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Faculty of Biosciences, Fisheries and Economics
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Atlantic salmon B cells- local and systemic responses to intraperitoneally administered salmonid alphavirus

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A dissertation for the degree of Philosophiae Doctor – December 2019

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Acknowledgments

…The Best Always Goes to the Most High…

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Shiferaw Jenberie, Henriette Nordli, Guro Strandskog, Linn Greiner-Tollersrud, Ma. Michelle D. Peñaranda, Jorunn B. Jørgensen and Ingvill Jensen

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Thesis summary

To date, infectious diseases are one of the main challenges for further expansion of the aquaculture industry. Vaccination has made a remarkable impact on reducing disease burden and antibiotic use and thereby improving fish welfare. Despite vaccination, fish viral diseases are still a major challenge calling the efficacy of viral vaccine into question. Disease prevention by vaccination relies on the induction of protective and long lasting humoral immunity mediated by B cells. In Atlantic salmon (A. salmon) or in teleost fish in general, there is scarcity of information regarding the basic biology of B cells and the mechanistic orchestration of the immune response against viral antigens.

The main goal of this thesis was to characterise B cell populations from A. salmon and study their responses after *in vitro* and *in vivo* stimulation. Previous works have shown that the TLR-ligand CpG is a promising molecular vaccine adjuvant for A. salmon. In this thesis, the immunostimulatory role of CpG was further investigated using sorted B cells. A. salmon B cells transcribed both TLR genes sensing CpG in teleost species, TLR9 and TLR21, providing the basis for further studies of CpG effect on A. salmon B cells. CpG stimulation upregulated transcription of surface IgM (sIgM) gene and enhanced secretion of IgM *in vitro* suggesting that CpG directly affects the differentiation of B cells into antibody secreting cells (ASC). Beyond their adaptive immune function, B cells from peripheral blood, spleen and HK constitutively transcribed high levels of MHCII gene indicating Ag-presenting functions. To further expand the understanding of the IgM$^+$ B cell and ASC responses in A. salmon, *in vivo* experiments were undertaken by intraperitoneal (IP) injection of inactivated (inSAV) or virulent (wtSAV3) salmonid alphavirus (SAV) with the objective of comparing the local and systemic responses. In steady state A. salmon, the frequency of IgM$^+$ B cells was over two-times higher in the systemic immune sites compared to the PerC, while PerC had over 2-times higher total ASC frequency than the systemic immune sites showing a previously unnoticed uneven distribution of B cells. Compared to IgM$^+$ B cells, ASC exhibited a lower frequency across the three sites suggesting that they represent either a small fraction of the IgM$^+$ cells or a different population with minimal/ no surface IgM. IP SAV3 challenge induced two IgM$^+$ B cell populations, IgM^{high} and IgM^{low}, with a marked shift towards IgM^{low} in the PerC suggesting the presence of a niche in the PerC that supports B cell differentiation. The peak IgM$^+$ B cells and total ASC frequency at 6 wpc in the PerC of wtSAV3 infected fish indicate that

the B cell response is more important at later stage. inSAV induced a significantly lower frequency of Ag-specific ASC compared to wtSAV3 at 13 wpc; by then, the response was barely detected in the HK and spleen. Within the wtSAV3 infected fish, the frequency of Ag-specific ASC in the PerC increased from 6 to 13 wpc while frequency of total ASC decreased during the same period suggesting an independent regulation of these cells in A. salmon. Comparing the three sites in the wtSAV3 infected fish, the PerC had the highest frequency of Ag-specific ASC suggesting on local importance of these cells. Additionally, the peak total ASC frequency in the PerC of the wtSAV3 infected fish correlated positively with the anti-SAV E2 and virus neutralizing antibody responses in serum. However, whether the PerC Ag-specific ASC contribute to the serum specific antibody response remains unclear and needs further investigation.

Abbreviations

A. salmon	Atlantic salmon	MHC	Major histocompatibility complex
Ab	Antibody	mIgM	Membrane bound IgM
Ag	Antigen	NALT	Nasopharynx associated lymphoid tissue
AID	Activation-induced cytidine deaminase	NF-κB	Nuclear factor kappa B
APC	Antigen-presenting cell	NOD	Nucleotide-binding oligomerization domain
ASC	Antibody secreting cells		
BAFF	B cell activating factor	nsP	Non-structural protein
BCR	B cell receptor	PAMP	Pathogen associated molecular pattern
Beff	Effector B cell	PAS	Pulse adherent cell supernatant
Breg	Regulatory B cell	PD	Pancreas disease
CAS	Control adherent cell supernatant	PerC	Peritoneal cavity
CD	Cluster of differentiation	PRR	Pattern recognition receptor
CSR	Class switch recombination	RAG	Recombinase activating gene
DNA	Deoxyribonucleic acid	RIG	Retinoic acid-inducible gene
dpi	Days post-infection	SAV	Salmonid alphavirus
ELISA	Enzyme linked immunosorbent assay	SHM	Somatic hypermutation
ELISpot	Enzyme-linked immunospot assay	sIgM	Secreted IgM
FACS	Fluorescent activated cell sorting	TLR	Toll-like receptor
HK	Head kidney	TNF	Tumor necrosis factor
IFN	Interferon	VN	Virus neutralization
Ig	Immunoglobulin	wpc	Weeks post-challenge
IL	Interleukin	wpi	Weeks post-injection
IP	Intraperitoneal		
ITAM	Immunoreceptor tyrosine-based activation motif		
LRR	Leucine-rich repeat		
MACS	Magnetic activated cell sorting		
mAb	Monoclonal antibody		
MALT	Mucosal-associated lymphoid tissue		
MAMP	Microbial associated molecular pattern		

Introduction

1. Teleost's immune system: Primitive yet efficient and complex

Fish represent the most diverse group of vertebrates that appeared in evolution after adaptive radiation (Rauta et al., 2012). Despite some interesting anatomical differences, the immune system of teleost fish functions in a similar way as in higher vertebrates when it comes to basic mechanisms involved in protection (Uribe et al., 2011). Teleosts have not experienced the last major advance in the immune system evolution that fostered formation of lymph nodes and germinal centres in higher vertebrates (Neely and Flajnik, 2016). This recent evolutionary leap that has enabled higher vertebrates to embark on canonical germinal centre (GC) reactions such as antibody class switching and memory immune responses. Lacking the last leap in the immune system evolution, teleosts are regarded as having a more primitive type of immune system compared to higher vertebrates.

The innate and adaptive arms of the immune system in jawed vertebrates coevolved in such a way that they accomplished a highly specialized task while tightly interacting each other to regulate the outcome of an immune response against a pathogen (Cooper and Alder, 2006). Invertebrates lack an adaptive immune system based on B and T cell receptors and hence, rely solely on their innate immunity (Flajnik and Kasahara, 2010). Although the basic innate immune mechanisms operating in both invertebrates and vertebrates are conserved, it seems that the variability and diversity are much higher among invertebrates (Hibino et al., 2006, Flajnik and Kasahara, 2010). As for invertebrates, teleosts possess a greater expansion and unique constellation of pattern recognition receptor (PRR) families to compensate for the limitation of their adaptive immune system (Flajnik and Kasahara, 2010, Palti, 2011). On the other hand, higher vertebrates reach the same or even greater level of versatility by fine-tuning their adaptive immune system (Magnadottir, 2006). This has led to the hypothesis that the acquisition of a highly specialized adaptive immunity in mammals may have relieved some pressure on their innate mechanisms (Tort et al., 2003, Buchmann, 2014). By the virtue of their unique evolutionary position, teleost fish draw the attention of comparative immunologists finding insights into the early events on how the adaptive immune system has been innovated (Zhu et al., 2013).

1.1. The innate immune system

The innate arm is evolutionarily the most ancient part of the immune system. It is based on germ-line encoded non-rearranging receptors, called PRRs, expressed on different subsets of leukocytes including macrophages, granulocytes, dendritic, B cells, natural killer cells, etc. (Zhu et al., 2013). Ligand binding by PRRs activates rapid antimicrobial effector mechanisms that involve phagocytosis, proteolytic activation cascades and synthesis of potent antimicrobial molecules (Medzhitov, 2007). The PRRs recognize conserved microbial associated molecular patterns (MAMPs). The innate immune system is the earliest to respond following recognition of non-self in a manner irrespective of previous history of exposure and activates the adaptive immune response. Hence, it plays an important instructive role on the adaptive immune system (Tort et al., 2003, Medzhitov, 2007, Uribe et al., 2011, Rauta et al., 2012). PRRs such as Toll-like receptors (TLRs), RIG-I like receptors (RLRs), NOD-like receptors (NLRs), and C-type lectin receptors (CLRs) have been characterized in several fish species (Uribe et al., 2011).

The innate immune system in fish is commonly divided into three compartments: the epithelial/mucosal barrier, the humoral and the cellular components (Magnadottir, 2010). The fact that fish are free-living organisms from the embryonic stage of life in their aquatic environment, which is also home for numerous potential pathogens, necessitates for an efficient and early functioning immune system. Additionally, compared with mammalian counterparts, the adaptive immune response of fish takes longer to develop emphasizing further the importance of an early functioning innate immune system. Hence, teleost fish, both immature and adult life forms, rely on the innate immune system for an extended period until the adaptive arm is kicked off (Uribe et al., 2011).

1.2. The adaptive immune system

The addition of the adaptive immunity to the pre-existing innate immunity is believed to foster existential advantage for vertebrates. The adaptive immune response is executed following recognition of MAMPs by the innate immune cells with subsequent intracellular killing and presentation in an MHC-II restricted manner (Uribe et al., 2011). The apparently unlimited variability that B cell receptors (BCRs) and T cell receptors (TCRs) present to the adaptive immunity enables it to recognize any antigen (Ag) (Parra et al., 2013). Originating from

different tissues but from the same common lymphoid progenitor, B and T cells constitute the main cell types of the adaptive immunity. These cells work in concert to produce the desired fine-tuned adaptive responses. To date, based on the Ag-binding repertoire, two types of adaptive immune strategies have known to exist in vertebrates: the variable lymphocyte receptor (VLR) in agnathans and BCR/TCR in gnathostomes (Parra et al., 2013). Gnathostomes are evolutionary the oldest animals where adaptive immunity hallmark molecules such as BCR, TCR, MHCII and RAG can be traced back (Flajnik, 2018). However, teleost fish lack some machineries of somatic reorganization of the Ig gene. Accordingly, teleosts have weak adaptive immune response compared to higher vertebrates, which is characterised by limited repertoire of antibodies, weak Ab-affinity maturation and slow memory responses (Tort et al., 2003, Uribe et al., 2011).

2. B cells in teleost fish

Since their discovery in the mid-1960s in birds, B cells have been recognized for their ability to produce antibodies (Cooper et al., 1965, LeBien and Tedder, 2008). As in higher vertebrates, B cells in teleost fish can be defined as those lymphocytes that express immunoglobulin receptors on their surface that recognise specific antigenic epitopes and secrete specific antibody in response to antigenic stimuli (Kaattari, 1992). To date, B cells have been identified in all fish species examined thus far. However, their genesis, physical characteristics, distribution, form of response and antibody product have not precisely followed the mammalian paradigm (Kaattari, 1992). B cells are recognised for their versatile role in modulating the outcome of the immune response against a pathogen and indeed, they are not simply antibody making machines. B cells are required for normal development and maintenance of the immune system (Nolte et al., 2004). B cells are also important for optimal T cell activation (LeBien and Tedder, 2008). B cells function as professional Ag presenting cell (APC) (Zhu et al., 2014) and can prime naïve T cells during primary immune response (Zhu et al., 2014). In addition, B cells play an important immunomodulatory function via production of cytokines (Lampropoulou et al., 2012, Hamze et al., 2013). B cell research in fish remains largely on characterizing basic phenotypic characteristics, antibody products and their subsequent activation and differentiation after antigenic stimulation, while knowledge about their antibody independent immune-regulatory function is scarcely available.

2.1. Evolution of B cells

The emergence of cartilaginous fish, around 450 Mya, is a landmark in the evolution of the adaptive immunity (Flajnik, 2018) (Fig. 1). This existential innovation in vertebrae is thought to be driven by increased challenge from pathogens (Lee and Mazmanian, 2010). It is generally accepted that BCR and TCR genes share the same common ancestor as evidenced by their similar domain organization and reliance on the same repertoire diversification machinery (Flajnik and Kasahara, 2010). RAG transposon invasion and whole genome duplications are now widely believed to be the two macroevolutionary events that contributed for the relatively rapid emergence of the adaptive immunity. It is conceived that VDJ recombination, the whole essence of the adaptive immunity, arose because of a lateral gene-transfer event causing a RAG transposon-mediated disruption of a single gene. It seems likely that this transposon-targeted gene might encoded a cell-surface non-rearranging receptor involved in immune defence in invertebrates or lower vertebrates (Eason et al., 2004, Boehm, 2006, Pancer and Cooper, 2006, Boehm and Bleul, 2007, Huang et al., 2016). Homology studies show that TCR-like genes might have been the target of the RAG transposon insertion (Hernandez Prada et al., 2006, Boehm and Bleul, 2007) and BCR might have been evolved by gene duplication from this RAG-containing TCR-like gene cluster (Parra et al., 2013). It is also probable that an ancestral Ig super family (IgSF) gene with a characteristic of V-set domain would be diversified into the BCR and TCR genes (Hernandez Prada et al., 2006).
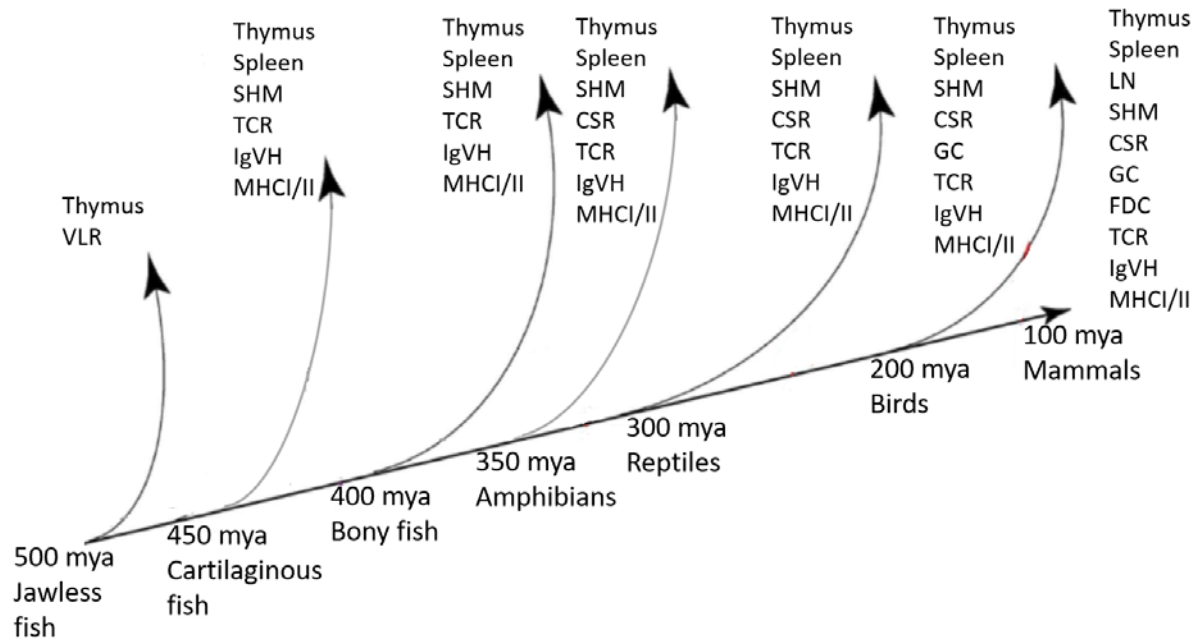
Figure 1. Evolution of key immune tissues, mechanisms and adaptive molecules in the vertebrate.
mya, million years ago; CSR , class switch recombination; FDC, follicular dendritic cells; IgVH,
immunoglobulin heavy chain variable region; SHM, somatic hypermutation; GC, germinal centre; TCR,
T cell receptor; LN, lymph node. Figure adapted from (Flajnik, 2018).

2.2. B cell lineages in teleost fish

Although our understanding of the B cells' lineage, phenotype and function continues to evolve, thus far, three distinct B cell lineages have been known to evolve in higher vertebrates (Kantor et al., 1992, Tung et al., 2006). Reconstitution studies have falsified the earlier B cell lineage paradigm that places all B cells within the same lineage. According to this long held paradigm, antigen stimulation and selection late in differentiation, after IgH rearrangement, drive B cell to assume one of the phenotypes (Borghesi and Gerstein, 2004, Montecino-Rodriguez et al., 2006). To date, at least, three independent B cell progenitors have known to exist in higher vertebrates; namely, B-1a, B-1b and B-2, that are diverged phenotypically well before the V to DJ rearrangement and expression that could be used for Ag-dependent selection (Kantor et al., 1992, Montecino-Rodriguez et al., 2006, Tung et al., 2006). In essence, these phenotypically and functionally distinct B cell progenitors belong to distinct developmental lineages that gave rise to the three B cells phenotypes, i.e. B-1a, B-1b and B-2 B cells. Additionally, the B-2 B cells make an important cell fate decision to develop into either follicular B cells (FB) or marginal zone B cells (MZB) in the spleen, which depends on signalling

through the different surface receptors (Allman and Pillai, 2008, Pillai and Cariappa, 2009). The antibody repertoires on these three distinct B cell lineages correlate well with hypothesis that B cell lineages reflect the existence of an evolutionarily layered immune system. In this regards, the immune system is sequentially evolved to a more dominant and sophisticated B-2 B cells response leaving behind the innate like B-1 B cells response as a minority (Hetzenberg and Herzenberg, 1989, Kantor et al., 1992, Tung et al., 2006).

B cell lineage studies have been far from being an agenda to fish immunologists owing to lack of appropriate reagents. Despite this, based on some phenotypic characteristics and functional resemblance, teleost B cells are considered functional homologue of mammalian B-1 B cells. These include production of polyreactive but poor-affinity natural antibody (Uribe et al., 2011), express wide range of PRRs (Abos et al., 2013) and surface molecules that mark mammalian B-1 B cells (Castro et al., 2015, Abos et al., 2018a) and high phagocytic capability (Li et al., 2006). However, the tendency to consider teleost B cells residing in different sites including in the peritoneal cavity (PerC) as a homogeneous lineage needs further investigations. Indeed, resembling the mammalian B-1 and B-2 B cells compartment, rainbow trout IgM$^+$ B cells in the PerC and spleen demonstrate different cytokine requirement for survival (Soleto et al., 2017, Tafalla et al., 2017). In addition, several important phenotypic and functional characteristics of progenitor B cells have not been yet addressed in fish.

