher, H. I., & van Binnendijk, R. S. (2009). Long-term presence of memory B-cells specific for different vaccine components. *Vaccine, 28*(1), 179-186. doi:10.1016/j.vaccine.2009.09.102

Castigli, E., Scott, S., Dedeoglu, F., Bryce, P., Jabara, H., Bhan, A. K., . . . Geha, R. S. (2004). Impaired IgA class switching in APRIL-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America, 101*(11), 3903-3908. doi:10.1073/pnas.0307348101

Cerutti, A., Puga, I., & Cols, M. (2011). Innate control of B cell responses. *Trends in Immunology, 32*(5), 202-211. doi:10.1016/j.it.2011.02.004

Chang, C. J., Gu, J., & Robertsen, B. (2017). Protective effect and antibody response of DNA vaccine against salmonid alphavirus 3 (SAV3) in Atlantic salmon. *Journal of Fish Diseases, 40*(12), 1775-1781. doi:10.1111/jfd.12644

Chang, S. K., Arendt, B. K., Darce, J. R., Wu, X. S., & Jelinek, D. F. (2006). A role for BLyS in the activation of innate immune cells. *Blood, 108*(8), 2687-2694. doi:10.1182/blood-2005-12-017319

ChemoMetec. (2015). NucleoCounter® User's Guide Revision 1.3

Chomczynski, P., & Sacchi, N. (1987). Single-Step Method of Rna Isolation by Acid Guanidinium Thiocyanate Phenol Chloroform Extraction. *Analytical Biochemistry, 162*(1), 156-159. doi:Doi 10.1016/0003-2697(87)90021-2

Chu, V. T., Enghard, P., Riemekasten, G., & Berek, C. (2007). In vitro and in vivo activation induces BAFF and APRIL expression in B cells. *Journal of Immunology, 179*(9), 5947-5957. doi:DOI 10.4049/jimmunol.179.9.5947

Chu, V. T., Enghard, P., Schurer, S., Steinhauser, G., Rudolph, B., Riemekasten, G., & Berek, C. (2009). Systemic Activation of the Immune System Induces Aberrant GAFF and APRIL Expression in B Cells in Patients With Systemic Lupus Erythematosus. *Arthritis and Rheumatism, 60*(7), 2083-2093. doi:10.1002/art.24628

Chung, N. P. Y., Matthews, K., Klasse, P. J., Sanders, R. W., & Moore, J. P. (2012). HIV-1 gp120 Impairs the Induction of B Cell Responses by TLR9-Activated Plasmacytoid Dendritic Cells. *Journal of Immunology, 189*(11), 5257-5265. doi:10.4049/jimmunol.1201905

Claudio, E., Brown, K., Park, S., Wang, H. S., & Siebenlist, U. (2002). BAFF-induced NEMO-independent processing of NF-kappa B2 in maturing B cells. *Nature Immunology, 3*(10), 958-965. doi:10.1038/ni842

Crane, M., & Hyatt, A. (2011). Viruses of Fish: An Overview of Significant Pathogens. *Viruses-Basel, 3*(11), 2025-2046. doi:10.3390/v3112025

Craxton, A., Magaletti, D., Ryan, E. J., & Clark, E. A. (2003). Macrophage- and dendritic cell-dependent regulation of human B-cell proliferation requires the TNF family ligand BAFF. *Blood, 101*(11), 4464-4471. doi:10.1182/blood-2002-10-3123

Cui, X. W., Li, J. F., Xiao, W., Xuan, Y., Tian, A. Y., Xu, X. Z., & Zhang, S. Q. (2012). Molecular cloning, expression and functional analysis of TNF13b (BAFF) in Japanese sea perch, Lateolabrax japonicus. *International Immunopharmacology, 12*(1), 34-41. doi:10.1016/j.intimp.2011.10.009

Czerkinsky, C. C., Nilsson, L. A., Nygren, H., Ouchterlony, O., & Tarkowski, A. (1983). A Solid-Phase Enzyme-Linked Immunospot (Elispot) Assay for Enumeration of Specific Antibody-Secreting Cells. *Journal of Immunological Methods, 65*(1-2), 109-121. doi:Doi 10.1016/0022-1759(83)90308-3

Dalmo, R. A., Ingebrigtsen, K., Sveinbjornsson, B., & Seljelid, R. (1996). Accumulation of immunomodulatory laminaran (beta(1,3)-D-glucan) in the heart, spleen and kidney of Atlantic cod, Gadus morhua L. *Journal of Fish Diseases, 19*(2), 129-136. doi:DOI 10.1111/j.1365-2761.1996.tb00691.x

Dalmo, R. A., Seternes, T., Arnesen, S. M., Jorgensen, T. O., & Bogwald, J. (1998). Tissue distribution and cellular uptake of Aeromonas salmonicida lipopolysaccharide (LPS) in some marine fish species. *Journal of Fish Diseases, 21*(5), 321-334. doi:DOI 10.1046/j.1365-2761.1998.00108.x

Darce, J. R., Arendt, B. K., Chang, S. K., & Jelinek, D. F. (2007). Divergent effects of BAFF on human memory B cell differentiation into Ig-secreting cells. *Journal of Immunology, 178*(9), 5612-5622. doi:DOI 10.4049/jimmunol.178.9.5612

Darce, J. R., Arendt, B. K., Wu, X. S., & Jelinek, D. F. (2007). Regulated expression of BAFF-binding receptors during human B cell differentiation. *Journal of Immunology, 179*(11), 7276-7286. doi:DOI 10.4049/jimmunol.179.11.7276

Das, S., Sutoh, Y., Hirano, M., Han, Q. F., Li, J. X., Cooper, M. D., & Herrin, B. R. (2016). Characterization of Lamprey BAFF-like Gene: Evolutionary Implications. *Journal of Immunology, 197*(7), 2695-2703. doi:10.4049/jimmunol.1600799

Dillon, S. R., Gross, J. A., Ansell, S. M., & Novak, A. J. (2006). An APRIL to remember: novel TNF ligands as therapeutic targets. *Nature Reviews Drug Discovery, 5*(3), 235-246. doi:10.1038/nrd1982

Do, R. K. G., & Chen-Kiang, S. (2002). Mechanism of BLyS action in B cell immunity. *Cytokine & Growth Factor Reviews, 13*(1), 19-25. doi:Doi 10.1016/S1359-6101(01)00025-9

Eason, D. D., Cannon, J. P., Haire, R. N., Rast, J. P., Ostrov, D. A., & Litman, G. W. (2004). Mechanisms of antigen receptor evolution. *Seminars in Immunology, 16*(4), 215-226. doi:10.1016/j.smim.2004.08.001

Edholm, E. S., Bengten, E., Stafford, J. L., Sahoo, M., Taylor, E. B., Miller, N. W., & Wilson, M. (2010). Identification of Two IgD(+) B Cell Populations in Channel Catfish, Ictalurus punctatus. *Journal of Immunology, 185*(7), 4082-4094. doi:10.4049/jimmunol.1000631

El Shikh, M. E. M., El Sayed, R. M., Sukumar, S., Szakal, A. K., & Tew, J. G. (2010). Activation of B cells by antigens on follicular dendritic cells. *Trends in Immunology, 31*(6), 205-211. doi:10.1016/j.it.2010.03.002

El Shikh, M. E. M., El Sayed, R. M., Szakal, A. K., & Tew, J. G. (2009). T-Independent Antibody Responses to T-Dependent Antigens: A Novel Follicular Dendritic Cell-Dependent Activity. *Journal of Immunology, 182*(6), 3482-3491. doi:10.4049/jimmunol.0802317

EMD-Millipore. (2012). Elispot assays: state-of-the-art tools for functional analysis of cellular immunology. Retrieved from https://www.emdmillipore.com/Web-CA-Site/en_CA/-/CAD/ShowDocument-Pronet?id=201306.9753

COMMISSION IMPLEMENTING DECISION of 27.6.2017 granting marketing authorisation under Regulation (EC) No 726/2004 of the European Parliament and of the Council for "CLYNAV - Salmon pancreas disease vaccine (recombinant DNA plasmid)", a veterinary medicinal product,, (2017).

Evans, D. L., Leary, J. H., & Jaso-Friedmann, L. (2001). Nonspecific cytotoxic cells and innate immunity: regulation by programmed cell death. *Developmental and Comparative Immunology, 25*(8-9), 791-805. doi:Doi 10.1016/S0145-305x(01)00036-2

Evensen, O., & Leong, J. A. C. (2013). DNA vaccines against viral diseases of farmed fish. *Fish & Shellfish Immunology, 35*(6), 1751-1758. doi:10.1016/j.fsi.2013.10.021

Fagarasan, S., & Honjo, T. (2004). Regulation of IgA synthesis at mucosal surfaces. *Current Opinion in Immunology, 16*(3), 277-283. doi:10.1016/j.coi.2004.03.005

Fesik, S. W. (2000). Insights into programmed cell death through structural biology. *Cell, 103*(2), 273-282. doi:Doi 10.1016/S0092-8674(00)00119-7

Fillatreau, S., Six, A., Magadan, S., Castro, R., Sunyer, J. O., & Boudinot, P. (2013). The astonishing diversity of Ig classes and B cell repertoires in teleost fish. *Frontiers in Immunology, 4*. doi:UNSP 28

10.3389/fimmu.2013.00028

Georgopoulou, U., & Vernier, J. M. (1986). Local Immunological Response in the Posterior Intestinal Segment of the Rainbow-Trout after Oral-Administration of Macromolecules. *Developmental and Comparative Immunology, 10*(4), 529-537. doi:Doi 10.1016/0145-305x(86)90174-6

Glenney, G. W., & Wiens, G. D. (2007). Early diversification of the TNF superfamily in teleosts: Genomic characterization and expression analysis. *Journal of Immunology, 178*(12), 7955-7973. doi:DOI 10.4049/jimmunol.178.12.7955

Godahewa, G. I., Perera, N. C. N., Umasuthan, N., Wan, Q., Whang, I., & Lee, J. (2016). Molecular characterization and expression analysis of B cell activating factor from rock bream (Oplegnathus fasciatus). *Developmental and Comparative Immunology, 55*, 1-11. doi:10.1016/j.dci.2015.10.004

Gomez-Casado, E., Estepa, A., & Coll, J. M. (2011). A comparative review on European-farmed finfish RNA viruses and their vaccines (vol 29, pg 2657, 2011). *Vaccine, 29*(21), 3826-3826. doi:10.1016/j.vaccine.2011.03.072

Graham, D. A., Fringuelli, E., Rowley, H. M., Cockerill, D., Cox, D. I., Turnbull, T., . . . Mc Loughlin, M. F. (2012). Geographical distribution of salmonid alphavirus subtypes in marine farmed Atlantic salmon, Salmo salar L., in Scotland and Ireland. *Journal of Fish Diseases, 35*(10), 755-765. doi:10.1111/j.1365-2761.2012.01401.x

Graham, D. A., Frost, P., McLaughlin, K., Rowley, H. M., Gabestad, I., Gordon, A., & McLoughlin, M. F. (2011). A comparative study of marine salmonid alphavirus subtypes 1-6 using an experimental cohabitation challenge model. *J Fish Dis, 34*(4), 273-286. doi:10.1111/j.1365-2761.2010.01234.x

Granja, A. G., Castro, R., Gonzalez, L., Soleto, I., & Tafalla, C. (2016). Teleost B cell-activating factor (BAFF) diferentially regulates B cell activity in lymphoid and mucosal tissues. *Fish & Shellfish Immunology, 53*, 84-85.