2.3. B cell subpopulations based on surface Ig expression

B cells can be classified into several subsets based on different criteria (Sagaert and De Wolf-Peeters, 2003). In the past few decades, advances in technological platforms combined with immunophenotyping techniques enabled immunologists to dissect B cells into complex subsets based on their surface markers (Maecker et al., 2012). However, lack of population specific surface markers and functional assays have slowed progresses of fish immunology and hence, compared to mammals, B cells in fish are poorly characterized (Peñaranda et al., 2019). In fish, B cells are commonly classified based on their surface Ig expression. The availability of mAbs against surface Ig from some teleost species makes this classification method popular in fish. To date, four subsets of B cells have been reported from teleost species. Of these, a subset co-expressing IgM and IgD has been found in most teleost species. Three other subsets of B cells solely expressing IgM, Ig D or IgT/IgZ have also been reported only from few teleost

species (Edholm et al., 2010, Zhang et al., 2010, Granja and Tafalla, 2017). Consistent with the above classification, analysis of trout (Hansen et al., 2005) and zebrafish (Danilova et al., 2005) genome revealed that the locus encoding Dτ-Jτ-Cτ (for IgT) is sandwiched between array of V gene segments upstream and the (DJC)μ cluster (for IgM) downstream showing a mutually exclusive expression of these heavy chain Ig isotypes in teleost fish. Indeed, this has partly explained the absence of class switch recombination in teleost species. This arrangement of the IgH locus, with minor variabilities, has been observed in all fish species examined (Fillatreau et al., 2013).

2.3.1. IgM+/IgD+ B cells: Double positive

Naïve mature B cells in most teleost fish co-express IgM and IgD on their surface. Despite the build-up of knowledge on IgM+ B cells, the better described B cell subset in fish, distinction between the double positive and IgM-only B cells have not been made in most studies. The IgM+ B cells are the major subset in the systemic (e.g. spleen and kidney) and peripheral sites (e.g. peripheral blood and peritoneal cavity) (Parra et al., 2016). Morphologically, the channel catfish double positive and IgM-only B cells are indistinguishable from each other and have typical lymphocyte characteristics, i.e. small in size and agranular (Edholm et al., 2011). Currently, it is not clear if other fish species have the IgM-only and the IgD-only B cell subsets as described for channel catfish and rainbow trout. Therefore, the IgM+ B cells phenotype and function described for most fish species cannot be assigned to either the double positive or the IgM-only subset without ambiguity. Nonetheless, IgM+ B cells, as named in most studies, in the systemic sites and peritoneal cavity differentiate into ASC-like phenotype and secrete IgM in response to Ag stimulation (Kaattari et al., 2002, Bromage et al., 2004, Zwollo et al., 2005, Castro et al., 2017, Granja and Tafalla, 2017).

2.3.2. IgT+ (in trout) or IgZ+ (in cyprinids) B cells

IgT+ B cells are the main B cell subset in the mucosal-associated lymphoid tissue (MALT) (Zhang et al., 2010, Olsen et al., 2011). In teleost species, MALT comprise gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT), the gill-associated lymphoid tissue (GIALT) and nasopharynx-associated lymphoid tissue (NALT) (Salinas, 2015). IgT+ B cells represent about 61% and 54% of the B cell population in SALT (Xu et al., 2013b) and GALT (Zhang et al., 2010) of naïve rainbow trout, respectively. However, in channel catfish, where

IgT^{+} B cells have not been identified thus far, the MALT is populated with resident IgM^{+} B cells (Hebert et al., 2002). The number of IgT^{+} cells increases in trout mucosal surfaces surviving gut parasite infection, while IgM^{+} B cells number is not affected indicating that IgT^{+} B cells are specialized in defending mucosal and skin surfaces (Zhang et al., 2010, Xu et al., 2013b). The IgT^{+} B cells are also the predominant B-cell subset in nasopharynx associated lymphoid tissue (NALT) in rainbow trout (Tacchi et al., 2014). Like the IgM^{+} B cells, IgT^{+} B cells have potent phagocytic and bactericidal capability suggesting that both B cell lineages in fish could have ancestral relationship with the B1-B cells of mammals (Li et al., 2006, Zhang et al., 2010). This functional plasticity of teleost fish B cells as APCs has positioned them at a crossroad in the evolution of the innate and adaptive immunity. Further, IgT^{+} B cells demonstrate comparable proliferation and immunoglobulin secretion as IgM^{+} B cells in response to microbial stimulation (Zhang et al., 2010).

2.3.3. IgD^{+} B cells: IgD-only

At sequence level, the IgD locus of teleosts shares many similarities to that of the mammalian counterpart including sequence homology, location immediately downstream of IgM locus and expression by alternative splicing (Edholm et al., 2011, Sun et al., 2012, Olivieri and Gambon Deza, 2018). In fish, as in higher vertebrates, IgD is a chimeric product containing rearranged VDJ and Cμ1 followed by Cδ suggesting an unusual pathway of transcript processing machinery (Hordvik et al., 1999). The inclusion of Cμ1 into the IgD H-chain is believed to be important for covalent association with IgL-chains (Edholm et al., 2010). The existence of two populations of IgD-expressing B cells is evolutionarily conserved between fish and mammals. However, in fish the IgD[+]/IgM[−] (IgD-only) B cells are so far reported only in catfish and rainbow trout (Edholm et al., 2010, Castro et al., 2014). Unlike the double positive B cells, the catfish IgD-only B cells display a plasmablast morphology and have a higher cytoplasm to nucleus ratio (Edholm et al., 2010). Further, in contrary to human IgD-only B cells, which represent about 0.5-1% of the circulating CD19^{+} B cells in the peripheral blood and 1.5-5% of the CD19^{+} B cells in tonsils (Chen et al., 2009), catfish IgD-only B cells can represent up to 60-80% of the total peripheral blood B cells (Edholm et al., 2010). The absence of IgD-only B cells and secreted IgD in the intestine or gut secretions indicates compartmentalization of IgD-only B cells in fish (Parra et al., 2016). As for the IgM-only subsets, functional studies describing the role of the IgD-only B cells have been hardly available in fish. However, it is

hypothesized that catfish membrane IgD functions as a typical Ag binding receptor (Edholm et al., 2011). The binding of IgD to granulocytes via a putative IgD-binding receptor further suggests it might play an immune function (Edholm et al., 2011).

2.4. Development and distribution of B cells

In marine teleosts, the major lymphoid organ to develop first is the kidney followed by spleen and, finally, the thymus, while in fresh water species the thymus develops first (Josefsson and Tatner, 1993, Pulsford et al., 1994, Pados and Crespo, 1996). However, the embryonic origin, time of appearance and distribution of IgM$^+$ B cells in the above major lymphoid tissues is still contentious in fish. Indeed, it is difficult to compare different lymphocyte ontology studies in fish and draw unified conclusion owing to differences in methodological approaches and preference to different lymphoid tissues. However, it is undeniable that early stage B cell ontology varies among the different teleost fish species mainly due to the differences in developmental stages at time of hatching and general development thereafter (Solem and Stenvik, 2006, Zapata et al., 2006, Parra et al., 2013). Of note, the ontogeny of the IgD-only and the IgT/IgZ bearing B cells is largely unknown (Salinas et al., 2011).

A pioneer study aiming at identifying the site of early embryonic lymphogenesis in teleost fish has shown limited or no migration of immature lymphocytes between the pronephros and thymus suggesting that hematopoietic stem cells in these organs might be acquired and maintained independently (Tatner, 1985). The same study also demonstrated that early thymectomy has no effect on the number of lymphocytes in the pronephros, which further shows the hematopoietic function of this organ (Tatner, 1985). It is now well established that the head kidney is the primary lymphoid organ for B cells development and maturation both in embryonic and adult life as demonstrated by a high expression of Ikaros (Hansen et al., 1997), TdT (Hansen, 1997), RAG-1 (Hansen and Kaattari, 1995), and RAG-2 (Hansen and Kaattari, 1996). Accordingly, lymphocytes seed the thymus first (T-cells), then the head kidney (B cells) followed by the spleen and finally, the mucosal-associated lymphoid tissues (MALTs) (Razquin et al., 1990, Solem and Stenvik, 2006). However, a study, based on RAG-1 expression and VDJ rearranged Ig gene, proposed zebrafish B cells appear fist in the pancreas before they seed the head kidney (Danilova and Steiner, 2002). A recent study in zebrafish, using RAG-2^{GFP} expressing larvae showed pronephros as the first extrathymic site of RAG-2 expression, which

stand in sharp contrast with the earlier report. Although data regarding involvement of pancreas in B cell genesis during embryonic life of teleost fish is limited to the above report, further study using mutant zebrafish that lack normal pancreas development (e.g. *slim jim*) (Trede et al., 2004) is needed to resolve these conflicting views. This discrepancy might however be due to differences in the sensitivity of the techniques used or lack of complete knowledge on the expression and functional significance of RAG-1/2 genes as RAG genes are also expressed by non-lymphoid tissues in zebrafish (e.g. olfactory placode) (Jessen et al., 2001) and higher vertebrates (e.g. mouse brain) (Chun et al., 1991).

2.5. Activation and differentiation of B cells

Despite bony fish being evolutionary at the base of adaptive radiation possessing the key elements of the adaptive immunity, many aspects of B cells activation and differentiation remain to be explored; mainly due to lack of B cell subset specific markers (Sunyer, 2012b, Abos et al., 2015, Castro et al., 2017, Peñaranda et al., 2019). Unlike T cells, B cells are produced both in embryonic as well as adult life from hematopoietic stem cells (HSCs) through complex processes that involve fate decision at several checkpoints to either maintain or delete the newly formed cells (Melchers, 2015). Central to B cell development and clonal selection is BCR signalling, which in combination with a network of tyrosine-based signalling molecules and accessory molecules guide the B cell to maturation and activation or deletion of potentially self-reactive B cells (Gauld et al., 2002, Melchers, 2015). Phenotypic characteristics delineating naïve mature B cells from activated forms, plasmablasts and plasma cells, have been described in several teleost fish (Zwollo et al., 2005, Ye et al., 2011b, Ma et al., 2013). However, except in rainbow trout and zebrafish the early developmental stages such as the common lymphoid progenitor (CLP), the pro-B cell (progenitor B cell) and pre-B cells (precursor B cells) are largely unknown (Zwollo et al., 2010, Page et al., 2013). In mammals, foetal B cell development (B-1 B cells) occurs in the liver with a subsequent shift to the bone marrow in neonatal and adult life (B-2 B cells) (Duber et al., 2009), while head kidney continues as sole source of B cells for all life forms in teleost fish (Zwollo et al., 2010). Although teleost fish B cells are suggested to be functional homologues of the mammalian B-1 B cells (Abos et al., 2018a, Peñaranda et al., 2019), it is not clear whether their sustained genesis in the same tissue both in embryonic and adult life forms has contributed towards this homology.

During an immune response, activation of naïve mature B cell is triggered via BCR and/or TLRs binding specific Ag and/or MAMPs (Tarlinton et al., 2008). Upon receiving the proper stimulatory signals, B cells increase in size, expand clonally and undergo differentiation (Tarlinton et al., 2008). In its own, this extensive genetic reprogramming is insufficient to ensure fate of the resulting differentiated B cells (Elgueta et al., 2010). The outcome of the activation does not merely depend on the sum total of signals delivered through BCR and TLRs, instead depends on a complex scenario that involve the nature of the Ag, the B cell subset, the costimulatory signals provided, the quality of T cell help and other microenvironment-derived factors (Elgueta et al., 2010). The signals triggered are mediated by recruitment and activation of intra-cellular kinases. Ultimately, signals are translocated to the nucleus, where they are integrated to regulate gene expression. One of the main transcriptional activators is the nuclear factor (NF)-κB, which regulates cellular processes leading to activation, differentiation or apoptosis (Gauld et al., 2002, Elgueta et al., 2010). In teleost species, the activation of B cells culminates with the generation of three fate committed effector B cells that play a central role in humoral immunity. These are plasmablasts (replicating, low and affinity-immature antibody producers, bearing minimal BCR and short-lived), memory B cells (replicating, high but affinity-immature antibody producers, bearing minimal BCR and long-lived) and plasma cells (non-replicating, terminally differentiated, high and affinity-mature antibody producers, bearing no BCR and long-lived) (Zwollo et al., 2005, Ma et al., 2013). Contrasting this, long-lived plasma cells expressing low level of IgM has been reported recently in channel catfish (Wu et al., 2019a). In fish, it has been proposed that the head kidney provides a niche for long-term maintenance of plasma cells or long-lived plasma cells (Ye et al., 2011b). However, the unique organization of the immune system coupled with the marked difference in the adaptive immune response constrained direct functional comparison of the fate committed B cells between fish and higher vertebrates. Accordingly, as opposed to mammals, secondary immune responses in fish is not accompanied by a significant boost in Ab titer, Ab-affinity maturation and switched memory (Ma et al., 2013).

2.6. B cell surface receptors

In a typical immune response against infection, B cells operate in consortium with other cells of the immune system for optimal activation of both the innate and adaptive immune response. Whereas, BCR signalling is vital for virtually all aspects of B cell development and

functionality, B cells integrate signals from multiple other receptors to decide fate and fine tune the type and magnitude of immune response (Borriello et al., 1997). In this regard, crosstalk between BCR and TLRs or co-stimulatory molecules, CD40 and B7 molecules, have been implicated in a number of B cell physiology as well as pathological conditions (Borriello et al., 1997, Mizuno and Rothstein, 2005). In higher vertebrates, it has been shown that TLR-induced increase in actin dynamics lowers the threshold for signalling via BCR (Freeman et al., 2015). Signal integration from CD40 helps BCR-activated B cells to bypass the key signalling pathway for NF-κB activation (Mizuno and Rothstein, 2005). Crosstalk between B7 molecules and BCR is also critical for effective adaptive immune responses, which include germinal centre formation and Ig class switching (Borriello et al., 1997). In teleost species examined thus far, several of these key costimulatory molecules with varying gene synteny have been identified indicating that costimulation is conserved in ancient vertebrates (Hansen et al., 2009, Zhang et al., 2009, Lagos et al., 2012, Iliev et al., 2013b, Zhu et al., 2014, Mo et al., 2017, Zhang et al., 2018). Despite this, data on functional studies are scarcely available.

2.6.1. B cell antigen receptors (BCRs)

In mammals, except for the IgD-only B cells that represent the minority, mature B cells co-express two BCRs, IgM and IgD, which have identical Ag-binding specificity and are composed of membrane-bound immunoglobulin (mIg) with a short intracellular domain of three amino acids: lysine, valine, lysine (KVK) (Treanor, 2012). Hence, in itself, BCRs lack signalling motifs but is instead linked to the CD79a (Igα)/CD79b (Igβ) heterodimer, which forms the signalling subunits of the BCR complex (Treanor, 2012). The signalling subunits each carries a phosphorylatable tyrosine-based motif at the cytoplasmic tail (immunoreceptor tyrosine-based activation motif, ITAM) that plays a central role in downstream signalling during B cell activation (Tsourkas et al., 2012, Fiala et al., 2013, Klasener et al., 2014). The IgM and IgD BCRs differ from each other only in the constant parts of their respective heavy chains, while the Igα/Igβ subunit is identical for the two BCR classes, apart from minor differences in the glycosylation of Igα (Campbell et al., 1991). In knockout and transgenic mice models, IgD displays no specific immune function suggesting that BCRs possess overlapping or redundant function (Roes and Rajewsky, 1993).

Binding of Ag to the BCRs sets off a multitude of downstream signalling events and changes on the conformation and location of the BCRs itself on the B cell surface, which collectively culminate in B cell activation. B cells bind Ag over a wide affinity range and the strength of BCR signalling increases with affinity, a process known as 'affinity discrimination' (Tsourkas et al., 2012). Recent findings have demonstrated preformed oligomeric clusters of BCRs on resting B cells suggesting that it is more the dissociation (dissociation model) than the aggregation (cross-linking model) of the BCRs that drives B cell activation (Yang and Reth, 2010, Klasener et al., 2014, Maity et al., 2015). Consistent with these findings, the recruitment of spleen tyrosine kinase (Syk) by ITAM following BCR ligation opens up the BCR by an inside-out signalling mechanism that amplifies BCR signalling (Yang and Reth, 2010, Tsourkas et al., 2012, Fiala et al., 2013, Maity et al., 2015, Volkmann et al., 2016).

B cells in teleost fish and mammals share many important similarities. As has been described in mammals, BCR complex in catfish comprises BCRs and BCR accessory molecules, CD79a and CD79b, with their cytoplasmic signalling tail containing ITAM (Sahoo et al., 2008). Additionally, CD79 genes have been described from several teleost fish species and recently in A. salmon, suggesting that BCR signalling via the BCR accessory molecules is an ancient mechanism (Huang et al., 2015, Liu et al., 2017, Peñaranda et al., 2019). CD22, another B cell surface receptor known to downregulate the activation of B cells in mammals, has also been reported on the surface of Atlantic salmon B cells (Peñaranda et al., 2019). However, compared to mammals, in teleost fish much less has been known about BCR oligomerization and spatial organization, and the subsequent downstream signalling that governs intrinsic and extrinsic function of the Ag interacting B cells.

2.6.2. Toll-like receptors (TLRs): First responders to danger signal

TLRs are hallmarks of the innate immune system that are conserved evolutionary from invertebrates to vertebrates (Takeda et al., 2003, Palti, 2011, Jimenez-Dalmaroni et al., 2016). It has come clear that B cells express and respond to signalling via a variety of TLRs. Although signalling via TLRs on B cells activates both the innate and adaptive immune responses, it can also limit the magnitude of the immune response to the level required for the clearance of the danger (Takeda et al., 2003, Jimenez-Dalmaroni et al., 2016). These opposing roles of TRLs indicate TLR signalling in B cells is largely context dependent. TLRs have been reported from

several teleost fish species (Pietretti and Wiegertjes, 2014). TLR repertoires show interesting variability between teleost fish and mammals. Thus far, nine "mammalian" (TLR1-5 and TLR7-9, 13) and nine 'non-mammalian' (TLR14, 18-23, TLR25 and TLR26) TLRs have been reported in teleost fish (Quiniou et al., 2013), while thirteen (TLR1-13) TLRs have been identified in mammals (Yang and Reth, 2010, Palti, 2011). The recent fish-specific whole genome duplication events have believed to contribute to the appearance of paralogue TLRs with partitioned functions of the ancestral gene (Lynch and Force, 2000, Cresko et al., 2003). The discovery that at least one gene representing each of the six major TLR families, TLR1, TLR3, TLR4, TLR5, TLR7 and TLR11, in most vertebrates suggests evolutionary conservation from teleost to higher vertebrate (Roach et al., 2005, Pietretti and Wiegertjes, 2014). The leucine-rich repeat (LRR) ectodomain displays low sequence homology between teleost fish and mammals calling for further functional studies to determine ligand specificity of fish TLRs as these data have been lacking in most teleost fish (Quiniou et al., 2013, Pietretti and Wiegertjes, 2014). Nonetheless, several studies have revealed that fish-specific TLRs share functional similarity with their mammalian counterparts (Rebl et al., 2010, Abos et al., 2015, Iliev et al., 2013a).