Granja, A. G., Holland, J. W., Pignatelli, J., Secombes, C. J., & Tafalla, C. (2017). Characterization of BAFF and APRIL subfamily receptors in rainbow trout (Oncorhynchus mykiss). Potential role of the BAFF/APRIL axis in the pathogenesis of proliferative kidney disease. *PLoS One, 12*(3). doi:ARTN e0174249

10.1371/journal.pone.0174249

Granja, A. G., & Tafalla, C. (2017). Different IgM(+) B cell subpopulations residing within the peritoneal cavity of vaccinated rainbow trout are differently regulated by BAFF. *Fish Shellfish Immunol*. doi:10.1016/j.fsi.2017.10.003

Groom, J. R., Fletcher, C. A., Walters, S. N., Grey, S. T., Watt, S. V., Sweet, M. J., . . . Mackay, F. (2007). BAFF and MyD88 signals promote a lupuslike disease independent of T cells. *Journal of Experimental Medicine, 204*(8), 1959-1971. doi:10.1084/jem.20062567

Grove, S., Austbo, L., Hodneland, K., Frost, P., Lovoll, M., McLoughlin, M., . . . Rimstad, E. (2013). Immune parameters correlating with reduced susceptibility to pancreas disease in experimentally challenged Atlantic salmon (Salmo salar). *Fish & Shellfish Immunology, 34*(3), 789-798. doi:10.1016/j.fsi.2012.12.014

Hahne, M., Kataoka, T., Schroter, M., Hofmann, K., Irmler, M., Bodmer, J. L., . . . Tschopp, J. (1998). APRIL, a new ligand of the tumor necrosis factor family, stimulates tumor cell growth. *Journal of Experimental Medicine, 188*(6), 1185-1190. doi:DOI 10.1084/jem.188.6.1185

Hansen, J. D., Landis, E. D., & Phillips, R. B. (2005). Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. *Proceedings of the National Academy of Sciences of the United States of America, 102*(19), 6919-6924. doi:10.1073/pnas.0500027102

Hardenberg, G., Planelles, L., Schwarte, C. M., van Bostelen, L., Le Huong, T., Hahne, M., & Medema, J. P. (2007). Specific TLR ligands regulate APRIL secretion by dendritic cells in a PKR-dependent manner. *European Journal of Immunology, 37*(10), 2900-2911.

Hatzoglou, A., Roussel, J., Bourgeade, M. F., Rogier, E., Madry, C., Inoue, J., . . . Tsapis, A. (2000). TNF receptor family member BCMA (B cell maturation) associates with TNF receptor-associated factor (TRAF) 1, TRAF2, and TRAF3 and activates NF-kappa B, Elk-1, c-Jun N-terminal kinase, and p38 mitogen-activated protein

kinase. *Journal of Immunology, 165*(3), 1322-1330. doi:DOI 10.4049/jimmunol.165.3.1322

Haugarvoll, E., Bjerkas, I., Nowak, B. F., Hordvik, I., & Koppang, E. O. (2008). Identification and characterization of a novel intraepithelial lymphoid tissue in the gills of Atlantic salmon. *Journal of Anatomy, 213*(2), 202-209. doi:10.1111/j.1469-7580.2008.00943.x

He, B., Chadburn, A., Jou, E., Schattner, E. J., Knowles, D. M., & Cerutti, A. (2004). Lymphoma B cells evade apoptosis through the TNF family members BAFF/BLyS and APRIL. *Journal of Immunology, 172*(5), 3268-3279. doi:DOI 10.4049/jimmunol.172.5.3268

Hedfors, I. A., Bakke, H., Skjodt, K., & Grimholt, U. (2012). Antibodies recognizing both IgM isotypes in Atlantic salmon. *Fish & Shellfish Immunology, 33*(5), 1199-1206. doi:10.1016/j.fsi.2012.09.009

Heid, C. A., Stevens, J., Livak, K. J., & Williams, P. M. (1996). Real time quantitative PCR. *Genome Research, 6*(10), 986-994. doi:DOI 10.1101/gr.6.10.986

Higuchi, R., Fockler, C., Dollinger, G., & Watson, R. (1993). Kinetic Pcr Analysis - Real-Time Monitoring of DNA Amplification Reactions. *Bio-Technology, 11*(9), 1026-1030. doi:DOI 10.1038/nbt0993-1026

Hikima, J. I., Jung, T. S., & Aoki, T. (2011). Immunoglobulin genes and their transcriptional control in teleosts. *Developmental and Comparative Immunology, 35*(9), 924-936. doi:10.1016/j.dci.2010.10.011

Hildebrand, J. M., Xie, P., & Bishop, G. A. (2010). Signal Transduction by Receptors for BAFF and APRIL. In M. P. Cancro (Ed.), *BLyS Ligands and Receptors* (pp. 93-114). Totowa, NJ: Humana Press.

Hjortaas, M. J., Skjelstad, H. R., Taksdal, T., Olsen, A. B., Johansen, R., Bang-Jensen, B., . . . Sindre, H. (2013). The first detections of subtype 2-related salmonid alphavirus (SAV2) in Atlantic salmon, Salmo salar L., in Norway. *J Fish Dis, 36*(1), 71-74. doi:10.1111/j.1365-2761.2012.01445.x

Hodneland, K., & Endresen, C. (2006). Sensitive and specific detection of Salmonid alphavirus using real-time PCR (TaqMan (R)). *Journal of Virological Methods, 131*(2), 184-192. doi:10.1016/j.jviromet.2005.08.012

Huard, B., Arlettaz, L., Ambrose, C., Kindler, V., Mauri, D., Roosnek, E., . . . French, L. E. (2004). BAFF production by antigen-presenting cells provides T cell co-stimulation. *International Immunology, 16*(3), 467-475. doi:10.1093/intimm/dxh043

Hymowitz, S. G., Patel, D. R., Wallweber, H. J. A., Runyon, S., Yan, M. H., Yin, J. P., . . . Starovasnik, M. A. (2005). Structures of APRIL-receptor complexes - Like BCMA, TACI employs only a single cysteine-rich domain for high affinity ligand binding. *Journal of Biological Chemistry, 280*(8), 7218-7227. doi:10.1074/jbc.M411714200

Ingold, K., Zumsteg, A., Tardivel, A., Huard, B., Steiner, Q. G., Cachero, T. G., . . . Schneider, P. (2005). Identification of proteoglycans as the APRIL-specific binding partners. *Journal of Experimental Medicine, 201*(9), 1375-1383. doi:10.1084/jem.20042309

Inoue, J., Ishida, T., Tsukamoto, N., Kobayashi, N., Naito, A., Azuma, S., & Yamamoto, T. (2000). Tumor necrosis factor receptor-associated factor (TRAF) family: Adapter proteins that mediate cytokine signaling. *Experimental Cell Research, 254*(1), 14-24. doi:DOI 10.1006/excr.1999.4733

Ittah, M., Miceli-Richard, C., Gottenberg, J. E., Sellam, J., Eid, P., Lebon, P., . . . Mariette, X. (2008). Viruses induce high expression of BAFF by salivary gland epithelial cells

through TLR- and type-I IFN-dependent and -independent pathways. *Eur J Immunol, 38*(4), 1058-1064. doi:10.1002/eji.200738013

Iwasaki, A., & Medzhitov, R. (2004). Toll-like receptor control of the adaptive immune responses. *Nature Immunology, 5*(10), 987-995. doi:10.1038/ni1112

Jansen, M. D., Jensen, B. B., Taksdal, T., Sindre, H., & Lillehaug, A. (2015). Pankreassykdom hos laksefisk–en review med fokus på forebygging, kontroll og bekjempelse. *The Norwegian Veterinary Institute*.

Jenberie, S., Thim, H. L., Sunyer, J. O., Skjodt, K., Jensen, I., & Jorgensen, J. B. (2018). Profiling Atlantic salmon B cell populations: CpG-mediated TLR-ligation enhances IgM secretion and modulates immune gene expression. *Sci Rep, 8*(1), 3565. doi:10.1038/s41598-018-21895-9

Jensen, I., Larsen, R., & Robertsen, B. (2002). An antiviral state induced in Chinook salmon embryo cells (CHSE-214) by transfection with the double-stranded RNA poly I : C. *Fish & Shellfish Immunology, 13*(5), 367-378. doi:10.1006/fsim.2002.0412

Jerne, N. K., & Nordin, A. A. (1963). Plaque Formation in Agar by Single Antibody-Producing Cells. *Science, 140*(356), 405-&. doi:DOI 10.1126/science.140.3565.405

Johansen, L. H., Thim, H. L., Jorgensen, S. M., Afanasyev, S., Strandskog, G., Taksdal, T., . . . Krasnov, A. (2015). Comparison of transcriptomic responses to pancreas disease (PD) and heart and skeletal muscle inflammation (HSMI) in heart of Atlantic salmon (Salmo salar L). *Fish & Shellfish Immunology, 46*(2), 612-623. doi:10.1016/j.fsi.2015.07.023

Jorgensen, J. B., Johansen, A., Stenersen, B., & Sommer, A. I. (2001). CpG oligodeoxynucleotides and plasmid DNA stimulate Atlantic salmon (Salmo salar L.) leucocytes to produce supernatants with antiviral activity. *Developmental and Comparative Immunology, 25*(4), 313-321. doi:Doi 10.1016/S0145-305x(00)00068-9

Jorgensen, J. B., Johansen, L. H., Steiro, K., & Johansen, A. (2003). CpG DNA induces protective antiviral immune responses in Atlantic salmon (Salmo salar L.). *Journal of Virology, 77*(21), 11471-11479. doi:10.1128/Jvi.77.21.11471-11479.2003

Kaattari, S. L., Zhang, H. L. L., Khor, I. W., Kaattari, I. M., & Shapiro, D. A. (2002). Affinity maturation in trout: clonal dominance of high affinity antibodies late in the immune response. *Developmental and Comparative Immunology, 26*(2), 191-200. doi:Doi 10.1016/S0145-305x(01)00064-7

Kalyuzhny, A. E. (2012). *Handbook of ELISPOT : methods and protocols* (2nd ed.). New York: Humana Press.