In fish, data on basal TLR expression and function in defined leukocyte populations are scarcely available (Abos et al., 2015). In mammals, TLRs have been best described in myeloid cells, while their regulatory role in activation and differentiation of B cells has been relatively less explored (Buchta and Bishop, 2014). However, it is well established that mammalian B cells can receive activation or deletion signal through TLR and/or BCR during an immune response (Buchta and Bishop, 2014). This cross-talk between TLR, BCR and other costimulatory receptors, e.g. CD40, plays a pivotal role in B cell development, activation, effector function and development of autoimmunity, when not properly controlled (Buchta and Bishop, 2014, Suthers and Sarantopoulos, 2017). At transcript level, human B cells express all the ten TLRs described in human. However, different subset of B cells express varying amount of TLRs and respond differently to different TLR ligand ligation (Buchta and Bishop, 2014). *In vitro* stimulation of leukocytes with a putative TLR3 ligand (poly I:C) and viral haemorrhagic septicaemia virus induces upregulation of TLR3 in sorted IgM$^+$ splenic B cells in rainbow trout showing a functional conservation of TLR3 in fish (Abos et al., 2015). That rainbow trout study

has also reported other nucleic acid sensing TLR genes in IgM+ B including TLR7, 8, 9 and 22 (Abos et al., 2015).
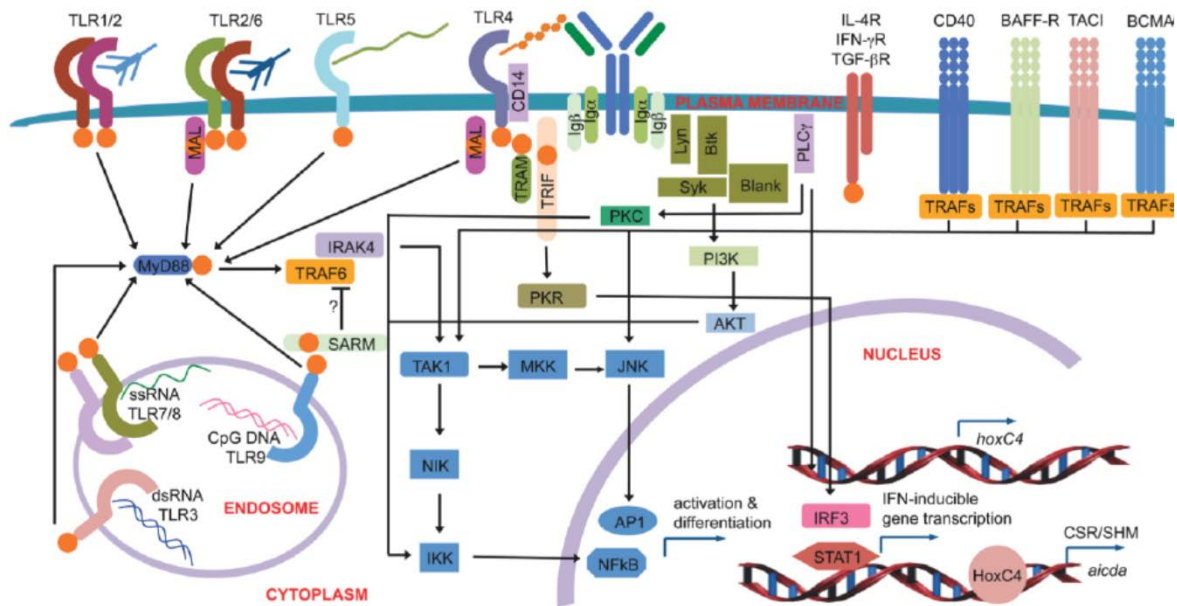
Figure 2. Naïve mature B cells integrate signal from TLRs, BCR, cytokine receptors and TNF family receptors to initiate activation and differentiate towards antibody secreting phenotype. TLR ligands are sensed by the extracellular or endosomal TLRs and signals are initially relayed to the nucleus via homotypic TIR-TIR interactions (orange spheres) with TIR adapters. These signals are integrated and initiate a response by inducing NF-κB and AP-1, inflammatory gene transcription, IFN-inducible gene transcription, and induction of AID activity, leading to CSR and SHM (Pone et al., 2010).

2.6.3. Costimulatory molecules: Gear-shifters

Costimulatory molecules are cell surface molecules that mediate cell-cell interactions thereby prompt intracellular signals in the interacting cells. Following BCR engagement, B cells should integrate signals from costimulatory molecules, including CD40, CD80 (B7-1) and CD86 (B7-2), in order to regulate the magnitude and duration of adaptive immune responses (Borriello et al., 1997, Crow, 2004, Elgueta et al., 2009). Signalling through BCR without costimulation leads to apoptosis or anergy indicating their role as a key checkpoint in B cell activation (Geisberger et al., 2006). B cells that have received signals via BCR and costimulatory molecules eventually integrate a third signal, cytokines from Th cells, and complete their terminal differentiation to ASC (Borriello et al., 1997). Costimulatory molecules on B cells, B7 molecules, are also involved in preventing chronic activation of the immune system via interaction with CTLA-4 (CD152) on activated T-cells. Secondary signalling via costimulatory molecules are therefore important in

maintaining the delicate balance between immune stimulation and suppression of autoimmunity (Crow, 2004). In fish, only one copy of B7 gene, CD80/86, has been discovered thus far (Abos et al., 2015, Zhu et al., 2014, Iliev et al., 2013b), which at gene homology level resembles more the B7-2 of mammals (Zhu et al., 2014). In A. salmon, CpG ODN induces very low transcript level of CD86 in IgM$^+$ B cells, while it transcribes a relatively high expression of CD40 (Iliev et al., 2013b). In contrast, a study in rainbow trout has reported a significantly higher expression of CD80/86 transcript in IgM$^+$ B cells following virus infection (Abos et al., 2015) suggesting as yet clearly unknown immune function of CD80/86 in teleost fish. CD5, a negative regulator of BCR and TCR signalling in higher vertebrates (Voisinne et al., 2018), has been shown to be expressed on the majority of rainbow trout IgM$^+$ B cells (Abos et al., 2018a). This finding opposes what is known in higher vertebrates where CD5 is a marker of the B1 B cells, which represents the minority of the B cell pool. However, whether teleost CD5 functions the same way as in higher vertebrates remains undefined.

2.7. Teleost fish immunoglobulins (Igs): No class switch despite functional AID

Igs produced by B cells are fundamental mediators of the humoral immune response (Fillatreau, 2018). The three IgH loci, namely μ, τ/ζ and δ, described above in connection with subtyping of B cells are also used to define the three Ig isotypes in teleost fish. Accordingly, three Ig isotypes, IgM, IgT/IgZ and IgD, have been identified from different fish species (Palenzuela et al., 1996, Danilova et al., 2005, Hansen et al., 2005 , Edholm et al., 2010, Zhang et al., 2010, Hordvik, 2015) emphasizing that teleost fish possess limited antibody isotypes compared to mammals. Diversification of Ig repertoire in higher vertebrates is possible due to the activities of the activation-induced cytidine deaminase (AID). AID catalyses somatic hypermutation (SHM) and class switch recombination (CSR) in higher vertebrates (Barreto et al., 2005, Ichikawa et al., 2006). However, despite the acquisition of functional AID, CSR has not been reported in teleost fish, which is partly explained by lack of cis-elements in the IgH gene (Wakae et al., 2006). Consequently, it is believed that CSR is invented late in the evolution of AID connected with the emergence of terrestrial vertebrates (Ichikawa et al., 2006). The fact that teleost can affinity mature (SHM) their Ig without CSR supports further the report that SHM and CSR appeared separately during vertebrate evolution (Kaattari et al., 2002, Ichikawa et al., 2006). Setting the stage for the hypothesis that the adaptive immune

response in teleosts lacks some features present in higher vertebrates (Kaattari et al., 2002, Solem and Stenvik, 2006, Ye et al., 2013).

Whereas IgT is secreted by IgT^{+} B cells, correlating the secretion of IgM or IgD to either IgM[+] or IgD[+] or double-positive B cell is difficult in teleost fish as little is known about the function of these different B cell subsets (Edholm et al., 2011, Ramirez-Gomez et al., 2012). Most Igs occur naturally as multimers of the monomeric subunits, which adds structural avidity for Ag compared to the monomeric forms (Sørensen et al., 1999). Ig multimerization in higher vertebrates, IgM and IgA, is possible due to a small intermolecular tethering peptide called joining chain (J-chain) (Davis and Shulman, 1989). The same peptide also mediates interaction of Ig with polymeric Ig receptor (pIgR) at the mucosal surface, which is important for trans-mucosal transport (Johansen et al., 2001). In teleost fish, pIgR has been characterized from several species (Hamuro et al., 2007, Rombout et al., 2008, Feng et al., 2009, Xu et al., 2013a, Sheng et al., 2018). As in mammals, teleost pIgR mediates trans-mucosal transport of secreted IgM and IgT into external body surfaces (Zhang et al., 2010, Sheng et al., 2018). In sharp contrast with higher vertebrates, however, teleost fish IgM lacks the J-chain and thus, tetramerization of IgM is by interchain disulphide bonds (Ye et al., 2013). The number of the disulphide linkages seems to affect the effector function of the Ig such as cytotoxicity, complement activation, opsonisation, etc. (Ye et al., 2011a). In turn, this effector function is thought to be determined by BCR affinity for specific Ag, where higher affinity tracks with more disulphide linkages (Ye et al., 2011a, Ye et al., 2013).

2.7.1. IgM: The most abundant Ig in serum

IgM is the first identified and well-characterized Ig isotype in fish (Mashoof and Criscitiello, 2016). It is a structural and functional homolog of the mammalian IgM (Warr, 1995, Bengten et al., 2006b). It is also the most ancient Ig evolutionarily and appears early in embryonic development (Castillo et al., 1993). In most teleost species, as in mammals, the *ighμ* gene is comprised of exons encoding four μ constant (Cμ) and two transmembrane regions (Fillatreau et al., 2013, Mashoof and Criscitiello, 2016). The secreted form of *ighμ* transcript consists of all the four Cμ domains while the membrane-bound form utilizes the first three Cμ domains (Mashoof and Criscitiello, 2016). However, medaka (*Oryzias latipes)* and Antarctic fish are exceptions in that they utilize the first two Cμ domains for mIgM (Magadan-Mompo et al.,

2011, Quiniou et al., 2011), while one single Cμ domain and a transmembrane region have been reported in zebrafish (Hu et al., 2011). In teleost fish, IgM is secreted by plasmablasts, memory cells and plasma cell-like cells (Ye et al., 2011b, Ma et al., 2013). Plasma cell-like cells that have survival niche mainly in the head kidney play a key role in eliciting sustained and unswitched IgM responses (Ye et al., 2011b).

IgM is the principal Ig isotype involved in systemic immune response and is by far the most abundant Ig in serum in all teleost fish examined thus far (Bengten et al., 2006a, Solem and Stenvik, 2006, Ye et al., 2013). In addition, its role in mucosal (gut and gills) and skin immunity has been reported in several teleost fish (Solem and Stenvik, 2006, Salinas et al., 2011). IgM is a tetramer in serum and mucus in most teleost species (~700-800 kDa), which is tethered together by intermolecular disulphide bonds without the J-chains (Salinas et al., 2011). IgM exists in different redox states depending on the nature and number of disulphide bonds between the monomeric and/or dimeric subunits (Kaattari et al., 1998). Generally, increase in the oxidation state is shown to be related to increase in BCR affinity, which in turn relates to a higher Ig affinity (Ye et al., 2010).

In *Salmonidae*, due to whole genome duplication events that resulted in ancestral tetraploidy, two highly similar Ig heavy chain gene complexes have been described; namely IgH-A and IgH-B (Hordvik, 1998, Yasuike et al., 2010, Hordvik, 2015). However, thus far, it is only in the genus *Salmo* (e.g. A. salmon and brown trout) that the two genes correspond to two distinct sub-variants of serum IgM, IgM-A and IgM-B, which can be sub-fractioned using gel filtration followed by ion exchange chromatography (Hordvik et al., 2002, Kamil et al., 2013). In the genus *Salmo*, IgH-A and IgH-B show remarkable structural differences indicating that the two loci have been evolved independently after the recent whole genome duplication. It is therefore conceivable to assume that establishment of disomic inheritance only in the genus *Salmo* Ig gene loci has contributed to the existence of these two IgM sub-variants in A. salmon (Hordvik, 1998, Hordvik et al., 2002). The expression of these IgM sub-variants is most likely regulated by a mechanism similar to allotypic exclusion (Hordvik, 2015). In both A. salmon and brown trout serum the two IgM sub-variants appeared in a roughly equimolar concentration (Hordvik, 1998). However, compared to IgM-A, IgM-B displays a higher degree of disulfide cross-linking, most likely due to the characteristic extra cysteine residue near the C terminal

part of Cμ4 (Yasuike et al., 2010, Kamil et al., 2013). Although the biological significance of having the two IgM sub-variants is far from being completely understood, biological activity of both variants has been reported in A. salmon (Hordvik et al., 2002). mAbs recognising both variants of IgM in A. salmon have now been developed revealing that most earlier studies in A. salmon based on anti-trout IgM antibodies consistently detected only one variant of IgM (Hedfors et al., 2012).

2.7.2. IgT: Specialized in mucosal immunity

In addition to increasing the Ig isotypes of teleost species, the discovery of IgT in teleosts has changed the evolutionary timescale of Ig compartmentalization, which was originally thought to be emerged in tetrapods (Danilova et al., 2005, Hansen et al., 2005). This Ig is named IgT in rainbow trout and IgZ in zebrafish (Danilova et al., 2005, Hansen et al., 2005). Unlike teleost IgM, but similar to mammalian IgA, IgT is monomeric (~180 kDa in rainbow trout) in serum and a multimer of ~4-5 monomers held together through non-covalent bonds in mucosal sites such as the gut, skin and gills (Zhang et al., 2010, Xu et al., 2013b, Xu et al., 2016). Except in rainbow trout, IgT is largely unknown at protein level due to the lack of an antibody against it. A genetic study has revealed secreted and membrane bound forms of IgT in A. salmon both containing four constant Ig domains, Cτ1-Cτ4 (Tadiso et al., 2011). IgT functions as an analogue of the mammalian IgA (Cerutti and Rescigno, 2008) by mediating the process of immune exclusion of commensal microbes at mucosal surfaces (Zhang et al., 2011). Despite functional similarities, the sequence homology between IgT and IgA is very low suggesting that they are evolved independently through convergent evolution (Zhang et al., 2010). The confinement of parasite specific IgT titre in the gut mucus (Zhang et al., 2010) or its detection in a much lesser level in serum compared to the gut (Xu et al., 2013b) after parasite infection shows its specialized role in gut mucosal response. Whereas IgT predominantly coats resident bacteria in SALT (Xu et al., 2013b), equal percentages of bacteria are coated with IgT and IgM in NALT of rainbow trout (Tacchi et al., 2014). A dominant immunological role of IgT against a parasitic disease called proliferative kidney disease (PKD), compared to IgM and IgD, has been documented outside the mucosal compartment of rainbow trout (Abos et al., 2018b).

The gene encoding for ightτ/ζ has been reported in several teleost fish and in all fish species analysed thus far, it is located upstream of the Cμ domain (Fig. 3). With some exceptions, the

constant regions (Cτ/ζ) of ightτ/ζ encodes four Cτ/ζ domains in the majority of teleost species (Savan et al., 2005, Gambon-Deza et al., 2010, Fillatreau et al., 2013, Giacomelli et al., 2015, Kato et al., 2015). Due to ancestral tetraploidy, the genome of A. salmon contains three intact τ genes and five τ pseudogenes (Yasuike et al., 2010). Medaka and catfish have no gene encoding for ightτ in the igh loci (Bengten et al., 2006b, Magadan-Mompo et al., 2011), while common carp has two separated ighζ loci encoding four (IgZ1) and two (IgZ2) Cζ domains (Ryo et al., 2010). IgT differs structurally from IgM in that it utilizes the whole ightτ/ζ domains in making both the secreted and membrane-bound forms (Fillatreau et al., 2013, Bengten and Wilson, 2015).
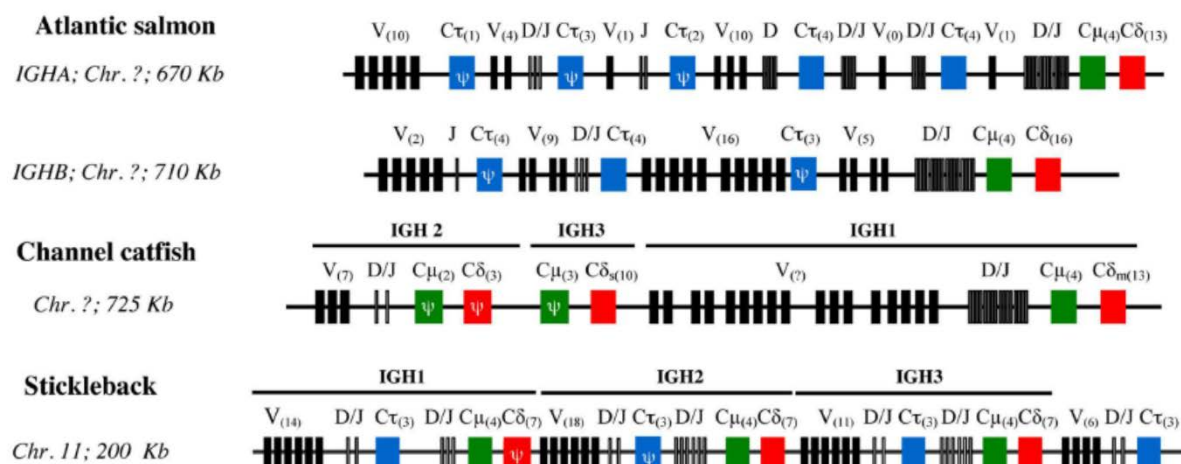
Figure 3. IgH loci in different teleost species with partial or complete duplications in different chromosomes (Chr.) (A. salmon) or in the same chromosome (channel catfish and three-spined stickleback). *Figure showing the genomic configuration of the V (black boxes), D/J (narrow gray boxes), and CH gene sets (Cμ- green boxes, Cδ- red boxes, and Cτ/ζ- blue boxes). Number within brackets indicate the number of in-frame V genes and CH exons. Schemes are not in scale. IgH Cδs and Cδm in catfish correspond to the secreted and membrane IgD coding genes, respectively. CH sequences with frameshift mutations are considered as pseudogenes (Ψ). "?" indicates lack of data. Figure adapted from (Fillatreau et al., 2013).*

2.7.3. IgD: Calls for more functional study

Like IgM, IgD is an ancient immunoglobulin class that has been cloned in several teleost species (Wilson et al., 1997, Hordvik et al., 1999, Hirono et al., 2003, Saha et al., 2004, Edholm et al., 2010). To date, what we know about IgD function is derived mostly from catfish and rainbow trout as these are the only teleost fish species where IgD is characterised at protein level (Edholm et al., 2011, Ramirez-Gomez et al., 2012). In these two species, the *ighδ* gene has

different structures and transcriptional regulations, warranting further studies in other representative teleost species to understand its biological significance. Unlike *ighµ*, the *ighδ* has shown marked variability among different fish species (Edholm et al., 2011, Ramirez-Gomez et al., 2012) and it is located immediately downstream of the *ighµ* gene (Bengten and Wilson, 2015) (Fig. 3). Despite a remarkable Cδ sequence diversity among fish species (Sun et al., 2011), a chimeric *ighδ* transcript composed of a rearranged VDJ segment followed by Cµ1 and Cδ domains is a common feature to all examined teleost species (Edholm et al., 2011, Sun et al., 2011). In catfish, two separate genes encode the secreted and membrane-bound forms of *ighδ*, while a single gene in rainbow trout is transcribed into both forms through alternative splicing (Bengten et al., 2006a, Edholm et al., 2011, Ramirez-Gomez et al., 2012).

Serum IgD appears to be monomeric in both catfish and rainbow trout (Edholm et al., 2011, Ramirez-Gomez et al., 2012). However, catfish secreted IgD differs from the rainbow trout IgD in that it lacks a VH region, a Cµ1 and a IgL. Transcript analysis of secreted IgD from peripheral blood IgD-only and double positive B cells revealed that the catfish secreted 'V-less' IgD is produced mainly by the IgD-only B cells (Edholm et al., 2011, Edholm et al., 2010). Two secreted IgD variants exist in the serum of catfish, ~130 kDa/ 180 kDa, and rainbow trout, ~240 kDa/ ~370 and ~400 kDa (Edholm et al., 2011, Ramirez-Gomez et al., 2012). Although secreted catfish IgD lacks Ag specific binding site, it is proposed that the Fc region functions as a PRR molecule (Edholm et al., 2010). Despite Ag binding site, pathogen-specific IgD responses are undetectable in the gill mucus and serum of trout (Hirono et al., 2003). Contrasting this, a gene expression study in rainbow trout has reported a faint splenic IgD response to viral infection (Castro et al., 2013). Although IgT plays a major role, a parasitic disease, PKD, induces IgD response in HK of rainbow trout suggsting an important immunological role of IgD in teleost species (Abos et al., 2018b). IgD appears to coat a small proportion of gill microbiota suggesting that it might play a role in immune exclusion (Xu et al., 2016). To date, no IgD protein has been found in the gut mucus (Parra et al., 2016) emphasizing that the immune function of IgD is still far from being completely understood.

2.8. B cells at a crossroad between the innate and adaptive immunity

Mounting a protective but at the same time an appropriate level of immune response against foreign Ag requires an extensive interaction and coordination between the innate and

adaptive immune system (Clark and Kupper, 2005, Lampropoulou et al., 2012). The co-evolution of the two arms of the immune system in vertebrates is believed to be driven by increased microbial challenge. Nevertheless, as has been described in jawed vertebrates, they have a check-and-balance role one over the other for the normal functioning of the immune system (Harris et al., 2000, Vazquez et al., 2015).

Immune cells that recognise pathogen-associated molecular patterns (PAMPs) via PRRs, internally process and present Ag to T-cells via a MCH-II restricted manner constitutes innate immune cells, while those that recognise antigen presented on the surface of innate cells using receptors (BCR/TCR) are traditionally assigned to the adaptive immunity (Magnadottir, 2006). Accordingly, lymphocytes, both B-and T-cells, are the founding members of cells of the adaptive immunity. Crossing this traditional boundary, however, studies in fish as well as in mammals have shown potent phagocytic, intracellular microbicidal and antigen presenting capabilities of B cells for soluble and non-specific particulate antigens (Li et al., 2006, Sunyer, 2012b, Zhu et al., 2014, Wu et al., 2019b). In doing so, B cells prime naïve T cells and regulate their differentiation in primary immune responses in the same way as innate cells such as professional APCs do (Harris et al., 2000). In addition, effector B cells, via differential expression of cytokines, regulate polarization of T cells response and modulate the overall immune responses; a task previously assigned to innate immune cells (Harris et al., 2000, Zhu et al., 2014, Vazquez et al., 2015). These increasingly exciting antibody-independent immunomodulatory functions of B cells position them at an important crossroad linking the adaptive and innate immunity (Zhu et al., 2014, Vazquez et al., 2015).

2.9. B cell cytokines: Mission beyond antibody production

Beyond their central role in humoral immunity by secreting antibodies, B cells regulate other essential functions of the immune system via production of cytokines (Clark and Kupper, 2005, LeBien and Tedder, 2008, Lampropoulou et al., 2012, Hamze et al., 2013). Cytokine production by B cells varies depending on their stage of maturation and activation conditions (Vazquez et al., 2015). Nonetheless, B cells, via their cytokines, are active modulators of immune response throughout their journey to plasma cells (Vazquez et al., 2015). Conversely, the terminal differentiation of B cells and fine-tuning of the antibody secretion are under a direct influence of cytokines derived from CD4 T cells and their microenvironment (Clark and Kupper, 2005).

This highly intricate crosstalk between B cells and their microenvironment requires high degree of control. Hence, for B cells to become cytokine secreting effector cells, additional signalling from their cytokine milieu is required beyond signal delivered via BCR (Vazquez et al., 2015).

B cells are quite diverse in terms of cytokine production and cytokine receptor expression (Lund, 2008). Cytokine-producing B cells are generally divided into regulatory (Breg) and effector (Beff) B cells in mammals (Hamze et al., 2013). This pool of regulatory B cells are largely unknown in teleost fish. However, in an attempt to shed light on the existence of Breg cells, IL-10 gene has been characterized in some fish species (Savan et al., 2003, Zou et al., 2003, Grayfer et al., 2011, Takizawa et al., 2013). In addition, goldfish recombinant IL-10 has shown functional conservation *in vitro* by supressing signature pro-inflammatory cytokine gene, IFNγ (Grayfer et al., 2011). In rainbow trout, a small percentage of IgM$^+$ and IgT$^+$ B cells expressing IL-10 has been reported (Takizawa et al., 2013). In higher vertebrates, except plasma cells, other stages of B cells including immature B cells, mature B cells and plasmablasts have the plasticity to differentiate into Breg or Beff cells. While BCR, CD40 and/or TLR signalling are well described for driving activated B cells to Breg cells phenotype (Mauri and Bosma, 2012, Rosser and Mauri, 2015), Beff cells development is mainly influenced by cytokine produced by T cells (Harris et al., 2000, León et al., 2012). To date, the Breg and Beff subpopulation have not been identified in teleost species.

An important family of cytokine having relevance to this thesis is interferon (IFN). In teleosts, IFNs mediate a potent innate antiviral response against viral infection. Researches in the past two decades have evidenced the complexity of the IFN system and their antiviral role in fish (Jensen and Robertsen, 2002, Robertsen et al., 2003, Zou et al., 2007, Verrier et al., 2011, Svingerud et al., 2012). A multitude of antiviral genes and diverse cellular pathways are turned on in a host cell in response to virus infection (Der et al., 1998, Yibing et al., 2003). As in higher vertebrates, teleost fish IFNs display a significant induction by virus infection and establish an antiviral state when overexpressed (Levraud et al., 2007, Zou et al., 2007, Yu et al., 2010). In higher vertebrates, IFNs are classified into three subfamilies, type I, II and III (Sadler and Williams, 2008). Type I and II IFN genes with some structural differences have been discovered from several teleost species (Zhang and Gui, 2012), while type III IFN gene is missing from the

genome of teleosts (Secombes and Zou, 2017). However, the current system of fish IFNs nomenclature does not follow the mammalian system and is not consistent across the different taxonomic fish groups, calling for a unified system. In A. salmon, for example, the type I IFN gene family encodes for at least four subtypes with varying antiviral activity; named, IFNa, IFNb, IFNc and IFNd (Sun et al., 2009). Although type the IFNs are known to be produced by B cells in mammals (Ref), their production by a defined subset of leukocyte, such as B cells, of teleost fish is poorly understood.

2.10. Peritoneal cavity (PerC) B cell response

As a peripheral immune site, as has been described in mammals (Kolaczkowska, 2010), the steady state PerC of teleost fish is expected to harbour a population of mature leukocytes that would be either resident or mobilised from primary sites to maintain the homeostasis of the microenvironment. Although there is a great variation in the proportion, the resident PerC leukocyte population of fish has been profiled to contain both lymphocytes and myeloid cells (Meseguer et al., 1993, Afonso et al., 1997, Vizzini et al., 2007, Moss et al., 2009, Tumbol et al., 2009, Korytar et al., 2013). Common to all the studied teleost fish, however, the proportion as well as the composition of this resident population changes markedly over time following intraperitoneal (ip) stimulation (Afonso et al., 1998, Afonso et al., 2000, Korytar et al., 2013, Castro et al., 2017). In fish, earlier studies have exclusively analyzed the myeloid populations assuming that these are the dominating resident populations defending the PerC (Afonso et al., 1998, Do Vale et al., 2002, Chaves-Pozo et al., 2005), while lymphocytes are largely ignored. A recent finding that lymphocytes, mainly IgM$^+$ B cells, are the dominating resident and induced population in trout PerC has raised a question about their role (Korytar et al., 2013) and opened up avenue for detail characterization and functional studies of this less understood subset of PerC B cells.

In mammals, three distinct B cell lineages have been identified; namely B-1a, B-1b and B-2 (Tung et al., 2006). The PerC is home for the majority of B-1 B cells with few co-inhabiting B-2 B cells (Baumgarth, 2011). Whereas the different B cell subsets have remained enigmatic in teleost fish, B cells are one of the main cell types recruited into the PerC in response to different stimuli (Korytar et al., 2013, Castro et al., 2017). In rainbow trout, IgM$^+$ B cells populate the PerC following a decline in the myeloid cell populations (Korytar et al., 2013,

Castro et al., 2017). However, the timing and proportion of the PerC recruited B cells vary considerably depending on the type of stimuli employed. Similar to the systemic IgM$^+$ B cells (Li et al., 2006), PerC IgM$^+$ B cells are highly phagocytic where in an experiment with ip *E.coli* stimulation the IgM$^+$ B cells represent about 80% of the cells that phagocytized E.coli (Castro et al., 2017). Furthermore, as has been described in mammals, the IgM$^+$ B cells that have engulfed *E.coli* show signs of activation and differentiation towards plasmablast- or plasma cell-like cells as demonstrated by increase in size and internal complexity (Castro et al., 2017, Granja and Tafalla, 2017). Along the same line, two subsets of IgM$^+$ B cells, one expressing high surface IgM (naïve phenotype) and the other one expressing low surface IgM (plasma cell phenotype) have been identified in the PerC of trout and A. salmon after ip stimulation (Granja and Tafalla, 2017). Collectively, these findings suggest that the PerC of fish provides the necessary niche for activation and differentiation of naïve B cells into plasma cell-like cells. Likewise, the trout PerC adipose tissue has played a role as peripheral immune site by retaining antigen and supporting differentiation of B cells into plasmablasts (Pignatelli et al., 2014). In mammals, unique secondary lymphoid tissues, called milky spots, have been well characterized in the PerC that support the terminal differentiation of B cells (Mebius, 2009). However, despite accumulating evidences demonstrating an important peripheral immune function that PerC might play by retaining antigens and supporting B cells differentiation, the precise anatomical location and structural architecture of a putative secondary immune tissue has not been yet characterized in the PerC of fish.

3. Pancreas disease (PD): A concern to the Norwegian A. salmon farming industry

Pancreas disease (PD) caused by *Salmonid alphavirus* (SAV) affects farmed A. salmon (*Salmo salar* L.) and rainbow trout in the seawater phase (Jansen et al., 2017). Since its incursion in Norway in 1980s, PD has incurred substantial economic loss to the salmonid farming industry (Aunsmo et al., 2010) with a potential of imposing trade restriction on salmon products exported from Norway (Jansen et al., 2017). Despite decades of efforts to improve vaccine efficacy and application of strict preventive biosecurity measures that range from movement control and vaccination (endemic areas) to stamping out (non-endemic zone), PD has remained a growing problem for the Norwegian A. salmon farming industry. Infected farmed salmonids being the main reservoir of infection, transmission between seawater cages occurs mainly by water current or through human activities (Boucher et al., 1995, Jansen et al., 2017).

The risk of vertical transmission of SAV is regarded negligible by the Norwegian Scientific Committee for Food Safety (OIE, 2018). As for most viral diseases, virus shedding occurs during the incubation period of PD (Graham et al., 2011, Skjold et al., 2016) making early diagnosis and instigation of control measures practically impossible.

3.1. Salmonid alphavirus (SAV): Virus subtypes circulating in Europe

SAV (also called salmon pancreas disease virus, SPDV) is a relatively new addition to the genus Alphavirus of the family Togaviridae (Weston et al., 1999, Weston et al., 2002). Molecular epidemiological studies show wide distribution of SAV in Europe with high genetic diversity of the circulating virus types (Weston et al., 2005, Graham et al., 2007, Fringuelli et al., 2008). The Alphavirus genome contains two open reading frames; one encoding the non-structural proteins (nsP1-4) and the other encoding capsid glycoproteins (E1, E2, E3 and 6K) (Powers et al., 2001). A study comparing 18 isolates based on partial sequence analysis of E1, nsP4 and nsP3 genes found three distinct subtypes, designated SAV1 to 3 (Weston et al., 2005). A subsequent study, however, on 48 isolates from farmed A. salmon and rainbow trout across Europe using partial sequence analysis of highly variable regions within E2 and nsP3 genes added three more subtypes to the existing three nailing the list down as known today to six (Fringuelli et al., 2008). SAV2 is the most widely distributed subtype and is further subdivided into the fresh- and marine-water variants (Fig. 4.). The fresh water SAV2 variant typically causes sleeping disease (SD) in freshwater rainbow trout in many countries in Europe (Graham et al., 2007, Fringuelli et al., 2008), while the marine variant typically causes PD in seawater A. salmon and rainbow trout in Ireland, UK and Norway (Graham et al., 2007, Jansen et al., 2017). SAV3 is endemic to Norway and affects A. salmon and rainbow trout in marine environment (Fringuelli et al., 2008).
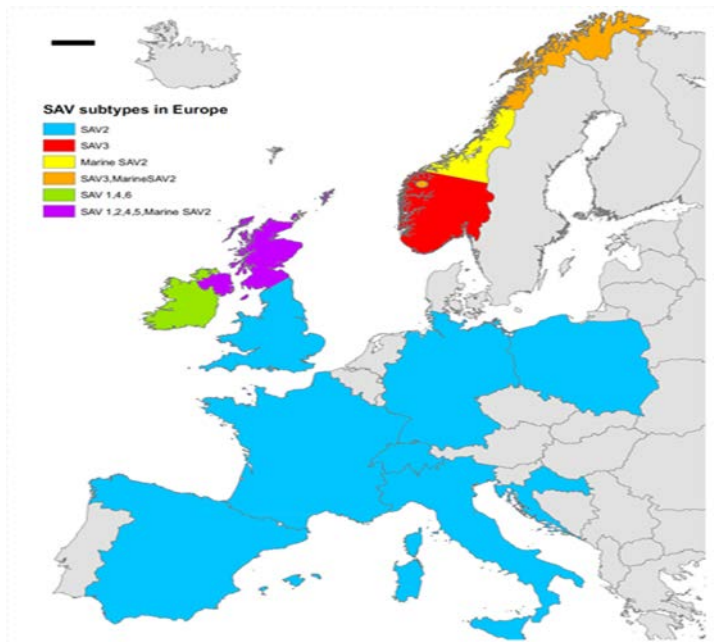
Figure 4. Distribution of the different subtypes of salmonid alphavirus (SAV) in Europe (Jansen et al., 2017).

3.2. Vaccines against PD

In today's open sea-based aquaculture system that is inevitably exposed to specific pathogens, vaccines remain as a cornerstone in preventing diseases of farmed fish. The early observation that fish surviving PD are resistance to reinfection suggests on a possibility of using vaccine in control strategy. In line with this, antisera raised in A. salmon against the six SAV subtypes show broad cross reactivity highlighting on a possibility of developing a monovalent vaccine (Graham et al., 2014). Commercial whole-virus inactivated PD vaccines, based on SAV1 subtype, have been introduced into the Norwegian A. salmon farming industry since 2002 (Karlsen et al., 2012). Despite these favourable conditions for developing effective vaccine, the efficacies of the available PD vaccines have been debated under field condition as increasing number of PD outbreaks hammer the industry in recent years (Karlsen et al., 2012). Nonetheless, it has been documented that the current inactivated vaccines have contributed for the economy of the industry by reducing the number of new outbreaks via reducing virus shedding from infected fish, decreasing cumulative mortality, improving the growth rate and minimizing the number of fish discarded at slaughter (Skjold et al., 2016, Deperasinska et al., 2018). The demand for a better PD vaccine is therefore hugely untapped in the Norwegian aquaculture industry that urges authorities to look for other options- DNA vaccination. This undoubtedly is a pivotal shift in fish vaccination against hard to combat viral diseases in the

future where the conventional approach fails to confer satisfactory protection (Lorenzen and LaPatra, 2005, Dalmo, 2018).