Katsenelson, N., Kanswal, S., Puig, M., Mostowski, H., Verthelyi, D., & Akkoyunlu, M. (2007). Synthetic CpG oligodeoxynucleotides augment BAFF- and APRIL-mediated immunoglobulin secretion. *European Journal of Immunology, 37*(7), 1785-1795. doi:10.1002/eji.200636800

Lamond, A. I. (2002). Molecular biology of the cell, 4th edition. *Nature, 417*(6887), 383-383. doi:DOI 10.1038/417383a

Li, R. G., Dooley, H., Wang, T. H., Secombes, C. J., & Bird, S. (2012). Characterisation and expression analysis of B-cell activating factor (BAFF) in spiny dogfish (Squalus acanthias): Cartilaginous fish BAFF has a unique extra exon that may impact receptor binding. *Developmental and Comparative Immunology, 36*(4), 707-717. doi:10.1016/j.dci.2011.11.010

Li, R. G., Redmond, A. K., Wang, T. H., Bird, S., Dooley, H., & Secombes, C. J. (2015). Characterisation of the TNF superfamily members CD40L and BAFF in the small-spotted catshark (Scyliorhinus canicula). *Fish & Shellfish Immunology, 47*(1), 381-389. doi:10.1016/j.fsi.2015.09.033

Liang, Z. N., Kong, Y., Luo, C. H., Shen, Y., & Zhang, S. Q. (2010). Molecular cloning, functional characterization and phylogenetic analysis of B-cell activating factor in zebrafish (Danio rerio). *Fish & Shellfish Immunology, 29*(2), 233-240. doi:10.1016/j.fsi.2010.03.006

Life-Technologies. (2012). Real-time PCR handbook. Retrieved from http://www.gene-quantification.com/real-time-pcr-handbook-life-technologies-update-flr.pdf

Liu, H. Z., Zhang, J. X., Li, J. F., Song, J. Y., & Zhang, S. Q. (2016). Molecular structure, distribution, and immunology function of TNFSF13B (BAFF) in Nile tilapia (Oreochromis niloticus). *Fish & Shellfish Immunology, 51*, 240-250.

Liu, Y. F., Hong, X., Kappler, J., Jiang, L., Zhang, R. G., Xu, L. G., . . . Zhang, G. Y. (2003). Ligand-receptor binding revealed by the TNF family member TALL-1. *Nature, 423*(6935), 49-56. doi:10.1038/nature01543

Locksley, R. M., Killeen, N., & Lenardo, M. J. (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell, 104*(4), 487-501.

Lopez-Fraga, M., Fernandez, R., Albar, J. P., & Hahne, M. (2001). Biologically active APRIL is secreted following intracellular processing in the Golgi apparatus by furin convertase. *Embo Reports, 2*(10), 945-951. doi:DOI 10.1093/embo-reports/kve198

Lovoll, M., Austbo, L., Jorgensen, J. B., Rimstad, E., & Frost, P. (2011). Transcription of reference genes used for quantitative RT-PCR in Atlantic salmon is affected by viral infection. *Veterinary research, 42*. doi:Artn 8

10.1186/1297-9716-42-8

Lugo-Villarino, G., Balla, K. M., Stachura, D. L., Banuelos, K., Werneck, M. B. F., & Traver, D. (2010). Identification of dendritic antigen-presenting cells in the zebrafish. *Proceedings of the National Academy of Sciences of the United States of America, 107*(36), 15850-15855. doi:10.1073/pnas.1000494107

Mackay, F., & Schneider, P. (2008). TACI, an enigmatic BAFF/APRIL receptor, with new unappreciated biochemical and biological properties. *Cytokine & Growth Factor Reviews, 19*(3-4), 263-276. doi:10.1016/j.cytogfr.2008.04.006

Mackay, F., & Schneider, P. (2009). Cracking the BAFF code. *Nature Reviews Immunology, 9*(7), 491-502. doi:10.1038/nri2572

Mackay, F., Schneider, P., Rennert, P., & Browning, J. (2003). BAFF and APRIL: A tutorial on B cell survival. *Annual Review of Immunology, 21*, 231-264. doi:10.1146/annurev.immunol.21.120601.141152

Magis, C., van der Sloot, A. M., Serrano, L., & Notredame, C. (2012). An improved understanding of TNFL/TNFR interactions using structure-based classifications. *Trends in Biochemical Sciences, 37*(9), 353-363. doi:10.1016/j.tibs.2012.06.002

Magnadottir, B. (2010). Immunological Control of Fish Diseases. *Marine Biotechnology, 12*(4), 361-379. doi:10.1007/s10126-010-9279-x

Matthes, T., Dunand-Sauthier, I., Santiago-Raber, M. L., Krause, K. H., Donze, O., Passweg, J., . . . Huard, B. (2011). Production of the plasma-cell survival factor a proliferation-inducing ligand (APRIL) peaks in myeloid precursor cells from human bone marrow. *Blood, 118*(7), 1838-1844. doi:10.1182/blood-2011-01-332940

McLoughlin, M. F., & Graham, D. A. (2007). Alphavirus infections in salmonids - a review. *Journal of Fish Diseases, 30*(9), 511-531. doi:DOI 10.1111/j.1365-2761.2007.00848.x

McLoughlin, M. F., Nelson, R. T., Rowley, H. M., Cox, D. I., & Grant, A. N. (1996). Experimental pancreas disease in Atlantic salmon Salmo salar post-smolts induced by salmon pancreas disease virus (SPDV). *Diseases of Aquatic Organisms, 26*(2), 117-124. doi:DOI 10.3354/dao026117

Medzhitov, R., & Janeway, C. A. (2002). Decoding the patterns of self and nonself by the innate immune system. *Science, 296*(5566), 298-300. doi:DOI 10.1126/science.1068883

Meng, F. Q., Sun, Y. N., & Xu, T. J. (2015). Comparative genomic of the BAFF and BAFF-like genes and immune response to bacteria of miiuy croaker (Miichthys miiuy). *Fish & Shellfish Immunology, 43*(1), 191-199. doi:10.1016/j.fsi.2014.12.022

Miller, N. W., & Clem, L. W. (1984). Microsystem for Invitro Primary and Secondary Immunization of Channel Catfish (Ictalurus-Punctatus) Leukocytes with Hapten-Carrier Conjugates. *Journal of Immunological Methods, 72*(2), 367-379. doi:Doi 10.1016/0022-1759(84)90006-1

Min, C., Liang, Z. N., Cui, X. W., Wang, Q., Chen, Y. Q., & Zhang, S. Q. (2012). Molecular cloning and partial functional characterization of a proliferation inducing ligand (APRIL) in zebrafish (Danio rerio). *Developmental and Comparative Immunology, 37*(1), 202-206. doi:10.1016/j.dci.2011.12.002

Moore, P. A., Belvedere, O., Orr, A., Pieri, K., LaFleur, D. W., Feng, P., . . . Hilbert, D. M. (1999). BLyS: Member of the tumor necrosis factor family and B lymphocyte stimulator. *Science, 285*(5425), 260-263. doi:DOI 10.1126/science.285.5425.260

Morel, F., Doussiere, J., & Vignais, P. V. (1991). The Superoxide-Generating Oxidase of Phagocytic-Cells - Physiological, Molecular and Pathological Aspects. *European Journal of Biochemistry, 201*(3), 523-546. doi:DOI 10.1111/j.1432-1033.1991.tb16312.x

Moriette, C., LeBerre, M., Boscher, S. K., Castric, J., & Bremont, M. (2005). Characterization and mapping of monoclonal antibodies against the Sleeping disease virus, an aquatic alphavirus. *Journal of General Virology, 86*, 3119-3127. doi:10.1099/vir.0.81030-0

Moriette, C., LeBerre, M., Lamoureux, A., Lai, T. L., & Bremont, M. (2006). Recovery of a recombinant Salmonid alphavirus fully attenuated and protective for rainbow trout. *Journal of Virology, 80*(8), 4088-4098. doi:10.1128/Jvi.80.8.4088-4098.2006

Mukhopadhyay, A., Ni, J., Zhai, Y. F., Yu, G. L., & Aggarwal, B. B. (1999). Identification and characterization of a novel cytokine, THANK, a (T)under-barNF (H)under-baromologue that activates (A)under-barpoptosis, nuclear factor-kappa B, and c-Jun NH2-terminal (K)under-barinase. *Journal of Biological Chemistry, 274*(23), 15978-15981. doi:DOI 10.1074/jbc.274.23.15978

Munang'andu, H. M., Fredriksen, B. N., Mutoloki, S., Dalmo, R. A., & Evensen, O. (2013). Antigen dose and humoral immune response correspond with protection for inactivated infectious pancreatic necrosis virus vaccines in Atlantic salmon (Salmo salar L). *Veterinary research, 44*. doi:Artn 7

10.1186/1297-9716-44-7

Murphy, J. E., Tedbury, P. R., Homer-Vanniasinkam, S., Walker, J. H., & Ponnambalam, S. (2005). Biochemistry and cell biology of mammalian scavenger receptors. *Atherosclerosis, 182*(1), 1-15. doi:10.1016/j.atherosclerosis.2005.03.036

Mutoloki, S., Jørgensen, J. B., & Evensen, Ø. (2014). The adaptive immune response in fish. *Fish Vaccination*, 104-115.