Confirming the success stories of DNA vaccines in fish, a DNA vaccine, Clynav from Elanco, has gained market authorization for use in European A. salmon farming in 2017. However, whether Clynav provides a better protection over the traditional whole-inactivated vaccines under the Norwegian aquaculture condition has remained to be evaluated in the near future. DNA vaccines against fish viral diseases have shown promising results (Lorenzen and LaPatra, 2005, Collins et al., 2019). A DNA vaccine for A. salmon against infectious hematopoietic necrosis virus (IHNV) has been commercially available in Canada (Marta and Leong, 2013). In addition, under experimental conditions, several other fish DNA vaccines confer satisfactory protection. A bivalent DNA vaccine constructed with the glycoprotein gene of IHNV and VP2-VP3 gene of IPNV has conferred significant protection against IHNV and IPNV in salmon and trout (Xu et al., 2017). Another DNA vaccine based on viral hemagglutinin esterase Ag with type I interferon as a molecular adjuvant has induced effective protection against ISAV in A. salmon (Chang et al., 2017). Recently, a DNA vaccine against PD using the complete SAV3 structural cassette has shown superior protection as measured by the absence of pathological lesions, elevated neutralizing antibody titer compared to a commercial inactivated PD vaccine in A. salmon (Chang et al., 2017). However, these proofs of concepts have not been progressed to large-scale field efficacy trials that might partly be due to the current tough hurdles in bringing DNA vaccines into the market.

3.3. CpG ODNs: Potential molecular adjuvants

A short oligodeoxynucleotide (ODN) sequence, approximately 20 nucleotides long, containing one or more indispensable dinucleotide core of unmethylated cytosine-guanine (C-G) motifs joined by a phosphate backbone (C-p-G) are generally called CpG ODNs. CpG ODNs are known for their potent immunostimulatory capability both *in vitro* and *in vivo* (Thim et al., 2012, Cheng et al., 2016, Su et al., 2017). Although CpG is a necessary requirement for immunostimulatory ODNs, their immunogenicity is markedly affected by sequences flanking 5' and 3' ends of the CpG motif (Krieg, 2002). Synthetic CpGs are unstable and hence, rapidly degraded *in vivo*. To prolong their half-life, they are generally made with a modified nuclease-resistant phosphorothioate (PS) backbone. In teleost fish, GTCGTT (Kanellos et al., 1999) and

AACGTT (Jorgensen et al., 2003) motifs have been reported to activate the immune system by boosting Ag-specific antibody response and inducing protection against infection, respectively. Recently, a GTCGTT motif has been shown to induce expression of immune genes in sorted IgM$^+$ cells from A. salmon (Iliev et al., 2013b).

CpG ODNs have shown promising adjuvant effects when administered with immunodominant epitopes against a wide range of infectious diseases (Thim et al., 2012, Cheng et al., 2016, Su et al., 2017). They are also indicated to compensate for the loss of immune function in elderly, new-borns and immunosuppressed individuals against vaccine antigens (Millan et al., 1998, Qin et al., 2004, Sen et al., 2006). After decades of research, several CpG-adjuvanted vaccines have been progressed to clinical trials in human, while hepatitis B vaccine containing CpG as an adjuvant being the only one gaining market authorization (Bode et al., 2011). Although there are issues concerning induction of autoimmunity and toxic shock, clinical studies have shown that CpGs have good safety profile at their recommended dose (Bode et al., 2011). In fish, the use of CpG ODNs as an adjuvant together with different antigens have shown protection by boosting both the innate and adaptive immune responses under experimental conditions (Kanellos et al., 1999, Thim et al., 2012, Thim et al., 2014, Su et al., 2017). However, there are no CpG ODN adjuvanted vaccine commercially available thus far for veterinary use.

Aim of study

The enormous potential that the aquaculture industry holds to feed the future has been seriously constrained by diseases, where diseases caused by viruses account for a great deal of economic loss. Standing against decades of efforts, the demand for more efficacious viral vaccines is still hugely unsatisfied in the global aquaculture industry. Immunological memory, essentially mediated by B and T cells, form the biological basis for protection of disease by vaccination. To contribute towards the process of designing better vaccines based on improved understanding of the mechanisms of protection, this work aimed at enhancing knowledge on B cells in A. salmon.

General objective

To better profile the B cell population from systemic and peripheral immune sites and study the B cell response after *in vitro* and *in vivo* stimulation in A. salmon.

Specific objectives

- Develop functional assays to characterize B cells from A. salmon
- Profile B cell populations from different immune sites of A. salmon
- Sorting B cells from different immune sites of A. salmon and undertaking *in vitro* stimulation studies to profile regulation of selected immune genes and secretion of IgM
- Investigating the dynamics of IgM+ B cells, total ASC and Ag-specific ASC responses in the PerC and systemic immune sites, HK and spleen, of A. salmon after IP challenge with salmonid alphavirus

Summary of papers

Paper I:

Profiling Atlantic salmon B cell populations: CpG-mediated TLR ligation enhances IgM secretion and modulates immune gene expression

Shiferaw Jenberie, Hanna L. Thim, J. Oriol Sunyer, Karsten Skjødt, Ingvill Jensen, Jorunn B. Jørgensen. SCIENTIFIC Reports (2018), 8:3565

Disease prevention by vaccination represents the most feasible measure currently available for the aquaculture industry. Understanding B cell responses are vital for a rational vaccine design, which in Atlantic salmon (A. salmon) is just started being investigated. In view of this, we studied IgM$^+$ B cells distribution and *in vitro* activation from systemic and peripheral immune sites of A. salmon. IgM$^+$ B cells were the dominating B cell population in the head kidney (HK), posterior kidney, spleen and peripheral blood compared to IgT$^+$ B cells. Gene expression analyses of magnetic-activated cell sorting (MACS) purified IgM$^+$ B cells showed a varying basal level transcriptions of nucleic acid sensing Toll-like receptors (TLRs) including TLR9, 21, 3, 22 and 8a. Of the two TLRs sensing CpG DNA in teleosts, TLR9 was expressed over tenfold higher compared to TLR21. Despite this, the biological significance of having two sets of TLR genes sensing CpG remains undefined in A. salmon. Upon *in vitro* CpG stimulation, MACS sorted IgM$^+$ B cells upregulated the transcription of secreted IgM (sIgM) and enhanced the secretion of IgM protein suggesting that CpG drives activation of naïve B cells towards antibody secreting cells (ASC) phenotype. The constitutively high expression of MHCII gene in sorted B cells indicates antigen-presenting function. B cells stimulated with CpG exhibited upregulation of type I interferon (IFN) genes proposing an IFN response. This work demonstrates that IgM$^+$ B cells are the dominating B cell populations and upon CpG stimulation, IgM$^+$ B cells enhance secretion of IgM and upregulate transcription of a signature cytokine of innate antiviral role.

Paper II:

Salmonid alphavirus subtype 3 induces prolonged local B cell responses in peritoneal cavity of Atlantic salmon (*Salmo salar*) after intraperitoneal challenge

Shiferaw Jenberie, Ma. Michelle D. Peñaranda, Hanna L. Thim, Morten B. Styrvold, Guro Strandskog, Jorunn B. Jørgensen and Ingvill Jensen

In teleost fish, studies that have involved the peritoneal cavity (PerC) mainly focus on describing intraperitoneal (IP) response typically occurring within the first two weeks following IP immune insult. To our knowledge, there is no data showing prolonged local B cell responses in the PerC of teleost species. In steady state Atlantic salmon (A. salmon), we found uneven distribution of IgM$^+$ B cells and antibody secreting cells (ASC) between the PerC and systemic immune sites warranting further investigation to understand what drives this intrinsic difference in B cell localization. Upon IP salmonid alphavirus subtype 3 (SAV3) infection, the PerC demonstrated increased total leukocyte count for the duration of the experiment while a decline in leukocyte counts were evident in systemic sites. Flow cytometry analysis of this virus-induced leukocyte population at 14 days post-challenge (dpc) revealed a significant increase in frequency of IgM$^+$ B cells in the PerC, head kidney (HK) and spleen. Further analysis of this IgM$^+$ B cell population at 14 dpc exhibited two distinct populations, IgM^{high} and IgM IgM^{low}, with a remarkable shift towards IgM^{low} cells in the PerC showing emergence of plasmablast-like cells. Additionally, the SAV3 infection induced a significantly higher frequency of IgM$^+$ B cells in the PerC for the duration of the experiment compared to control fish. ELISpot analyses revealed an increase in total ASC in the PerC at all sampling points, while a decrease in total ASC was found in the systemic sites, HK and spleen. The peak total ASC response in the PerC at 6 wpc coincided with and correlated positively to the detection of virus specific anti-E2 antibody and neutralizing antibody response in serum. Whether the PerC of A. salmon mounts virus specific ASC response or if so, the contribution of the PerC ASC to serum antibody response remains enigmatic and needs further investigation. Altogether, for the first time in teleost species, this study demonstrates prolonged IgM$^+$ B cell and ASC responses in the PerC suggesting an active immunological role of the PerC that likely influence the outcome of the immune response to IP administered antigen.

Paper III

Antigen specific antibody secreting cells reside in the peritoneal cavity and systemic sites of Atlantic salmon (*Salmo salar*) challenged intraperitoneally with salmonid alphavirus

Shiferaw Jenberie, Henriette Nordli, Guro Strandskog, Linn Greiner-Tollersrud, Ma. Michelle D. Peñaranda, Jorunn B. Jørgensen and Ingvill Jensen

In paper II, we have shown that the PerC of A. salmon promotes prolonged antibody secreting cells (ASC) response to IP SAV3 infection. However, whether the PerC micromilieu of teleost species supports the formation and maintenance of antigen (Ag) specific ASC remains unknown. Here, for the first time in teleost species, we reported Ag-specific ASC response in the PerC of A. salmon after IP injection with virulent salmonid alphavirus subtype 3 (wtSAV3) or inactivated SAV (inSAV). Comparing the two antigens, the wtSAV3 induced a significantly higher frequency of Ag-specific ASC at 13 wpi across the three sites; by then, the response induced by the inSAV was barely detectable. Comparing the three sites of wtSAV3 injected fish, the PerC displayed the highest frequency of Ag-specific ASC at 13 wpi suggesting on local importance of these cells. Despite detection of Ag-specific ASC by ELISpot, virus specific antibody was not detected in sera samples from inSAV group using ELISA. The weak induction of Ag-specific ASC response coupled with the low IgM secretion rate of Ag-specific ASC from inSAV group likely to justify the negative ELISA result. In the PerC of wtSAV3 injected fish, the frequency of Ag-specific ASC increased significantly from 6 to 13 wpi while the frequency of total ASC declined during the same period suggesting an independent regulation of the two B cell populations in A. salmon. This work provides an evidence of the presence of Ag-specific ASC in the PerC of a teleost species after IP challenge. However, whether the Ag-specific ASC are formed locally in the PerC or recruited from other immune sites remains poorly understood and warrants further investigation.

Discussion of the findings

Despite emerging technological breakthroughs, many aspects of B cell biology are still inadequately understood in teleost fish. Advancing our knowledge of teleost fish B cell biology has a great relevance in designing better fish vaccines. However, among others, lack of customized reagents for fish has seriously constrained the progress of fish immunology. Functional assays such as ELISpot, both total and Ag-specific, magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS) have been developed and employed in this project to better characterize B cells from A. salmon.

B cell lineage in teleost species: Questioning the current paradigm

Reconstitution studies coupled with immunophenotyping techniques have led to the discovery of three developmentally distinct B cell lineages in mammals: B-1a, B-1b and B-2 (Kantor et al., 1992, Tung et al., 2006). In mammals, evolution favors the more dominant B-2 B cells, leaving the B-1 B cells behind as a minority. Despite being rare events in the secondary lymphoid tissues, the B-1 B cells are the majority in body cavities such as the PerC and are the major source of natural antibody in mammals (Hayakawa et al., 1985, Jackson-Jones and Benezech, 2018). B cell lineage studies in teleost fish are at its infancy and the few studies available are mainly based on searching for phenotypic and functional similarities that define B cell lineages in mammals (Abos et al., 2018a). Accordingly, regardless of anatomical location, an increasing number of reports propose that B cells from teleost species have functional homology to the mammalian B-1 B cells (Sunyer, 2012a, Zhu et al., 2014, Abos et al., 2018a). Driven by this prevailing view, to date data comparing the IgM secreting capability of B cells from different immune sites of teleost fish are not available. At steady state, we found over 2-times higher frequency of IgM$^+$ B cells in the systemic sites, HK and spleen, than in the PerC (paper II). Given this information, one would intuitively expect higher frequency of ASC in the systemic sites. Interestingly, what we found was the other way round where the PerC had over 2-times higher frequency of ASC than in the systemic sites (paper II). However, whether this difference in ASC frequency between PerC and systemic sites is due to lineage differences as described in mammals or presence of chemoattractants that differentially sequester ASC in the PerC of A. salmon remains an open question and warrants further investigation. In mammals, the B-1 B cells are further divided into B-1a and B-1b lineages principally based on the expression of CD5 (Tung et al., 2006). About half of the B cell population in rainbow trout

spleen expresses CD5 (Abos et al., 2018a) while in normal mice spleen CD5$^+$ B cells represent only 1-5% of the B cell population (Hayakawa et al., 1985). In that rainbow trout study, IgM$^+$ B cells, irrespective of CD5 expression, show phenotypic characteristics resembling the mammalian B-1 B cells such as large size, surface IgM^{high}, surface IgD^{low} and extended survival *in vitro* (Abos et al., 2018a). However, whether CD5 expression by rainbow trout B cells defines a different B cell lineage as in mammals or has another biological role distinguishing the two B cell populations warrants further investigation. Further, resembling characteristics of the mammalian B-1 and B-2 B cell compartments, rainbow trout IgM$^+$ B cells from the PerC and spleen demonstrate different requirements for BAFF-family cytokines for survival (Soleto et al., 2017, Tafalla et al., 2017). In mice, spontaneous natural IgM production by PerC B-1 cells is a subject of debate. However, recent reports indicate that mice PerC B-1 B cells do not produce natural antibody *in vivo* or *ex vivo* at steady state (Baumgarth, 2011, Choi et al., 2012). Instead, after Ag encounter in the PerC, B-1 B cells rapidly migrate to the spleen for activation and differentiation to IgM ASC (Baumgarth, 2011, Choi et al., 2012). As opposed to mice or perhaps indicating an important functional difference, A. salmon PerC B cells produced IgM *ex vivo* at steady state (paper II). Hence, we believe that further studies in this direction will either consolidate the current paradigm or provide a new insight regarding B cell lineages in teleost fish.

A double set of dsDNA sensing TLR and response to CpG stimulation

Expression of Toll-like receptors (TLRs) in B cells provides a requirement for innate signals for optimal orchestration the adaptive immune response (Hua and Hou, 2013). Hence, profiling the constitutive TLR expression of B cells helps to understand how the B cells respond to different stimuli as TLR signaling modulates various aspects of B cell function. In mammals, CpG signaling via TLR9 affects antigen presentation, cytokine secretion, affinity maturation and survival of long-lived plasma cells and memory B cells (Bode et al., 2011, Hua and Hou, 2013). Unlike in mammals, data on the effect of CpG from a defined B cell population are scarcely available in teleost immunology. In paper I, B cells from different immune sites constitutively transcribed a double set of dsDNA (TLR9, 21) and dsRNA (TLR3, 22) sensing TLRs, a phenomenon that supposedly occurred secondary to the whole genome duplication events (Cresko et al., 2003). The finding of a high basal level transcription of TLR9 combined with our research group's interest to evaluate the potential of CpG as molecular vaccine adjuvant for

fish prompted us to study its effect on B cells. A. salmon TLR9 has shown functional and structural conservation with the mammalian counterpart (Skjaeveland et al., 2008, Iliev et al., 2013a). Both TLR9 and 21 respond to CpG in zebrafish (Yeh et al., 2013). However, whether TLR21 has a redundant/novel function or the biological significance of having a double set of TLRs remains enigmatic and calls for further investigation in A. salmon. CpG induces nonspecific innate antiviral mechanism that protects A. salmon from lethal viral challenge (Jorgensen et al., 2003). Upon CpG stimulation, sorted naïve B cells enhanced secretion of IgM (paper I) suggesting that it induces a polyclonal stimulation of B cells as the antibody response was not targeted toward a specific antigen. In our study, however, the possibility that BCR cross-linking by the sorting antibody that might help B cells to integrate signals from BCR and TLR to differentiate into ASC cannot be ruled out. In mammals, CpG induces bacteria-reactive antibodies that enhance phagocytosis and protect from lethal IP bacterial challenge suggesting on the polyclonal nature of the antibodies (Kim et al., 2018). In teleost species, demonstrating a potent adjuvant effect, CpG boost specific antibody response when co-administered with antigens (Thim et al., 2012, Pavan et al., 2016). Hence, a better understanding of the adjuvant property of CpG is crucial to make use of its ability to initiate an innate immune response that is required to design more efficacious fish vaccine.

B cell response to SAV3 challenge

Pancreas disease (PD) caused by salmonid alphavirus (SAV) is one of the main disease threats to the Norwegian A. salmon farming industry. Despite vaccination, increasing number of PD outbreaks have been reported in recent years, calling the efficacy of the inactivated vaccines into question. The immune response to SAV is mainly mediated by neutralizing antibodies secreted by effector B cells (Lopez-Doriga et al., 2001). Hence, in addition to its economic significance, the choice of SAV as a model in the current study was to harness its capability of inducing B cell responses. Recent rainbow trout studies have proposed an active immunological role of the PerC that might influence the outcome of the immune response to IP injected antigens (Pignatelli et al., 2014, Veenstra et al., 2018). In paper II and III, we studied B cell responses against SAV focusing mainly on the PerC. A general increase in the frequency of IgM^{+} B cells and ASC was observed in the PerC of infected fish suggesting that B cells are key responders to viral infection. IgM^{+} B cells with high phagocytic capability have been previously reported in A. salmon (Overland et al., 2010). Hence, the recruitment and/or

differentiation of IgM^{+} B cells at the site of infection in our study suggests their active involvement in the clearance of the infection (paper II). Despite *ex vivo* IgM secretion by PerC ASC, *in vivo* IgM secretion in the PerC of teleost species has not yet been elucidated. Hence, it is an open question whether the prolonged total ASC response in the PerC of infected fish (paper II and III) contributes to the serum antibody response and/or local IgM secretion that promotes virus clearance and the microenvironment homeostasis. This prolonged PerC B cell response appeared to be induced due to retention of the virus in the PerC as trace amounts of virus RNA were detected in PerC leukocytes and adipose tissue for at least 6 wpc (Own data, not shown), but this needs further clarification. Interestingly, despite infected fish had fewer numbers of total ASC in their systemic immune sites compared to control, they had high total IgM protein in serum (paper II) suggesting either a high IgM secretion rate by ASC or a source of serum IgM outside of the systemic compartment, which in this case could be the PerC. Consistent with that, a positive correlation was found between the PerC total ASC response and the virus specific antibody response in serum (paper II).

An issue that has never been addressed before in teleost species is whether the PerC of teleost fish mounts Ag-specific ASC response. Indeed, PerC of A. salmon possessed Ag-specific ASC after IP infection (paper III). However, many aspects of this PerC Ag-specific ASC response, such as local formation versus recruitment from other sites, regulation and local IgM secretion, are still poorly understood. In mice, the generation of PerC Ag-specific ASC is route dependent as they are detected following only IP, but not intravenous, injection of antigen (Jones et al., 2015). Additionally, in mice lacking spleen, lymph nodes and Peyer's patches, PerC ASC serve as source of serum antigen specific IgM (Jones et al., 2015). Hence, it is likely that the PerC Ag-specific ASC in our study are generated locally in the PerC and could contribute to the local and systemic antibody responses; however, this needs further elucidation.

Teleost PerC: A peripheral immunological site?

In mammals, the B-1 B cells residing in the serous cavities migrate to the milky spot (MS) or fat-associated lymphoid clusters (FALCs) and other secondary immune sites for self-renewal and secretion of IgM (Jackson-Jones and Benezech, 2018). Milky spots play important peripheral immunological function in mammals (Mebius, 2009). Although many aspects

related to cellular organization and function remain undefined, structures resembling the milky spot of mammals have been recently described in the PerC adipose tissue of a teleost species (Pignatelli et al., 2014). In addition, PerC adipose tissue of rainbow trout differentially transcribes a wide range of genes known to play important immune function upon IP challenge (Pignatelli et al., 2014, Veenstra et al., 2018). A. salmon (paper II) and rainbow trout (Pignatelli et al., 2014, Castro et al., 2017, Granja and Tafalla, 2017) studies also demonstrate that PerC IgM$^+$ B cells differentiate towards plasmablast phenotype in response to IP immune insult. Despite buildup of information on an active immunological role of the PerC, what structure in the PerC provides a mechanical support or niche for the differentiation of B cells has not been elegantly demonstrated and is an interesting area of future study. The finding that PerC of A. salmon possessed Ag-specific ASC further corroborates the existence of an immunological site in the PerC (paper III). The previously proposed models for the distribution B cells in teleost species do not describe the contribution of the peripheral immune sites, such as the PerC, in B cell activation and differentiation (Bromage et al., 2004, Zwollo et al., 2005, Wu et al., 2019a). This limitation prompted us to propose a new model that shows the interplay between the PerC and the systemic sites with regard to B cell activation and maturation (Fig. 5). Whereas the PerC Ag-specific ASC were induced by the infection, their extended presence in the PerC appeared to be antigen independent (paper III). This is because the frequency of Ag-specific ASC increased from 6 to 13 wpi, while during the same period the virus RNA was undetected in the PerC AT (data not shown). Further, analysis of the PerC and systemic tissues from infected fish revealed uneven distribution of Ag-specific ASC with PerC having the highest frequency. This leads us to suggest the presence of a niche in the PerC that selectively favors their survival or a mechanism that sequesters Ag-specific ASC in the PerC. Consistent with this, in mammals, Ag-specific memory B-1a cells persist in the PerC indefinitely awaiting for subsequent infection (Yang et al., 2012). In teleost species, the discovery of ASC that are insensitive to hydroxyurea and secrete IgM *in vitro* for an extended period laid the scientific basis for the identification of HK, teleost's bone marrow analogue, as having a survival niche for long-lived plasma cells (Bromage et al., 2004). Despite this, the cellular composition and cytokine-micromilieu of the proposed survival niche in the HK remains poorly understood. Hence, whether a similar microenvironment exists in the PerC or whether PerC employs a different mechanism to promote a prolonged Ag-specific ASC response warrants further investigation. Based on the data presented in this thesis and previous reports in teleost species

(Bromage et al., 2004, Zwollo et al., 2005, Iliev et al., 2013b, Wu et al., 2019a), a hypothetical model of ASC migration within PerC, HK and spleen after IP infection is proposed (Fig. 5).

Legend:
- Immature B cell
- Mature naïve B cell
- Antibody secreting cell
- Long-lived antibody secreting cell
- Antigen presenting cell
- Antigen
- Antibody
- Lymphopoietic stroma
- Survival niche
- ? Lacking data
- PerC Peritoneal cavity
- AT Adipose tissue
- PB Peripheral blood
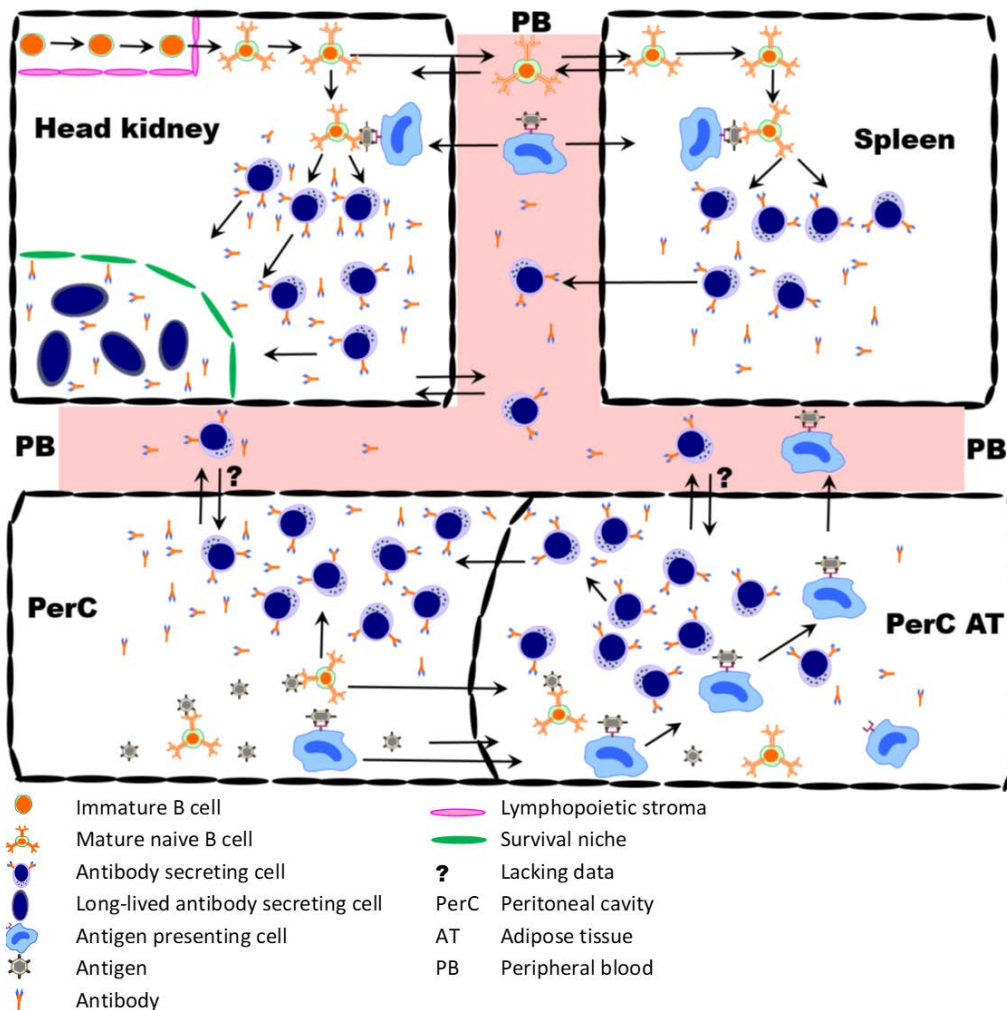
Figure 5. A hypothetical model of ASC migration within the PerC, HK and spleen of A. salmon after IP challenge. *B cells develop and mature in the HK from precursor B cells. Mature naïve, also called 'resting', B cells leave the HK and circulate/recirculate throughout the body via the blood in search of a bloodborne antigen. In addition, resident PerC B cells and other APCs sample antigens that are administered IP. Upon antigen encounter, resident PerC B cells mature into ASC either in the PerC or in the PerC AT. The AT ASC migrate back to the PerC or to the systemic immune sites for antibody secretion and in search of a survival niche in the HK to terminally differentiate into long-lived ASC. Similarly, antigen encountered PerC APCs migrate to the PerC AT to present antigen to AT resident naïve B cells or to transit to the systemic immune tissues. The Ag encountered APCs migrate from the PerC AT to the HK and spleen via the blood for antigen presentation and activation of naïve B cells. These B cells mature into ASC in the systemic compartments, secrete IgM and migrate to a survival niche in the HK or redistributed via the blood to the periphery, such as PerC, for antibody production.*

The PerC ASC response: Significance for vaccinology?

To date, except few intramuscularly injected DNA vaccines, most of the fish vaccines are inactivated whole-pathogen preparations that are administered IP. Historically, vaccine trials in fish involve IP injection of vaccine antigens, while indicators of efficacy are largely relied on findings in the systemic compartments. Consequently, knowledge of local PerC responses and the significance of these are limited. Here for the first time in teleost species we found a prolonged ASC response in the PerC of infected fish that correlated positively with the neutralizing antibody response in serum. To the contrary, injection of a massive amount of inactivated virus into the PerC induced a transient ASC response in the PerC that was not accompanied with specific antibody response in serum (paper III). It has been previously shown that ovalbumin and CpG injected IP in A. salmon were found exclusively in MHCII$^+$/IgM$^-$ cells in the HK and spleen (Iliev et al., 2013b). Given the highly phagocytic capability of B cells (Overland et al., 2010) and the presence of a significant number of IgM$^+$ B cells in the PerC of A. salmon (paper II), the above A. salmon study suggests that B cells that encounter Ag in the PerC remain in the PerC and differentiate into ASC in the PerC. Although the fate of these PerC ASC is not yet defined, it seems likely that they feed the specific antiviral antibody response both locally and in the systemic sites as has been demonstrated in mammals (Jones et al., 2015, Lue et al., 1994). Additionally, there appears to be a mechanism in the PerC that regulates B cell responses to different antigens as only live virus, and not inactivated virus, induces prolonged Ag-specific ASC response (paper III). Although little is known about this PerC regulatory mechanism, this could be due to the ability of the live virus to enter into the APCs that maximizes Ag-presentation and initiation of robust innate immune response, which culminates with the induction of strong and lasting ASC responses. In contrast, due to poor entry and cytosolic recognition, the inactivated virus induces weak innate immune response, which lead to weak activation of various signaling pathways that ultimately dictate the resulting weak ASC response. Taken together, these findings suggest a potent immunoregulatory activity of the PerC that can influence the outcome of the immune response against IP injected antigens. Further, it also implies that induction of a strong local ASC response in the PerC after IP vaccination could be used as an additional indicator of vaccine efficacy during vaccine development for fish. However, further studies are required particularly on how the PerC response correlates with disease protection and how it regulates the immune response to IP injected antigens to better formulate effective vaccines for fish.

Future perspectives

Lack of customized reagent for fish is still a serious hurdle ahead in advancing fish immunology. The assays that have been developed in this project enabled us to gain important insights regarding A. salmon B cells distribution and response underlining that fish immunology benefits a lot from endeavors of such kind in the future. Beyond adaptive immune function, B cells in higher vertebrates are increasingly seen as a key regulators of the immune response against a pathogen, a function that has not been studied in teleost species and calling for future investigation. Despite efforts made in this project to expand the knowledge on A. salmon B cells, there are still exciting questions awaiting immediate attention. These include:

✓ Understanding the impact of TLR signaling on B cell functionality

✓ Identifying surface pan-B cell markers based on molecules other than BCR and using that, establish analytical protocols for B cells

✓ Understanding molecular mechanisms that promote prolonged B cell responses in the PerC

✓ Determining whether the Ag-specific ASC are differentiated in the PerC or trafficked to the PerC from other immune sites.

✓ Given that differentiation of naïve B cells occurs in the PerC, characterizing a niche in the PerC that provides mechanical support for B cell differentiation

✓ Determining if PerC Ag-specific ASC secrete IgM locally and if so, the role that it plays in clearing an infection and maintaining homeostasis

✓ Studying whether the PerC Ag-specific ASC contribute to serum specific antibody response

Main conclusions

✓ Naïve B cells from A. salmon transcribe a wide range of nucleic acid sensing TLRs including TLR9, TLR21, TLR3, TLR22 and TLR8a

✓ CpG stimulation differentiates A. salmon B cell towards ASC phenotype and enhances IgM secretion *in vitro*

✓ A. salmon B cells upregulate transcription of type I IFN upon CpG stimulation, an innate antiviral immune signature

✓ IgM+ B cells are the dominating B cell population in HK, spleen, peripheral blood and the PerC of A. salmon

✓ ASC are distributed unevenly between the PerC and systemic immune sites, HK and spleen, in steady state A. salmon

✓ The PerC of A. salmon promotes prolonged IgM+ cell and ASC response after IP challenge with SAV3

✓ Ag-specific ASC reside in the PerC and systemic immune tissues, HK and spleen, of A. salmon after IP injection of virulent and inactivated SAV

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Paper I

Correction: Author Correction

OPEN

Profiling Atlantic salmon B cell populations: CpG-mediated TLR-ligation enhances IgM secretion and modulates immune gene expression

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While TLR-activated pathways are key regulators of B cell responses in mammals, their impact on teleost B cells are scarcely addressed. Here, the potential of Atlantic salmon B cells to respond to TLR ligands was shown by demonstrating a constitutive expression of nucleic-acid sensing TLRs in magnetic sorted IgM+ cells. Of the two receptors recognizing CpG in teleosts, *tlr9* was the dominating receptor with over ten-fold higher expression than *tlr21*. Upon CpG-stimulation, IgM secretion increased for head kidney (HK) and splenic IgM+ cells, while blood B cells were marginally affected. The results suggest that CpG directly affects salmon B cells to differentiate into antibody secreting cells (ASCs). IgM secretion was also detected in the non-treated controls, again with the highest levels in the HK derived population, signifying that persisting ASCs are present in this tissue. In all tissues, the IgM+ cells expressed high MHCII levels, suggesting antigen-presenting functions. Upon CpG-treatment the co-stimulatory molecules *cd83* and *cd40* were upregulated, while *cd86* was down-regulated under the same conditions. Finally, *ifna1* was upregulated upon CpG-stimulation in all tissues, while a restricted upregulation was evident for *ifnb*, proposing that salmon IgM+ B cells exhibit a type I IFN-response.

Adaptive immunity, present in all jawed vertebrates, is based on broad repertoires of antigen binding receptors expressed on B and T cells. In addition, B cells possess the capacity to directly sense and respond to pathogens through pattern recognition receptors (PRRs), including Toll-like receptors (TLRs). The development of different B cell subsets is determined in synergy between the B cell receptor, other receptor-derived signals and downstream signaling pathways[1]. These processes, as well as the various functionally distinct B cell subsets, are well studied in higher vertebrates, but less is known about the characteristics of teleosts B cells.

Teleosts and mammals diverged more than 350 million years ago; despite similarities between their immune systems, distinct differences are present. Lacking both bone marrow and lymph nodes, the anterior kidney (or head kidney; HK), and spleen are the organs involved in teleost B cell maturation and differentiation[2]. IgM, IgD and IgT are the only immunoglobulin (Ig) classes identified in fish[3,4]. IgM is regarded as a universal vertebrate Ig and was the first teleost Ig to be characterized[5]. Teleost IgM is a structural and functional homolog to mammalian IgM and the most prevalent isotype in fish serum present as a tetramer[6]. IgT (IgZ in cyprinids) is a unique teleost Ig[7–9] involved in mucosal immunity analogous to mammalian IgA[10–13]. Compared to IgM and IgT, teleost IgD functions are less examined, however, in channel catfish (*Ictalurus punctatus*) IgD is suggested to be a mediator of innate immunity[14]. Two B cell populations were initially described in rainbow trout (*Oncorhynchus mykiss*); one subset expressing both IgM and IgD and one expressing only IgT[13]. These two B cell types are localized differently,

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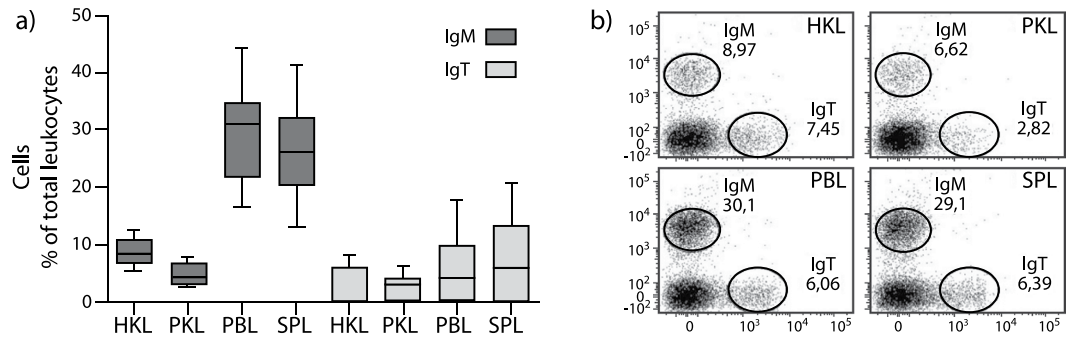
Figure 1. IgM$^+$ cells are the dominating B cell population in Atlantic salmon systemic lymphoid tissues. Flow cytometry analysis of Atlantic salmon head kidney (HKL), posterior kidney (PKL), peripheral blood (PBL) and spleen (SPL) leukocytes stained with trout anti-IgM and IgT mAbs. (**a**) Median frequencies of IgM$^+$ and IgT$^+$ B cells of total leukocytes (n = 12). The box indicates 25th and 75th percentiles and the bars min and max values. (**b**) Representative flow cytometry dot plots showing the IgM and IgT percentages in the systemic lymphoid tissues.

with IgM$^+$ cells being the main B cell population in spleen, kidney and blood[15], while IgT$^+$ cells dominate at mucosal sites[12,13,16]. Recently a third subset; expressing only IgD and homing mainly to the gills, was also identified in trout[17].