Nardelli, B., Belvedere, O., Roschke, V., Moore, P. A., Olsen, H. S., Migone, T. S., . . . Hilbert, D. M. (2001). Synthesis and release of B-lymphocyte stimulator from myeloid cells. *Blood, 97*(1), 198-204. doi:DOI 10.1182/blood.V97.1.198

Nelson, R. T., Mcloughlin, M. F., Rowley, H. M., Platten, M. A., & Mccormick, J. I. (1995). Isolation of a Toga-Like Virus from Farmed Atlantic Salmon Salmo-Salar with Pancreas Disease. *Diseases of Aquatic Organisms, 22*(1), 25-32. doi:DOI 10.3354/dao022025

Ng, L. G., Sutherland, A. P. R., Newton, R., Qian, F., Cachero, T. G., Scott, M. L., . . . Mackay, C. R. (2004). B cell-activating factor belonging to the TNF family (BAFF)-R is the principal BAFF receptor facilitating BAFF costimulation of circulating T and B cells. *Journal of Immunology, 173*(2), 807-817. doi:DOI 10.4049/jimmunol.173.2.807

Novak, A. J., Darce, J. R., Arendt, B. K., Harder, B., Henderson, K., Kindsvogel, W., . . . Jelinek, D. F. (2004). Expression of BCMA, TACI, and BAFF-R in multiple myeloma: a mechanism for growth and survival. *Blood, 103*(2), 689-694.

O'Connor, B. P., Raman, V. S., Erickson, L. D., Cook, W. J., Weaver, L. K., Ahonen, C., . . . Noelle, R. J. (2004). BCMA is essential for the survival of long-lived bone marrow plasma cells. *Journal of Experimental Medicine, 199*(1), 91-97. doi:10.1084/jem.20031330

Olsvik, P. A., Lie, K. K., Jordal, A. E., Nilsen, T. O., & Hordvik, I. (2005). Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. *BMC Mol Biol, 6*, 21. doi:10.1186/1471-2199-6-21

Palti, Y. (2011). Toll-like receptors in bony fish: From genomics to function. *Developmental and Comparative Immunology, 35*(12), 1263-1272. doi:10.1016/j.dci.2011.03.006

Pandit, N. P., Shen, Y. B., Wang, W. J., Chen, Y., & Li, J. L. (2013). Identification of TNF13b (BAFF) gene from grass carp (Ctenopharyngodon idella) and its immune response to bacteria and virus. *Developmental and Comparative Immunology, 39*(4), 460-464. doi:10.1016/j.dci.2013.01.004

Peñaranda, M. (2018). *A proliferation inducing ligand (APRIL) is a survival factor that plays an important role in the immune response of Atlantic salmon*.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research, 29*(9). doi:ARTN e45

DOI 10.1093/nar/29.9.e45

Phelan, P. E., Mellon, M. T., & Kim, C. H. (2005). Functional characterization of full-length TLR3, IRAK-4, and TRAF6 in zebrafish (Danio rerio). *Molecular Immunology, 42*(9), 1057-1071. doi:10.1016/j.molimm.2004.11.005

Pinto, D., Montani, E., Bolli, M., Garavaglia, G., Sallusto, F., Lanzavecchia, A., & Jarrossay, D. (2013). A functional BCR in human IgA and IgM plasma cells. *Blood, 121*(20), 4110-4114. doi:10.1182/blood-2012-09-459289

Pinto, R. D., Nascimento, D. S., Reis, M. I. R., do Vale, A., & dos Santos, N. M. S. (2007). Molecular characterization, 3D modelling and expression analysis of sea bass

(Dicentrarchus labrax L.) interleukin-10. *Molecular Immunology, 44*(8), 2056-2065. doi:10.1016/j.molimm.2006.09.014

Pongratz, G., Hochrinner, H., Straub, R. H., Lang, S., & Brunnler, T. (2013). B Cell Activating Factor of the Tumor Necrosis Factor Family (BAFF) Behaves as an Acute Phase Reactant in Acute Pancreatitis. *PLoS One, 8*(1). doi:ARTN e54297

10.1371/journal.pone.0054297

Poppe, T., Rimstad, E., & Hyllseth, B. (1989). Pancreas disease in Atlantic salmon (Salmo salar) postsmolts infected with infectious pancreatic necrosis virus (IPNV). *Bulletin of the European Association of Fish Pathologists, 9*(4), 83-85.

Pradet-Balade, B., Medema, J. P., Lopez-Fraga, M., Lozano, J. C., Kolfschoten, G. M., Picard, A., . . . Hahne, M. (2002). An endogenous hybrid mRNA encodes TWE-PRIL, a functional cell surface TWEAK-APRIL fusion protein. *Embo Journal, 21*(21), 5711-5720. doi:DOI 10.1093/emboj/cdf565

Press, C. M., & Evensen, O. (1999). The morphology of the immune system in teleost fishes. *Fish & Shellfish Immunology, 9*(4), 309-318. doi:DOI 10.1006/fsim.1998.0181

Qiagen. (2009). QuantiTect® Reverse Transcription Handbook. Retrieved from https://www.qiagen.com/at/resources/download.aspx?id=f0de5533-3dd1-4835-8820-1f5c088dd800&lang=en

Qiagen. (2012). RNeasy® Mini Handbook. Retrieved from https://www.qiagen.com/us/resources/resourcedetail?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24&lang=en

Rahman, Z. S., Rao, S. P., Kalled, S. L., & Manser, T. (2003). Normal induction but attenuated progression of germinal center responses in BAFF and BAFF-R signaling-deficient mice. *J Exp Med, 198*(8), 1157-1169. doi:10.1084/jem.20030495

Ramirez-Gomez, F., Greene, W., Rego, K., Hansen, J. D., Costa, G., Kataria, P., & Bromage, E. S. (2012). Discovery and Characterization of Secretory IgD in Rainbow Trout: Secretory IgD Is Produced through a Novel Splicing Mechanism. *Journal of Immunology, 188*(3), 1341-1349. doi:10.4049/jimmunol.1101938

Reichlin, A., Hu, Y., Meffre, E., Nagaoka, H., Gong, S. C., Kraus, M., . . . Nussenzweig, M. C. (2001). B cell development is arrested at the immature B cell stage in mice carrying a mutation in the cytoplasmic domain of immunoglobulin beta. *Journal of Experimental Medicine, 193*(1), 13-23. doi:DOI 10.1084/jem.193.1.13

Ren, W. H., Pang, S. Y., You, F. T., Zhou, L. D., & Zhang, S. Q. (2011). The first BAFF gene cloned from the cartilaginous fish. *Fish & Shellfish Immunology, 31*(6), 1088-1096. doi:10.1016/j.fsi.2011.09.013

Rijkers, G. T., Frederixwolters, E. M. H., & Vanmuiswinkel, W. B. (1980). Hemolytic Plaque-Assay in Carp (Cyprinus-Carpio). *Journal of Immunological Methods, 33*(1), 79-86. doi:Doi 10.1016/0022-1759(80)90084-8

Robertsen, B. (2006). The interferon system of teleost fish. *Fish & Shellfish Immunology, 20*(2), 172-191. doi:10.1016/j.fsi.2005.01.010

Robertsen, B. (2008). Expression of interferon and interferon-induced genes in salmonids in response to virus infection, interferon-inducing compounds and vaccination. *Fish & Shellfish Immunology, 25*(4), 351-357. doi:10.1016/j.fsi.2008.02.004

Robertsen, B. (2011). Can we get the upper hand on viral diseases in aquaculture of Atlantic salmon? *Aquaculture Research, 42*, 125-131.

Robertsen, B. (2018). The role of type I interferons in innate and adaptive immunity against viruses in Atlantic salmon. *Developmental and Comparative Immunology, 80*, 41-52. doi:10.1016/j.dci.2017.02.005

Robertsen, B., Bergan, V., Rokenes, T., Larsen, R., & Albuquerque, A. (2003). Atlantic salmon interferon genes: Cloning, sequence analysis, expression, and biological activity. *Journal of Interferon and Cytokine Research, 23*(10), 601-612. doi:Doi 10.1089/107999003322485107

Roca, F. J., Sepulcre, P., Lopez-Castejon, G., Meseguer, J., & Mulero, V. (2006). The colony-stimulating factor-1 receptor is a specific marker of macrophages from the bony fish gilthead seabream. *Molecular Immunology, 43*(9), 1418-1423. doi:10.1016/j.molimm.2005.07.028

Rolink, A. G., Tschopp, J., Schneider, P., & Melchers, F. (2002). BAFF is a survival and maturation factor for mouse B cells. *European Journal of Immunology, 32*(7), 2004-2010. doi:Doi 10.1002/1521-4141(200207)32:7<2004::Aid-Immu2004>3.0.Co;2-5

Rombout, J. H. W. M., Abelli, L., Picchietti, S., Scapigliati, G., & Kiron, V. (2011). Teleost intestinal immunology. *Fish & Shellfish Immunology, 31*(5), 616-626. doi:10.1016/j.fsi.2010.09.001

Rombout, J. H. W. M., Yang, G. W., & Kiron, V. (2014). Adaptive immune responses at mucosal surfaces of teleost fish. *Fish & Shellfish Immunology, 40*(2), 634-643. doi:10.1016/j.fsi.2014.08.020

Salinas, I. (2015). The Mucosal Immune System of Teleost Fish. *Biology (Basel), 4*(3), 525-539. doi:10.3390/biology4030525

Salinas, I., Zhang, Y. A., & Sunyer, J. O. (2011). Mucosal immunoglobulins and B cells of teleost fish. *Developmental and Comparative Immunology, 35*(12), 1346-1365. doi:10.1016/j.dci.2011.11.009

Sasaki, Y., Casola, S., Kutok, J. L., Rajewsky, K., & Schmidt-Supprian, M. (2004). TNF family member B cell-activating factor (BAFF) receptor-dependent and -independent roles for BAFF in B cell physiology. *Journal of Immunology, 173*(4), 2245-2252. doi:DOI 10.4049/jimmunol.173.4.2245

Scapini, P., Nardelli, B., Nadali, G., Calzetti, F., Pizzolo, G., Montecucco, C., & Cassatella, M. A. (2003). G-CSF-stimulated neutrophils are a prominent source of functional BLyS. *Journal of Experimental Medicine, 197*(3), 297-302. doi:10.1084/jem.20021343

Schiemann, B., Gommerman, J. L., Vora, K., Cachero, T. G., Shulga-Morskaya, S., Dobles, M., . . . Scott, M. L. (2001). An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science, 293*(5537), 2111-2114.