Besides mediating humoral adaptive responses, mammalian B cells secrete cytokines and present antigen to T cells[18,19]. Naïve mammalian B cells are generally divided into three subsets; follicular B cells, B-1 B cells and marginal zone (MZ) B cells. Mature follicular B cells home to follicles in the secondary lymphoid organs (lymph nodes and spleen), where they present T cell dependent antigens (TD) to T cells. MZ B cells and B-1 B cells, respectively, reside in the marginal zone of the spleen or in body cavities and both have important roles in T cell independent antibody (Ab) responses. These cells occupy a niche between innate and adaptive immunity, secreting broadly cross-reactive IgM Abs[20,21]. Upon innate signaling, e.g. TLR-ligands, both MZ- and B1-B cells rapidly differentiate into Ab-secreting plasma cells, while follicular B cells only do this in the context of prior BCR activation[22]. Whether teleost B cells fall into several functionally distinct subpopulations is presently unknown. However, some of the features that characterize mammalian B-1 cells are described for teleost B cells; for example a high phagocytic activity[23,24] and constitutive expression of PRRs[25]. Previous in *vivo* studies by our group showed that the combination of the TLR-ligand CpG and poly I:C, increased the neutralizing Ab response to a virus vaccine in Atlantic salmon (*Salmo salar*)[26,27]. This prompted us to use CpG as a model to investigate Atlantic salmon B cell biology, a topic of particular interest due to the high economic value of this species in the aquaculture industry and the urgent need for more efficient virus vaccines. A question of particular interest was whether the TLR-ligand CpG could directly stimulate B cells *in vitro* to secrete Abs and also to show enhanced antigen presentation functions stemming from increased expression of costimulatory molecules or MHC II molecules.

IgM$^+$ B cells that were obtained by magnetic activated cell sorting (MACS) were found to constitutively express nucleic acid sensing TLRs, providing a foundation for TLR ligands to aid in shaping salmon B cell responses. Indeed, upon CpG stimulation, IgM secretion was increased in IgM$^+$ cells; with the highest induction in HK compared to spleen and the lowest secretion in blood. In addition, gene expression analysis showed that the capacity of salmon IgM$^+$ cells to trigger type I interferon (IFN-I) responses and present antigen appeared to be modulated by CpG stimulation. The results presented here provide a platform for further in-depth studies, dissecting different B cell subsets in teleost fish and their functional capacities related to humoral immunity, antigen presentation and regulatory functions.

Results

IgM$^+$ B cells are the dominating B cell population in salmon kidney, blood and spleen.
The percentage of IgM$^+$ and IgT$^+$ B cells in relation to total leukocytes in salmon HK, posterior kidney (PK), peripheral blood (PB) and spleen were analyzed by flow cytometry using trout anti-IgM and anti-IgT mAbs (Fig. 1). For all tissues, the most abundant B cell population was the IgM$^+$ B cells (Fig. 1a,b). The IgM$^+$ population constituted about 30% of all leukocytes. In PB and spleen, and had a higher abundance compared to HK and PK (~5–10%). Both IgM$^+$ and IgT$^+$ cells showed a larger individual variation in PB (17 to 44% and 0.1 to 18%, respectively) and spleen (13 to 41% and 0.1 to 21%, respectively), that was not seen in the HK or PK. In four to five of the individuals analyzed, there were less than 2% IgT$^+$ cells, which was evident in all tissues.

Purity and viability of MACS sorted IgM$^+$ B cells from HK, spleen and PB.
To study B cell biology of salmon, cultures of IgM$^+$ cells were obtained by MACS. Before proceeding to further experiments, a basic characterization of these cells was done by purity and viability testing. As shown by flow cytometry, the purity of the IgM$^+$ B cells was >95% for PB and SP and >92% for HK (Fig. 2a). Viability was 98% after MACS and decreased to 78 and 35% after 24 and 48 hours in culture, respectively. Viability in CpG stimulated IgM$^+$ cells was in the same range as in unstimulated cells (Fig. 2b).

Since macrophages bind IgM through their Fc-receptor, there might be a possibility of macrophage contamination within the IgM$^+$ MACS purified cells. To test this, the expression levels of genes encoding the scavenger
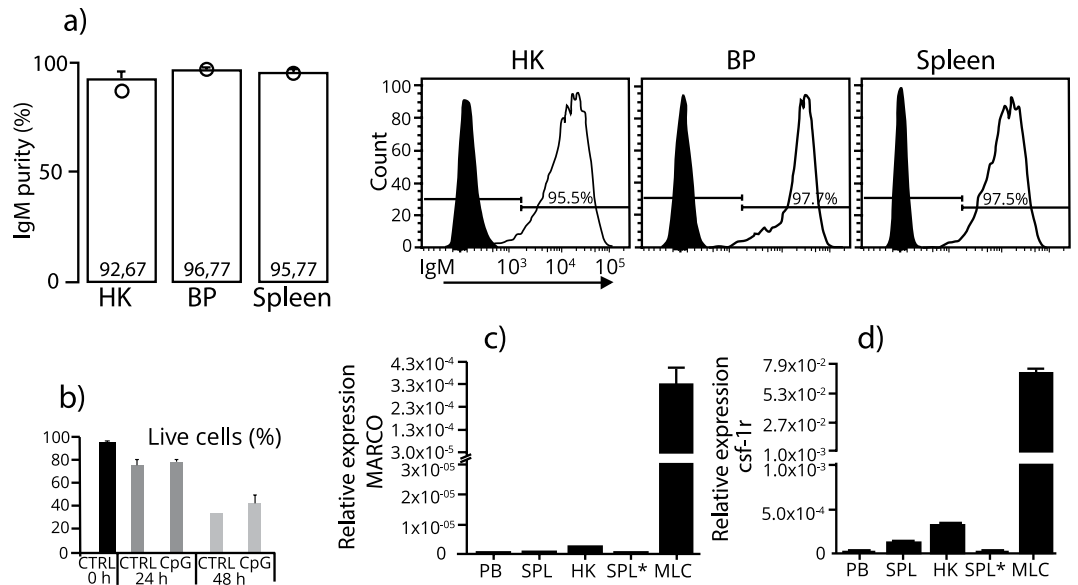
Figure 2. Purity and viability of IgM⁺ B cells sorted by magnetic activated cell sorting (MACS). (**a**) Upon sorting, the mean percentages of IgM⁺ cells from HK, PB and spleen (n = 3 for each tissue) were analysed by flow cytometry. The circle (○) represents total percentage of viable cells before gating for IgM⁺ events. Histogram represents one representative individual for each tissue, where IgM⁺ events are presented by the transparent peak and non-stained events by the black peak. (**b**) Viability of IgM⁺ cells kept in culture with or without CpG for 0, 12 and 24 hours. (**c** and **d**) The relative expression of MARCO and *csf-1r* in MACS and FACS sorted IgM⁺ cells, and in macrophage-like cells (MLC).
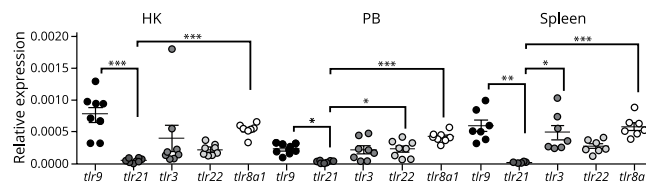

Figure 3. Relative expression of nucleic acid-sensing TLRs in Atlantic salmon IgM⁺ B cells derived from head kidney, peripheral blood (PB) and spleen. *tlr* expression was analyzed by RT-qPCR in IgM⁺ B cells sorted by MACS (n = 6 to 8). Data are presented as relative expression from individual fish (dots) where the bars indicate mean and s.e.m. Statistical significance between the *tlr* expression levels are indicated by brackets and the asterisks indicates the strength of significance: *$p < 0.05$ **$p < 0.01$ ***$p < 0.001$.

receptor MARCO and the *csf-1r*, known as markers of monocyte-macrophage lineages in mammals[28] and fish[29], were analyzed in freshly MACS-purified HK, spleen and PB B cell populations. For comparison, the expression of the same genes was tested in HK-derived untreated macrophage-like cells (MLC), and as expected high mRNA levels of both genes were seen (Fig. 2c,d). Notably, while MARCO expression was undetected in IgM⁺ cells derived from PB and spleen (Cq cut-off to 37.8), and at very low levels in the HK cells (Cq > 36), a modest *csf-1r* expression was apparent in cells from all three tissues (Cq = 30–34), and again, HK IgM⁺ cells yielded the highest expression (Supplementary Fig. S1). A comparison of the relative expression of MARCO and *csf-1r* between the IgM⁺ cells and the MLC are presented in Fig. 2c,d. A 324, 122, and 282 fold higher expression of MARCO was found in the MLC compared to PB, HK and spleen, respectively (Fig. 2c). In the same tissues, the *csf-1r* was 2690, 217 and 560 fold higher expressed in the MLC than in the IgM⁺ cells, respectively (Fig. 2d). In FACS-sorted splenic IgM⁺ cells (n = 5), both MARCO and *csf-1r* were expressed about the same level as in MACS-sorted cell, Cq > 37.8 and >32, respectively (Supplementary Fig. S1). Additionally, the expression of the T cell marker *cd4–2*[30] was explored and found to be undetectable across the tissues (Cq cut-off set to 38) (Supplementary Table S2). Our results indicate an absence of contaminating T cells in the sorted IgM⁺ populations, while traces of myeloid marker genes were detectable, most prominently in the HK derived cells.

Atlantic salmon IgM⁺ B cells express high levels of *tlr9* and *tlr8a1*. Characterization of the basal TLR expression in salmon B cells from lymphoid organs is key to understanding how TLR signaling may affect salmon B cell responses. Here, we focused on a set of nucleic acid recognizing TLRs shown to respond to ss-RNA (TLR8a1), ds-ssRNA (TLR3 and 22)[31] and CpG DNA (TLR9 and 21)[32]. *tlr3*, *8* and *9* are orthologues to mammalian TLRs, whereas *tlr21* and *22* are absent in mammals. Figure 3 shows that all five *tlr*s were expressed by
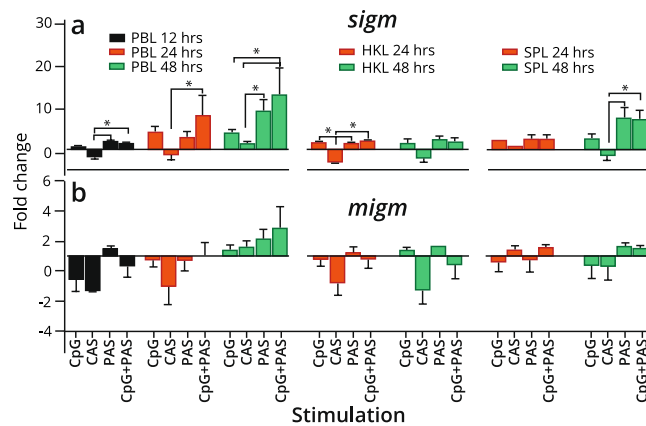
Figure 4. Expression of B cell markers genes in IgM$^+$ B cells treated with CpG and/or condition supernatants. IgM$^+$ B cells from peripheral blood (PB), head kidney (HK) and spleen leukocytes were MACS sorted and analyzed by RT-qPCR for expression of (**a**) secreted IgM (*sigm*) and (**b**) membrane IgM (*migm*). Gene expression data at each time point were normalized against the reference gene EF1aB and fold changes were calculated using the unstimulated sample at the same time point[74]. Data represent mean ± s.e.m. from at least three individuals (n = 3 to 6). The line intersecting the y-axis at 1 represents the unstimulated control that the fold change of the treatments are in relation to. Significant fold changes (p < 0.05) are indicated by *. CAS; Control adherent cell supernatant, PAS; Pulse adherent cell supernatant.

the IgM$^+$ B cell populations and that *tlr8a1* and *tlr9* had the highest relative expression, representative across all tissues. *tlr21* was the lowest expressed *tlr*-gene, and was respectively 18, 9 and 23-fold less expressed compared to *tlr9* in HK (p < 0.001), PB (p < 0.05) and spleen (p < 0.01) IgM$^+$ B cells. The individual variation of *tlr8a1*, one of several teleost *tlr8* isotypes[33], was low both within and across tissues. For the two *tlr*s recognizing dsRNA, the expression of the fish specific *tlr22*, was relatively stable across tissues and individuals, while the *tlr3* expression displayed a higher individual variation.

CpG-stimulation alters *sigm* transcript levels in IgM$^+$ B cells. Constitutive expression levels of both *tlr9* and 21 transcripts were evident when analyzing the MACS- sorted IgM$^+$ B cells; hence, this was followed up by investigating if their ligand CpG would affect the expression pattern of selected immune genes. For all the assayed genes, the effect of non-CpG treatment was negligible or minimal (Supplementary Fig. S2). The basal expression level of secreted IgM (*sigm*) was higher across the three tissues compared to membrane bound IgM (*migm*) (Supplementary Table 2). Higher basal levels of *sigm* transcripts (about 16-fold) were observed in IgM$^+$ HK (mean Cq = 18.2, SD = 0.3) and spleen (mean Cq = 18.3, SD = 0.1) compared to PB (Mean Cq = 22.5, SD = 0.2) B cells.

In general, the control supernatant, CAS, provided the weakest transcript induction, or even inhibited the expression of the B cell marker genes compared to the untreated control. Similarly, neither CpG nor PAS exhibited significant induction of *migm* in any of the tissues over time (Fig. 4b). For *sigm*, a gradual increase in induction was observed over time in B cells derived from PB and spleen (Fig. 4a). The CpG alone and the combination treatment, respectively, displayed fold inductions of 3.8 and 12.8 for PB, and 3.4 and 7.6 for spleen at 48 hours. Significant increase in *sigm* mRNA levels compared to the CAS treatment (p < 0.05) were observed in PB B cells stimulated with PAS or the combination at 12 hours, which was still significant in the combination treatment, at 24 and 48 hours (p < 0.05). The *sigm* transcript regulation was least affected by the stimulations in HK derived IgM$^+$ B cells, although a significant (p < 0.05) increase was apparent in all the three stimulations compared to CAS at 24 hours. Of notice, while HK response was unaffected over time, for spleen B cells the PAS and combination stimulation yielded a significant induction of *sigm* (p < 0.05) compared to CAS at 48 hours.

Induction of professional antigen presenting cell marker genes in IgM$^+$ B cells by CpG. The capabilities of B cells to phagocytose and present antigens (Ag) have been described in trout[34] and zebrafish[19]. Hence, we hypothesized that IgM$^+$ B cells from Atlantic salmon may express distinct transcripts of selected Ag presentation and co-stimulatory genes upon CpG stimulation. Accordingly, we measured the relative expression of *cd83, cd86, cd40* and *mhcII* in IgM$^+$ B cells obtained from the three tissues. CpG alone and the combination treatments augmented the expression of *cd83*, a marker for mature dendritic cells (DCs) and activated B cells[35]. For the three tissues, significant increase in *cd83* expression was evident in CpG and combination compared with CAS and PAS at 24 hours (Fig. 5a). However, in HK and spleen, *cd83* transcript levels slightly declined after 24 hours for the CpG and combination treatments (Fig. 5a), while the expression was still significantly maintained after 48 hours in PB IgM$^+$ B cells. *cd86* is one of the best-defined co-stimulatory molecules in mammals and plays a major role in providing co-stimulation to T cells by both DCs and B cells[36]. Recently, the involvement of CD86 in B cell-initiated adaptive immunity in a zebrafish model was reported[19]. Interestingly, except for CpG treated spleen B cells, the expression of *cd86* was downregulated compared to the control for all the treatment groups and time points in HK and PB (Fig. 5b). CD40 is a transmembrane glycoprotein and a member of the TNF receptor superfamily expressed by APCs[37]. Interactions between CD40 and its ligand, CD40L, holds a major role
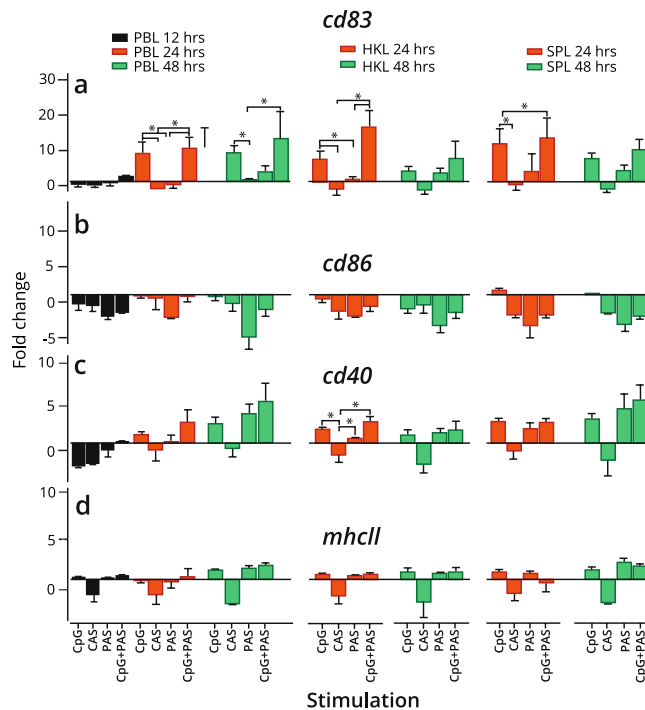
Figure 5. Expression of APC marker and co-stimulatory genes in IgM⁺ B cells treated with CpG and/or condition supernatants. IgM⁺ B cells from Atlantic salmon peripheral blood (PB), head kidney (HK) and spleen leukocytes were MACS purified, stimulated *in vitro* or left untreated and analyzed by RT-qPCR for expression of (**a**) *cd83*, (**b**) *cd86*, (**c**) *cd40* and (**d**) *mhcII*. Gene expression data at each time point were normalized against the endogenous control gene EF1aB and fold changes were calculated using the unstimulated sample at the same time point[74]. Data represent mean ± s.e.m from at least three individuals (n = 3 to 6). The line intersecting the y-axis at 1 represents the unstimulated control that the fold change of the treatments are in relation to. Significant fold changes (p < 0.05) are indicated by *. CAS; Control adherent cell supernatant, PAS; Pulse adherent cell supernatant.

in the cross-talk between T cells and APCs. CpG and PAS, both as standalone stimulations, and in combination, induced a modest, although comparable expression of *cd40* transcripts in sorted IgM⁺ B cells from the three tissues (Fig. 5c). Significantly (p < 0.05) higher induction of *cd40* was observed in CpG and PAS stimulated HK B cells at 24 hours compared to CAS. *mhcII* was constitutively expressed in IgM⁺ B cells from all the assayed tissues (basal level expression of Cq = 20.7, SD = 0.4 for PB; Cq = 20.9, SD = 0.8 for HK; Cq = 20.9, SD = 0.9 for spleen). At transcript level (Fig. 5d), the stimulations did not markedly affect the regulation of *mhcII* although the CAS simulation resulted in a more pronounced, yet insignificant, downregulation.