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C-T method. *Nature Protocols, 3*(6), 1101-1108. doi:10.1038/nprot.2008.73

Schneider, P. (2005). The role of APRIL and BAFF in lymphocyte activation. *Curr Opin Immunol, 17*(3), 282-289. doi:10.1016/j.coi.2005.04.005

Schneider, P., MacKay, F., Steiner, V., Hofmann, K., Bodmer, J. L., Holler, N., . . . Tschopp, J. (1999). BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. *Journal of Experimental Medicine, 189*(11), 1747-1756. doi:DOI 10.1084/jem.189.11.1747

Sedgwick, J. D., & Holt, P. G. (1983). A Solid-Phase Immunoenzymatic Technique for the Enumeration of Specific Antibody-Secreting Cells. *Journal of Immunological Methods, 57*(1-3), 301-309. doi:Doi 10.1016/0022-1759(83)90091-1

Seshasayee, D., Valdez, P., Yan, M. H., Dixit, V. M., Tumas, D., & Grewal, I. S. (2003). Loss of TACI causes fatal lymphoproliferation and autoimmunity, establishing TACI as an inhibitory BLyS receptor. *Immunity, 18*(2), 279-288. doi:Doi 10.1016/S1074-7613(03)00025-6

Shu, H. B., Hu, W. H., & Johnson, H. (1999). TALL-1 is a novel member of the TNF family that is down-regulated by mitogens. *Journal of Leukocyte Biology, 65*(5), 680-683.

Shulga-Morskaya, S., Dobles, M., Walsh, M. E., Ng, L. G., MacKay, F., Rao, S. P., . . . Scott, M. L. (2004). B cell-activating factor belonging to the TNF family acts through separate receptors to support B cell survival and T cell-independent antibody formation. *Journal of Immunology, 173*(4), 2331-2341. doi:DOI 10.4049/jimmunol.173.4.2331

Siwicki, A. K., & Dunier, M. (1993). Quantification of antibody secreting cells to Yersinia ruckeri by ELISPOT assay after in vivo and in vitro immunization of rainbow trout (Oncorhynchus mykiss). *Vet Immunol Immunopathol, 37*(1), 73-80.

Skjaeveland, I., Iliev, D. B., Strandskog, G., & Jorgensen, J. B. (2009). Identification and characterization of TLR8 and MyD88 homologs in Atlantic salmon (Salmo salar). *Developmental and Comparative Immunology, 33*(9), 1011-1017. doi:10.1016/j.dci.2009.04.007

Skjaeveland, I., Iliev, D. B., Zou, J., Jorgensen, T., & Jorgensen, J. B. (2008). A TLR9 homolog that is up-regulated by IFN-gamma in Atlantic salmon (Salmo salar). *Developmental and Comparative Immunology, 32*(6), 603-607. doi:10.1016/j.dci.2007.10.011

Smedsrod, B., Olsen, R., & Sveinbjornsson, B. (1995). Circulating Collagen Is Catabolized by Endocytosis Mainly by Endothelial-Cells of Endocardium in Cod (Gadus-Morhua). *Cell and Tissue Research, 280*(1), 39-48. doi:Doi 10.1007/Bf00304509

Soleto, I., Abos, B., Castro, R., Gonzalez, L., Tafalla, C., & Granja, A. G. (2017). The BAFF / APRIL axis plays an important role in virus-induced peritoneal responses in rainbow trout. *Fish Shellfish Immunol, 64*, 210-217. doi:10.1016/j.fsi.2017.03.023

Sommerset, I., Krossoy, B., Biering, E., & Frost, P. (2005). Vaccines for fish in aquaculture. *Expert Review of Vaccines, 4*(1), 89-101. doi:10.1586/14760584.4.1.89

St.meld. nr. 16 (2014-2015). (2015). *Forutsigbar og miljømessig bærekraftig vekst i norsk lakse- og ørretoppdrett*. Oslo: Det kongelige nærings- og fiskeridepartement.

Strandskog, G., Skjaeveland, I., Ellingsen, T., & Jorgensen, J. B. (2008). Double-stranded RNA- and CpG DNA-induced immune responses in Atlantic salmon: Comparison and synergies. *Vaccine, 26*(36), 4704-4715. doi:10.1016/j.vaccine.2008.06.054

Striz, I., Brabcova, E., Kolesar, L., & Sekerkova, A. (2014). Cytokine networking of innate immunity cells: a potential target of therapy. *Clinical Science, 126*(9-10), 593-612. doi:10.1042/Cs20130497

Sun, Y., & Sun, L. (2015). CsBAFF, a Teleost B Cell Activating Factor, Promotes Pathogen-Induced Innate Immunity and Vaccine-Induced Adaptive Immunity. *PLoS One, 10*(8), e0136015. doi:10.1371/journal.pone.0136015

Tafalla, C., Gonzalez, L., Castro, R., & Granja, A. G. (2017). B cell-activating Factor regulates Different aspects of B cell Functionality and is Produced by a subset of splenic B cells in Teleost Fish. *Frontiers in Immunology, 8*. doi:ARTN 295

10.3389/fimmu.2017.00295

Thermo-Scientific. (2010a). EZ-Link® NHS-PEG Solid Phase Biotinylation Kit – Pre-packed Column Retrieved from https://assets.thermofisher.com/TFS-

Assets/LSG/manuals/MAN0011500_EZ_NHSPEG_SolidPhaseBiotiny_Prepak_UG.pdf

Thermo-Scientific. (2010b). NanoDrop 1000 Spectrophotometer V3.8 User's Manual Retrieved from https://tools.thermofisher.com/content/sfs/manuals/nd-1000-v3.8-users-manual-8%205x11.pdf

Thim, H. L., Iliev, D. B., Christie, K. E., Villoing, S., McLoughlin, M. F., Strandskog, G., & Jorgensen, J. B. (2012). Immunoprotective activity of a Salmonid Alphavirus Vaccine: Comparison of the immune responses induced by inactivated whole virus antigen formulations based on CpG class B oligonucleotides and poly I:C alone or combined with an oil adjuvant. *Vaccine, 30*(32), 4828-4834. doi:10.1016/j.vaccine.2012.05.010

Thompson, J. S., Bixler, S. A., Qian, F., Vora, K., Scott, M. L., Cachero, T. G., . . . Ambrose, C. (2001). BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. *Science, 293*(5537), 2108-2111. doi:10.1126/science.1061965

Treml, L. S., Carlesso, G., Hoek, K. L., Stadanlick, J. E., Kambayashi, T., Bram, R. J., . . . Khan, W. N. (2007). TLR stimulation modifies BLyS receptor expression in follicular and marginal zone B cells. *Journal of Immunology, 178*(12), 7531-7539. doi:DOI 10.4049/jimmunol.178.12.7531

Uribe, C., Folch, H., Enriquez, R., & Moran, G. (2011). Innate and adaptive immunity in teleost fish: a review. *Veterinarni Medicina, 56*(10), 486-503.

Veterinærinstituttet. (2018). *Fiskehelserapporten 2017,* . Retrieved from https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2018/fiskehelserapporten-2017/_/attachment/download/fe9500ca-6520-4672-b84c-e1443c0c1cc6:f3d16157f27e2218bcd0862dba38b3f7c44e496b/fiskehelserapporten-enkeltsider_web.pdf

Vincent, F. B., Morand, E. F., Schneider, P., & Mackay, F. (2014). The BAFF/APRIL system in SLE pathogenesis. *Nature Reviews Rheumatology, 10*(6), 365-373. doi:10.1038/nrrheum.2014.33

von Bulow, G. U., van Deursen, J. M., & Bram, R. J. (2001). Regulation of the T-independent humoral response by TACI. *Immunity, 14*(5), 573-582. doi:Doi 10.1016/S1074-7613(01)00130-3

Whyte, S. K. (2007). The innate immune response of finfish - A review of current knowledge. *Fish & Shellfish Immunology, 23*(6), 1127-1151. doi:10.1016/j.fsi.2007.06.005

Wiens, G. D., & Glenney, G. W. (2011). Origin and evolution of TNF and TNF receptor superfamilies. *Developmental and Comparative Immunology, 35*(12), 1324-1335. doi:10.1016/j.dci.2011.03.031

Wilson, M., Bengten, E., Miller, N. W., Clem, L. W., DuPasquier, L., & Warr, G. W. (1997). A novel chimeric Ig heavy chain from a teleost fish shares similarities to IgD. *Proceedings of the National Academy of Sciences of the United States of America, 94*(9), 4593-4597. doi:DOI 10.1073/pnas.94.9.4593

Xiao, W., Long, W., Liu, G. Y., Sui, C. L., Guo, X. R., Tian, A. Y., . . . Zhang, S. Q. (2014). Molecular cloning, expression and functional analysis of B-cell activating.factor (BAFF) in yellow grouper, Epinephelus awoara. *Molecular Immunology, 59*(1), 64-70. doi:10.1016/j.molimm.2014.01.005

Xu, C., Guo, T.-C., Mutoloki, S., Haugland, Ø., & Evensen, Ø. (2012). Gene expression studies of host response to Salmonid alphavirus subtype 3 experimental infections in Atlantic salmon. *Veterinary research, 43*(1), 78.

Xu, C., Guo, T.-C., Mutoloki, S., Haugland, Ø., Marjara, I. S., & Evensen, Ø. (2010). Alpha interferon and not gamma interferon inhibits salmonid alphavirus subtype 3 replication in vitro. *Journal of Virology, 84*(17), 8903-8912.

Xu, C., Mutoloki, S., & Evensen, O. (2012). Superior protection conferred by inactivated whole virus vaccine over subunit and DNA vaccines against salmonid alphavirus infection in Atlantic salmon (Salmo salar L.). *Vaccine, 30*(26), 3918-3928. doi:10.1016/j.vaccine.2012.03.081

Xu, Z., Gomez, D., Parra, D., Takizawa, F., & Sunyer, J. O. (2013). IgT plays a prominent role in gill immune response of rainbow trout. *Fish & Shellfish Immunology, 34*(6), 1686-1686. doi:DOI 10.1016/j.fsi.2013.03.161

Xu, Z., Parra, D., Gomez, D., Salinas, I., Zhang, Y. A., Jorgensen, L. V., . . . Sunyer, J. O. (2013). Teleost skin, an ancient mucosal surface that elicits gut-like immune responses. *Proceedings of the National Academy of Sciences of the United States of America, 110*(32), 13097-13102. doi:10.1073/pnas.1304319110

Xu, Z. Y., Sun, B. J., Chang, M. X., & Nie, P. (2008). Characterization and expression analysis of TNFR-associated factor 1 (TRAF1) in grass carp Ctenopharyngodon idella. *Veterinary Immunology and Immunopathology, 121*(1-2), 44-57. doi:10.1016/j.vetimm.2007.08.001

Yan, M. H., Wang, M., Chan, B., Roose-Girma, M., Erickson, S., Baker, T., . . . Dixit, V. M. (2001). Activation and accumulation of B cells in TACI-deficient mice. *Nature Immunology, 2*(7), 638-643. doi:Doi 10.1038/89790

Ye, J. M., Kaattari, I. M., Ma, C. Y., & Kaattari, S. (2013). The teleost humoral immune response. *Fish & Shellfish Immunology, 35*(6), 1719-1728. doi:10.1016/j.fsi.2013.10.015

Zhang, Y. A., Salinas, I., & Sunyer, J. O. (2011). Recent findings on the structure and function of teleost IgT. *Fish & Shellfish Immunology, 31*(5), 627-634. doi:10.1016/j.fsi.2011.03.021

Zhu, L. Y., Nie, L., Zhu, G., Xiang, L. X., & Shao, J. Z. (2013). Advances in research of fish immune-relevant genes: A comparative overview of innate and adaptive immunity in teleosts. *Developmental and Comparative Immunology, 39*(1-2), 39-62. doi:10.1016/j.dci.2012.04.001

Zou, J., Clark, M. S., & Secombes, C. J. (2003). Characterisation, expression and promoter analysis of an interleukin 10 homologue in the puffer fish, Fugu rubripes. *Immunogenetics, 55*(5), 325-335. doi:10.1007/s00251-003-0580-y

Zwollo, P., Cole, S., Bromage, E., & Kaattari, S. (2005). B cell heterogeneity in the teleost kidney: Evidence for a maturation gradient from anterior to posterior kidney. *Journal of Immunology, 174*(11), 6608-6616. doi:DOI 10.4049/jimmunol.174.11.6608

Zwollo, P., Haines, A., Rosato, P., & Gumulak-Smith, J. (2008). Molecular and cellular analysis of B-cell populations in the rainbow trout using Pax5 and immunoglobulin markers. *Developmental and Comparative Immunology, 32*(12), 1482-1496. doi:10.1016/j.dci.2008.06.008

Zwollo, P., Mott, K., & Barr, M. (2010). Comparative analyses of B cell populations in trout kidney and mouse bone marrow: Establishing "B cell signatures". *Developmental and Comparative Immunology, 34*(12), 1291-1299. doi:10.1016/j.dci.2010.08.003

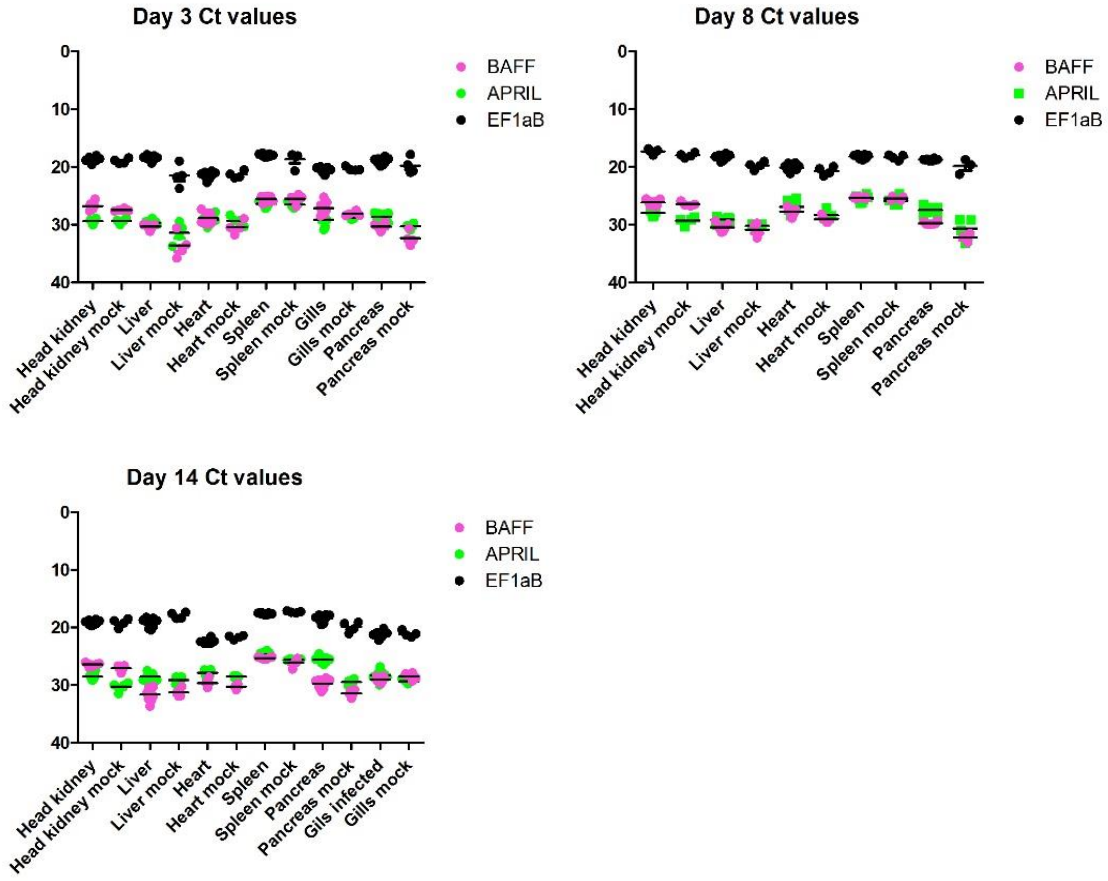
Appendix I

Reagents

Reagents	Manufacturer
Benzocain	Europharma
Bovine serum albumin	Sigma-Aldrich
Cell counting lysis buffer	Chemometec
Cell counting stabilizing buffer	Chemometec
CpG	Integrated DNA technologies
ELISPOT PDVF plates MSIPS4510	Merck Millipore
Ethanol (>99,8%)	Sigma-Aldrich
EZ-Link NHS-PEG Solid Phase Biotinylation kit	Thermo Scientific
Fast SYBR™green PCR master mix	Applied Biosystems, Life technologies
Fetal bovine serum, FBS	Biochrom AG
Heparin	LEO Pharma AS
Leibowitz's L-15 medium, L-15	Gibco™, life technologies
Methanol 99,8%	Sigma-Aldrich
Percoll	GE Healthcare
Phosphate buffered saline	Sigma-Aldrich
Poly I:C	Pharmacia biotech
Primers	Sigma-Aldrich
QuantiTect Reverse Transcription kit	Qiagen
RNA-later	Ambion™, Life technologies
RNeasy Mini Kit	Qiagen
Streptavidin-HRP conjugate	Mabtech
Streptomycin/Penicillin	Gibco™, life technologies
SYBR™green PCR master mix	Applied Biosystems, Life technologies
SYBR™Green PCR Master Mix	Applied Biosystems, Life technologies
Tetramethylbenzidine	Mabtech
Tween 20	Sigma-Aldrich
Ultra Pure Water	Biochrom AG

Appendix II

Ct values *in-vivo* experiments



Ct-values for BAFF, APRIL and EF1aB in mock- and SAV-infected Atlantic salmon at 3-, 8,- and 14 days post infection (*in-vivo*). Dot plots represent Ct values from day 3(A), day 8(B) and day 14(C). Lines represent mean values. n of SAV-infected fish = 8, n of mock-infected fish = 4. Ct data in gills at day 8 are excluded as they are unreliable (n=8).

Ct-values for BAFF, APRIL and EF1aB in untreated Atlantic salmon at 3-, 8,- and 14 days post infection (*in-vivo* experiment).The tables display highest-, lowest- and mean Ct-values of the given gene within stimulation groups.

		0 d.p.i	
Gene	Organ	Variation	Mean
Ef1aB	Gills	19,7402-20,67001	20,124
	Heart	19,07347-22,59141	21,197
	Head kidney	17,11969-18,72419	17,711
	Spleen	16,06136-17,41265	17,111
	Pancreas	17,36445-19,32514	18,581
	Liver	21,96217-19,07448	19,812
BAFF	Gills	28,782-29,375	29,183
	Heart	32,290-28,953	30,851
	Head kidney	26,881-28,167	27,350
	Spleen	24,153-25,012	24,675
	Pancreas	29,373-30,117	30,130
	Liver	30,75-21,376	31,512
APRIL	Gills	31,484-34-602	32,586
	Heart	35,253-36-989	36,348
	Head kidney	26,891-28,198	27,358
	Spleen	27,196-27,203	27,200
	Pancreas	30,115-31,075	30,663
	Liver	31,084-33,062	32,167

Ct-values for BAFF, APRIL and EF1aB in mock-infected Atlantic salmon at 3-, 8,- and 14 days post infection (*in-vivo* experiment).The tables display highest-, lowest- and mean Ct-values of the given gene within stimulation groups.

		3 d.p.i		8 d.p.i	
Gene	Organ	Variation	Mean	Variation	Mean
Ef1aB	Gills	19,80532-20,5373	20,313	17,504-19,604	18,787
	Heart	20,52262-21,25927	21,361	19,92862-21,58533	20,707
	Head kidney	18,42938-19,31392	19,001	17,49511-18,4825	17,998
	Spleen	17,892-20,693	18,698	17,94-18,394	18,325
	Pancreas	17,847-20,939	19,791	18,758-21,274	19,883
	Liver	18,969-23,703	21,486	19,019-20,615	19,748
BAFF	Gills	27,517-28,40695	28,111	21,47019-27,65506	23,817
	Heart	28,95543-31,53822	30,433	28,17939-29,57863	29,026
	Head kidney	27,13125-27,60363	27,451	25,8713-26,72388	26,415
	Spleen	24,768-26,845	25,532	25,097-25,7954	25,463
	Pancreas	30,63-33,568	32,345	31,513-33,128	32,230
	Liver	30,581-35,79	33,575	29,737-32,279	30,882
APRIL	Gills	29,09189-28,50698	28,767	20,63115-28,51533	24,083
	Heart	28,36657-30,3134	29,303	27,13384-28,94697	28,353
	Head kidney	28,83064-29,9664	29,353	28,69762-30335	29,303
	Spleen	25,943-27,145	26,523	24,593-26,624	25,880
	Pancreas	29,706-30,933	30,288	29,063-33,295	30,650
	Liver	29,458-33,703	31,420	29,868-30,922	30,221

		14 d.p.i	
Gene	Organ	Variation	Mean
Ef1aB	Gills	20,39547-21,64843	21,103
	Heart	21,37661-22,1432	21,682
	Head kidney	18,45879-20,20072	19,168
	Spleen	17,102-17,494	17,301
	Pancreas	19,039-21,037	19,892
	Liver	17,324-18,409	17,918
BAFF	Gills	27,86555-29,27467	28,474
	Heart	29,75967-30,79027	30,257
	Head kidney	27,92984-26,60902	27,072
	Spleen	25,342-27,22	26,074
	Pancreas	30,773-32,269	31,411
	Liver	30,251-31,908	31,281
APRIL	Gills	29,89545-29,28428	29,338
	Heart	28,20679-28,77541	28,510
	Head kidney	29,56709-31,45839	30,255
	Spleen	25,394-25,968	25,621
	Pancreas	28,897-30,126	29,495
	Liver	28,549-29,848	29,103

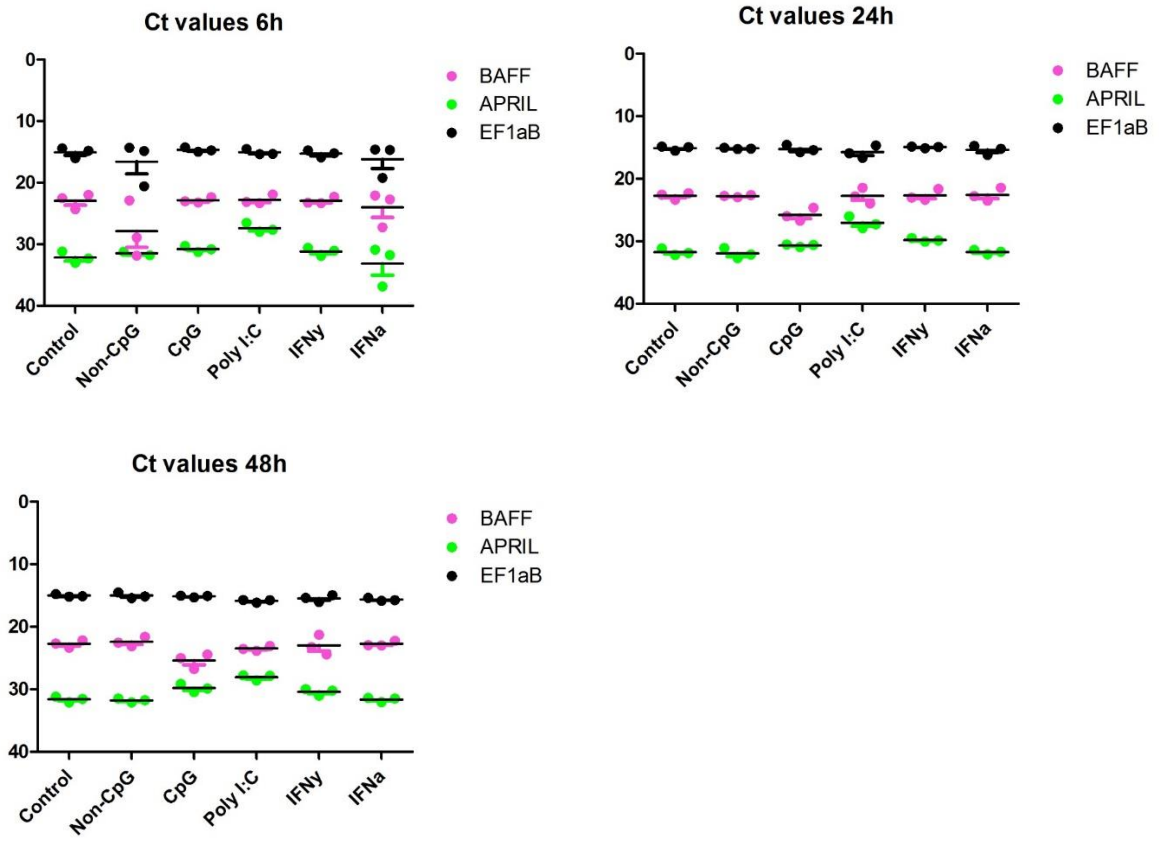
Ct-values for BAFF, APRIL and EF1aB in SAV-infected Atlantic salmon at 3-, 8,- and 14 days post infection (*in-vivo* experiment).The tables display highest-, lowest- and mean Ct-values of the given gene within stimulation groups.

		3 d.p.i		8 d.p.i	
Gene	Organ	Variation	Mean	Variation	Mean
Ef1aB	Gills	19,99084-21,45287	20,507	18,63071-20,29599	19,472
	Heart	20,70813-22,70078	21,453	19,2806-21,14328	20,122
	Head kidney	18,01263-19,5796	18,760	16,84924-17,9445	17,314
	Spleen	17,576-18,278	17,986	17,809-18,796	18,203
	Pancreas	18,148-19,835	19,017	18,447-18,943	18,755
	Liver	17,866-19,355	18,504	17,66-18,777	18,295
BAFF	Gills	27,92673-20,86175	29,160	27,22369-28,62415	27,734
	Heart	27,77017-3046707	29,189	26,77042-28,88968	27,728
	Head kidney	29,19279-30,02246	29,407	27,18718-28,50882	27,936
	Spleen	25,051-26,408	25,546	24,821-26,353	25,457
	Pancreas	29,579-31,187	30,364	26,47-28,289	27,482
	Liver	29,902-31,153	30,318	29,324-31,297	30,461
APRIL	Gills	27,92673-30,86175	29,160	26,62724-28,62415	27,734
	Heart	28,55788-30,46707	29,189	26,77042-28,88968	27,728
	Head kidney	29,19279-30,02246	29,407	27,18718-28,50882	27,936
	Spleen	25,936-27,016	26,426	24,986-26,207	25,486
	Pancreas	29,579-31,187	30,364	26,47-28,389	27,482
	Liver	28,904-30,019	29,681	28,708-29,974	29,151

		14 d.p.i	
Gene	Organ	Variation	Mean
Ef1aB	Gills	20,14588-22,20197	21,197
	Heart	21,50952-22,79704	22,456
	Head kidney	18,57948-19,68743	19,147
	Spleen	17,389-17,839	17,608
	Pancreas	17,813-19,507	18,420
	Liver	18,23-20,45	19,194
BAFF	Gills	26,80941-29,96016	28,268
	Heart	27,32079-28,47809	27,865
	Head kidney	27,30666-29,14415	28,546
	Spleen	25,082-25,517	25,329
	Pancreas	24,565-26,424	25,621
	Liver	30,203-33,647	31,623
APRIL	Gills	26,80941-29,96016	28,268
	Heart	27,32079-28,47809	27,865
	Head kidney	27,30666-29,14415	28,546
	Spleen	24,0-25,446	24,674
	Pancreas	24,565-26,424	25,621
	Liver	27,444-29,314	28,564

Appendix III

Mean Ct values of BAFF and APRIL in adherent cells



Ct-values for BAFF, APRIL and EF1aB in adherent head kidney leukocytes under different stimulations and timepoints. Dot plots represent Ct values from 6(A), 24(B) and 48(C) hours post stimulation. Lines represent mean values (n=3).

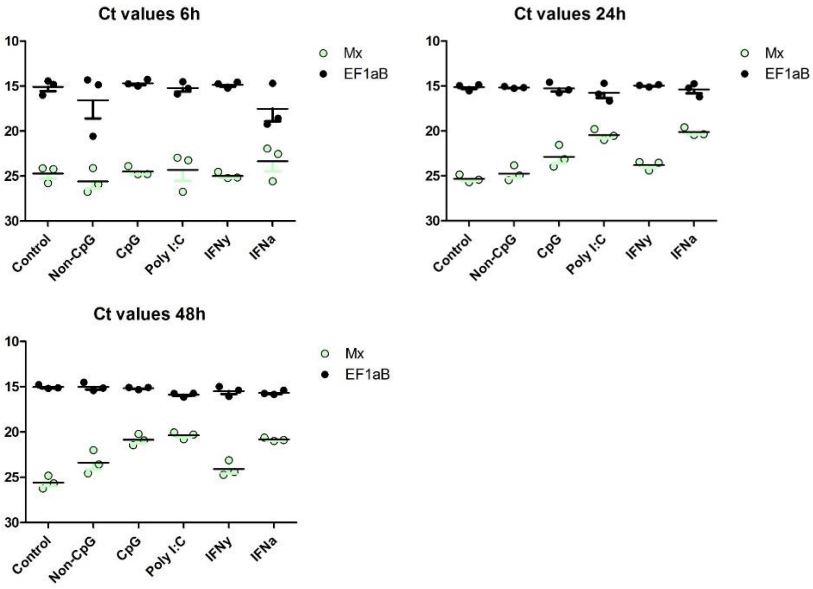
Ct-values for BAFF, APRIL and EF1aB in adherent head kidney leukocytes under different stimulations and timepoints. The tables display highest, lowest and mean Ct-values of the given gene within stimulation groups.

	6 hours post stimulation					
	Ef1aB		BAFF		APRIL	
Stimulation	Variation	Mean	Variation	Mean	Variation	Mean
Control	14,422-16,015	15,07933	21,978-24,333	15,07933	31,170-33,013	22,93767
Non-CpG	14,308-20,578	16,58133	22,874-31,836	16,58133	31,223-31,777	27,87733
CpG	14,255-14,976	14,66333	22,337-23,231	14,66333	30,278-31,234	22,86533
Poly I:C	14,507-15,339	15,04867	21,922-23,322	15,04867	26,505-27,987	22,79
IFNy	14,741-15,868	15,27867	22,294-23,316	15,27867	22,294-23,316	22,95667
IFNa	14,694-19,200	16,17267	22,092-27,266	16,17267	22,092-27,266	24,029

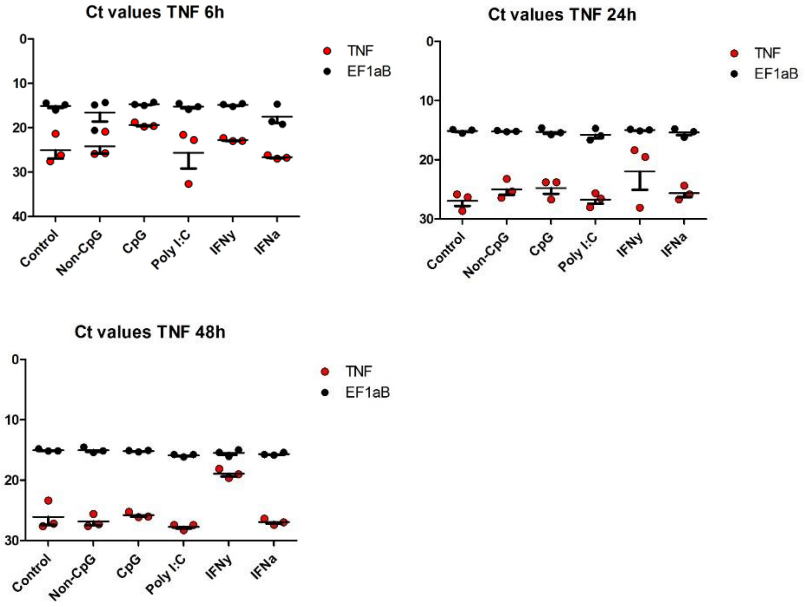
	24 hours post stimulation					
	Ef1aB		BAFF		APRIL	
Stimulation	Variation	Mean	Variation	Mean	Variation	Mean
Control	14,862-15,524	15,11167	22,313-23,346	22,73167	31,162-32,170	31,73767
Non-CpG	15,034-15,259	15,15767	22,612-22,955	22,77	31,074-32,683	31,95433
CpG	14,572-15,765	15,25333	24,656-26,727	25,79033	30,526-30,904	30,67467
Poly I:C	14,690-16,661	15,751	21,451-23,948	22,74067	26,018-27,869	27,05233
IFNy	14,841-15,112	14,95967	21,642-23,340	22,668	29,500-30,036	29,80467
IFNa	14,739-16,191	15,38533	21,435-23,519	22,58233	31,369-32,070	31,71133

	48 hours post stimulation					
	Ef1aB		BAFF		APRIL	
Stimulation	Variation	Mean	Variation	Mean	Variation	Mean
Control	14,862-15,524	15,11167	22,313-23,346	22,73167	31,162-32,170	31,73767
Non-CpG	15,034-15,259	15,15767	22,612-22,955	22,77	31,074-32,683	31,95433
CpG	14,572-15,765	15,25333	24,656-26,727	25,79033	30,526-30,904	30,67467
Poly I:C	14,690-16,661	15,751	21,451-23,948	22,74067	26,018-27,869	27,05233
IFNy	14,841-15,112	14,95967	21,642-23,340	22,668	29,500-30,036	29,80467
IFNa	14,739-16,191	15,38533	21,435-23,519	22,58233	31,369-32,070	31,71133

Mean Ct values of Mx and TNF in adherent cells



Ct-values for Mx and EF1aB in adherent head kidney leukocytes under different stimulations and timepoints. Dot plots represent Ct values from 6(A), 24(B) and 48(C) hours post stimulation. Lines represent mean values (n=3)



Ct-values for TNF and EF1aB in adherent head kidney leukocytes under different stimulations and timepoints. Dot plots represent Ct values from 6(A), 24(B) and 48(C) hours post stimulation. Lines represent mean values (n=3).

Ct-values for TNF and EF1aB in adherent head kidney leukocytes under different stimulations and timepoints. The tables display highest, lowest and mean Ct-values of the given gene within stimulation groups.

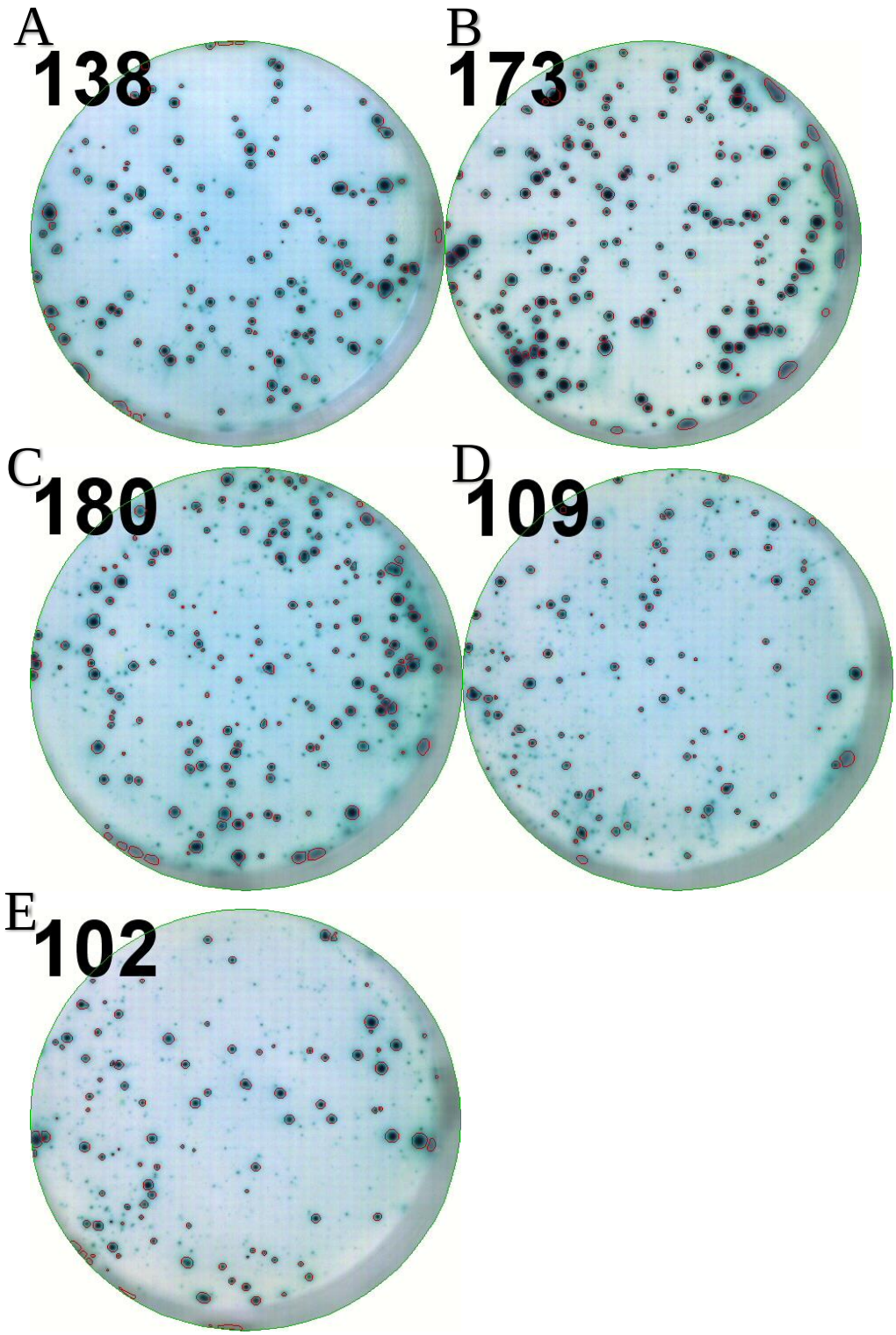
	6 hours post stimulation					
	Ef1aB		TNF		Mx	
Stimulation	Variation	Mean	Variation	Mean	Variation	Mean
Control	14,422-16,015	15,07933	21,356-27,561	25,03467	24,127-25,791	24,71233
Non-CpG	14,308-20,578	16,58133	20,884-25,89	24,167	24,109-26,749	25,596
CpG	14,255-14,976	14,66333	18,775-19,762	19,37733	23,896-24,8	24,49267
Poly I:C	14,507-15,339	15,04867	21,571-32,649	25,653	22,96-26,746	24,31667
IFNy	14,741-15,868	15,27867	22,32-22,992	22,75467	24,533-25,165	24,97133
IFNa	14,694-19,200	16,17267	26,187-26,966	26,63033	21,938-25,574	23,34933

	24 hours post stimulation					
	Ef1aB		TNF		Mx	
Stimulation	Variation	Mean	Variation	Mean	Variation	Mean
Control	14,862-15,524	15,11167	25,837-28,643	26,933	24,837-25,693	25,31633
Non-CpG	15,034-15,259	15,15767	23,203-26,411	24,98067	23,827-25,462	24,742
CpG	14,572-15,765	15,25333	23,792-26,703	24,762	21,55-23-96	22,883
Poly I:C	14,690-16,661	15,751	25,649-28,008	26,73233	19,788-20,944	20,44033
IFNy	14,841-15,112	14,95967	18,357-28,093	21,98533	23,462-24,393	23,79433
IFNa	14,739-16,191	15,38533	24,355-26,683	25,62167	19,59-20,436	20,12733

	48 hours post stimulation					
	Ef1aB		TNF		Mx	
Stimulation	Variation	Mean	Variation	Mean	Variation	Mean
Control	14,862-15,524	15,11167	23,358-27,619	26,056	24,819-26,229	25,57067
Non-CpG	15,034-15,259	15,15767	25,59-27,585	26,82367	21,996-24,555	23,374
CpG	14,572-15,765	15,25333	25,242-26,113	25,786	20,2-21,456	20,86167
Poly I:C	14,690-16,661	15,751	27,413-28,27	27,70067	20,037-20,794	20,366
IFNy	14,841-15,112	14,95967	18,103-19,624	18,90967	23,103-24,724	24,077
IFNa	14,739-16,191	15,38533	26,369-27,396	26,90533	20,605-20,993	20,83033

Appendix IV

ELISPOT assay



Raw images of ELISPOT wells containing HK MLCs extracted from the same fish including control(a), LPS(b), CpG(c). BAFF(d), and APRIL(e) treatment groups 5 days post stimulation. Counted spots encircled in red. BAFF, APRIL and CpG display a higher number of uncounted spots.