CpG-stimulation induce antibody secretion in HK and spleen IgM⁺ B cells.

To validate the qPCR results, the IgM content in cell culture supernatants and the MHCII expression in cell lysates from MACS-purified B cells were analyzed by Western blot. In line with the transcript data, translational results revealed that IgM secretion was enhanced in CpG treated cells compared to untreated controls (Fig. 6a,b). A pronounced individual variation in IgM secretion was observed for both controls and stimulated cells. Of notice, significantly higher levels of secreted IgM were detected in CpG-stimulated HK B cells when compared to the spleen cells (Fig. 6b). The lowest levels of secreted IgM were detected in PB B cell culture supernatants (Supplementary Fig. S4). At protein level, CpG-treatment affected MHCII expression modestly, although not significantly different, compared with the control cells (Fig. 6c).

CpG-stimulation induces type I *ifn* gene expression in IgM⁺ B cells.

To assess the capability of salmon IgM⁺ B cells to induce type I IFN responses, the expression of type I *ifn* genes were measured. CpG alone significantly (p < 0.05) up-regulated *ifna1* transcripts in B cells derived from the systemic tissues. The combination of CpG and PAS, however, induced a more pronounced expression than the standalone stimulation, which suggests an additive effect (Fig. 7a). Over time, *ifna1* expression patterns were similar for HK and spleen B cells and declined at 48 hours post stimulation, while the expression in PB IgM⁺ B cells was kept significantly higher at the same time point.

Upregulation of *ifnb* transcripts was observed only in IgM⁺ B cells obtained from HK and spleen at 24 hours for the two CpG treatments and was highly significant (p < 0.01) compared to CAS, while the expression was undetectable in PB IgM⁺ B cells (Fig. 7b). The basal expression of *ifnb* was undetected in IgM⁺ B cells obtained from all the tissues (Cq cut-off set to 36), while the expression of *ifnc* (Supplementary Table S2) was undetectable both in controls and stimulated IgM⁺ B cells.
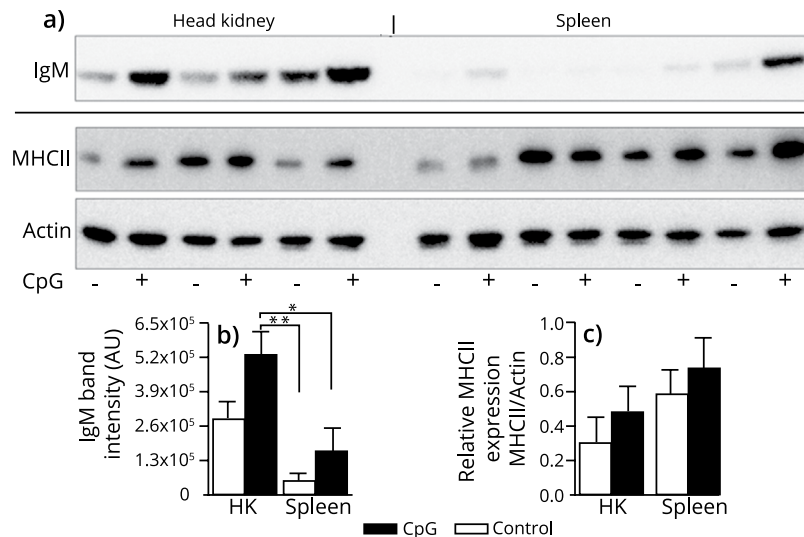
Figure 6. Protein level analysis of IgM and MHCII expression by Western blot. MACS purified IgM$^+$ B cells from head kidney (HK) and spleen were treated with CpG (+; 2 μM) or left untreated (−) for 72 hours before harvest of supernatants and cell lysates. Figures present results from two independent experiments. (**a**) Representative Western blot analysis showing IgM secretion in supernatants (upper panel), and MHCII expression in cell lysates (middle panel). An anti-actin Ab was applied as loading control (lower panel). MHCII and actin were run on the same gel; after MHCII detection, the gel was washed and reprobed with the anti-actin Ab. Bar graphs showing the mean band intensity (AU) of (**b**) secreted IgM and (**c**) MHCII relative expression in HK and spleen B cells (n = 6). Significantly higher IgM secretion is indicated by * where *p < 0.05 and **p < 0.01. The original full blot images can be found in Supplemental Fig. S5.
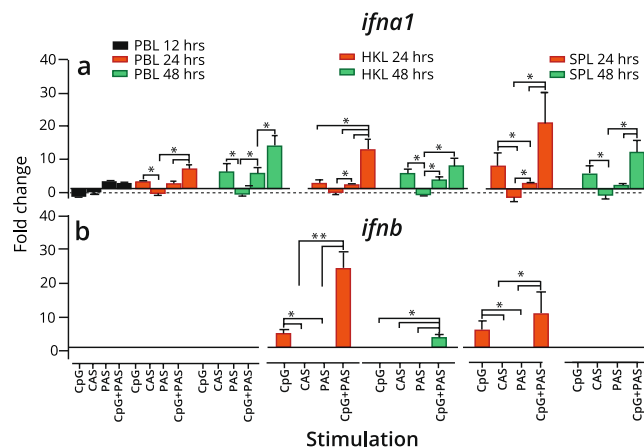

Figure 7. Expression of type I IFN genes in IgM$^+$ B cells treated with CpG and/or condition supernatants. IgM$^+$ B cells from Atlantic salmon peripheral blood (PB), head kidney (HK) and spleen (SP) leukocytes were MACS sorted, stimulated *in vitro* or left untreated and analyzed by RT-qPCR for expression of (**a**) *ifna1* and (**b**) *ifnb* genes. Gene expression data at each time point were normalized against the endogenous control EF1aB and fold changes were calculated using unstimulated cells at the same time point[74]. Data represent mean ± s.e.m from at least three individuals (n = 3 to 6). The line intersecting the y-axis at 1 represents the unstimulated control that the fold change of the treatments are in relation to. Significant fold changes, p < 0.05 or p < 0.01, are indicated by * or **, respectively. CAS; Control adherent cell supernatant, PAS; Pulse adherent cell supernatant.

Discussion

A better understanding of basic B cell biology, such as responsiveness to potential vaccine adjuvants and the mechanisms mediating their activity, is crucial for the development of new vaccines for farmed fish. For Atlantic salmon, many aspects of B cell functionality are unknown, partly due to the lack of population specific mAbs. Here we have used mAbs available against trout IgM and IgT combined with flow cytometry to separate and quantify B cell subpopulations in systemic immunological organs of non-immunized salmon. The monoclonal IgF1–18 (6-1-18) anti-trout IgM Ab is earlier reported to recognize both salmon IgM isotypes[38], while the cross-reactivity of the anti-trout IgT mAb to salmon IgT is reported herein (Supplementary Fig. S3)

Single IgM and IgT positive cells were identified in total leukocytes from PB, spleen, HK and PK at percentages varying from three to 44% for IgM and between 0.1 to 21% for IgT. The data are in accordance with a similar study in trout[13] demonstrating markedly higher abundances of IgM$^+$ B cells compared to IgT$^+$ B cells in systemic lymphoid tissues. As reported previously in several other teleost fish species[13,39,40] and also in salmon[38], PB and spleen here harbored the highest number of IgM$^+$ cells, ~30% of total leukocytes. The percentages of IgT$^+$ cells were in general low, and across the tissues investigated, several individuals had less than two percent of IgT$^+$ B cells. The trout anti-IgT mAb is directed against the constant heavy chain domains two to four of IgT subclass 1[13] and was recently shown to cross-react with the other two trout IgT subclasses[41]. Three IgT subclasses are present in salmon as well, showing ̰80% identity both to each other and to trout IgT[9]. Western blot analysis of a Flag-tagged CH4-domain of salmon IgT overexpressed in HEK293 cells verified cross-reactivity of the trout IgT Ab to salmon IgT (Supplementary Fig. S3). Whether the trout Ab is able to recognize all three salmon IgT$^+$ B cell subclasses is presently unknown, thus we cannot conclude if one or all three salmon IgT subclasses are present in the investigated tissues.

Induction of protective Ab responses is a major goal for the development of prophylactic vaccines for farmed fish. Studies by us and other groups have reported the potential of CpG-based adjuvants for fish vaccines[26,27,42]. A combination of the TLR-ligands CpG and poly I:C formulated in a whole-virus vaccine for salmon induced high titer-protective Abs after vaccination[26,27]. However, how these TLR ligands potentiate Ab responses in bony fish remain poorly understood. One important scope of this study was to investigate the direct effects of TLR engagement on salmon B cell functions. In this context, we used purified IgM$^+$ B cells obtained by MACS purification. While these cells were devoid of T cell marker genes, traces of well-known monocyte-macrophage lineage marker genes were detected. However, since their numbers were very low, their contribution to the overall mRNA levels expressed in the cultivated cells is most likely modest. Of notice, macrophage marker-gene expression were comparable between MACS and FACS purified IgM$^+$ cells, suggesting that the levels of B cell purity by the two methods are comparable. Since bony fish B cells are suggested to have a myeloid origin[34], one could speculate on whether myeloid restricted proteins like CSF-1R are totally absent in B cells.

To date, the expression pattern of TLRs in fish has mainly been monitored in whole tissues[43–45], while knowledge regarding their expression in defined leukocyte subsets are scarce. Here, transcripts of the five nucleic-acid binding TLRs were detected in the purified IgM$^+$ B cells from the three tissues. This suggests that salmon B cells have the potential to respond directly to CpG DNA and ds/ssRNA. The relative expression levels for each TLR showed little variability across the different tissues; slightly elevated levels of *tlr9* and t*lr8a1* were detected compared to the other TLRs, while *tlr21* was the least expressed TLR. A similar study in trout reported higher constitutive levels of *tlr9* and *tlr8a2* compared to *tlr3* in IgM$^+$ positive B cell populations from blood, kidney and spleen[25], although such differences were less apparent in our data. Variations in fish species, age and cell sorting procedures may explain the dissimilarities between these results. As in salmon, the fish specific receptor, *tlr22*, was abundant in trout B cells from all three tissues[25]. The most profound difference reported here is between *tlr9* and *21*, where *tlr9* had 10 to 30-fold higher basal expression levels compared to *tlr21*. TLR21 is a non-mammalian TLR, first identified in chicken, with functions resembling TLR9[46,47]. Later, *tlr21* genes have been identified in several teleost species including salmonids[48,49]. In zebrafish, both TLR9 and 21 respond to CpG-ODNs and slight differences in their ligand recognition profiles are shown with TLR9 possessing a broader sequence specific range compared to TLR21[32]. Whether this holds true for other fish species, including salmonids, warrants further investigations.

An important question for this study was whether CpG stimulation could activate salmon B cells to become Ab secreting cells (ASCs). Secreted IgM protein was detected in non-stimulated IgM$^+$ cells from spleen and HK, with markedly higher levels in HK derived B cells compared to spleen (Fig. 5a,b). In contrast, IgM secretion from PB IgM$^+$ cells were hardly detected (Supplementary Fig. S4). RT-qPCR correspondingly demonstrated that non-treated IgM$^+$ cells from PB expressed the lowest levels of secreted *igm* transcripts (mean Cq of 22.5), while basic levels in HK and spleen derived cells were about 16-fold higher (mean Cq of 18.2 and 18.3, respectively). The presence of Ig-secreting cells in the absence of immune activation suggest that plasmablasts/plasma cells might be present in these organs, and probably at a much higher abundance in HK compared to spleen and blood. This observation is in agreement with previous reports for other teleosts[50,51] and suggest that ASCs may persist from previous antigenic exposures or that these cells spontaneously secrete IgM, thus resembling the mammalian B-1 cells. In our study, the levels of secreted IgM protein increased upon *in vitro* CpG stimulation, especially for HK derived cells. The levels of s*igm* transcripts in the IgM$^+$ cells from the three tissues interrelated with the levels of secreted IgM in cell supernatants, where only secreted Ig transcripts derived from HK cells were significantly higher than the controls. The lack of salmon plasma cell markers impeded further characterization of the ASCs. For B cells activated through the BCR prior to cell harvest, CpG may contribute to terminal differentiation towards plasma cells. Whether CpG alone exerts an effect on naïve B cells, or, alternatively, that the combination of BCR triggering by the anti-IgM Ab and TLR signaling elicits differentiation into ASCs, is still an open question.

In addition to promote Ab responses, TLR signaling is involved in B cell cytokine secretion and antigen presentation[52]. In our data, the *mhcII* gene-expression pattern in IgM$^+$ cells was similar across all the three tissues, and its levels were non-significantly upregulated (~2-fold) by the stimulations (Fig. 4d). The detection of MHCII protein by Western blotting in lysates from IgM$^+$ cells validated the finding (Fig. 5b). In general, information about the role of teleost B cells as APCs is lacking. It has been reported that a population of IgM$^+$/MHC-II$^+$ cells from salmon HK has the ability to accumulate ovalbumin antigen *in vitro*[53], thus, supporting a role of HK-derived B cells in the antigen-presenting process. In this study, *cd83* and *cd40* transcript levels were slightly upregulated in CpG-treated HK-derived IgM$^+$ cells (Fig. 5a,c), while at the same time *cd86* levels decreased (Fig. 5b). These results were observed for all tissues. However, in contrast to the earlier study[53], where the B cells were cultivated and stimulated within a heterogeneous leukocyte population and subsequently FACS-sorted, the purified B cells were here directly stimulated. Our current data show that CpG alone, or possibly in combination with BCR

stimulation, is sufficient to affect the gene expression pattern of central antigen presenting markers on salmon B cells. The rapid up-regulation of *cd83* transcripts in the B cells described herein is also observed in murine B cells after TLR-engagement or BCR ligation[54]. While *cd83* mRNA expression was solely upregulated in CpG-treated B cells, both CpG alone and PAS induced elevated *cd40* mRNA levels in all the tissues when compared to the CAS, although significantly different only for HK at 24 hours. This is similar to the situation in mammals where both TLR signaling and cytokines derived from myeloid cells regulate *cd40* expression in B cells[55]. Results based on micro-array data, have shown that a suite of genes encoding secreted proteins, which includes cytokines, chemokines and TNF receptor superfamily members, are expressed in salmon adherent leukocytes stimulated with the same CpG ODN as used here[56]. It is therefore likely that PAS contains molecules with a potential to selectively upregulate different B cell transcripts.

The high phagocytic capacity of teleost B cells[24,57], combined with the upregulation of different APC markers reported here and by others[19,53,58], suggest that TLR-mediated activated B cells are capable of modulating T cell responses. As in our study, high expression of surface MHCII levels and *cd83* transcripts for LPS-stimulated trout splenic B cells has been reported elsewhere[58], underscoring their role as APCs. However, the down-regulation of *cd86* in our study contradicts the upregulation of this gene obtained in the previous study[58]. This may suggest that B cells show both similar and contrasting responses to the two microbial molecules CpG and LPS, which may influence their functions. Another important aspect is that the LPS-stimulation in the previous work[58] was not performed on purified B cells, and hence, the activation by other cell types and their secretory products may directly or indirectly influence B cell responses. The enhanced effect of the PAS supernatant when used with CpG, as reported herein, underscores that molecules derived from other cell types significantly shape the B cell responses to TLR ligation.

Trout peritoneal IgM+ cells, upon *in vivo* exposure to *E. coli* or VHSV, are shown to differentiate towards IgM-secreting cells, and at the same time these cells show decreased levels of MHCII surface expression compared to non-treated controls[59]. As the terminal differentiation of mammalian plasma cells is shown to be accompanied by the loss of MHCII expression[29], these authors argue that decreased MHCII surface levels may be a coincident indicator for trout plasma cell differentiation. This somewhat differs from our data where CpG treatment of IgM+ cells did not significantly affect MHCII levels, while increased IgM secretion clearly indicated CpG-induced differentiation towards plasma cells. However, the specific stage of differentiation of these cells towards the terminal plasma cell was not identified. Most of these cells may be at the plasmablast stage and might not have accomplished MHCII downregulation after 72 hours *in vitro* CpG treatment.

In our data, high individual variations in IgM secretion and MHCII proteins levels were apparent for both controls and stimulated IgM+ cells. This heterogeneity in responses suggest variations in the B cell composition between the individual donor fish. As a result, the secreted IgM may reflect both the presence of previously activated plasmablast/plasma cells and naïve B cells responding to the TLR-ligation. The impact of CpG treatment on MHCII levels may be different on naïve versus previously activated B cells. This proposed heterogeneity in the B cell populations restricts the usage of our data to evaluate if any association between plasma cell development and MHCII levels exists. Anyhow, whether active suppression of class II genes is an underlying mechanism for bony fish plasma cell differentiation or not, is an interesting question that calls for future investigation only attainable using subpopulation specific mAbs.

In general, information on CD40 signaling in teleost B cells is limited. However, studies in both zebrafish[60] and salmon[61] have reported that structural characteristics of CD40 and its ligand (CD40L or CD154) are conserved between mammals and fish. Salmon *cd40* was upregulated in cultivated HK leucocytes after exposure to different PAMPs including CpG[61], which is in accordance with the present data. In zebrafish, CD40/IgM double positive lymphocytes were reported[60], suggesting that CD40 is present in teleost B cells. The present work further support this, showing that salmon HK, spleen and PB B cells express considerable levels of *cd40* (Supplementary Table S2). Opposite to *cd40* and *cd83*, decreased levels of *cd86* were apparent upon CpG treatment across the tissues, which may suggest a mechanism to avoid prolonged T cell activation[62].

Teleost fish possess a well-developed type I IFN system encoded by multiple IFN genes. Six IFN-I subtypes are identified in the salmon genome, where the subtypes *ifna*, *ifnb* and *ifnc* are the best-defined representatives[63]. Here, CpG-stimulated B cells derived from HK and spleen showed increased transcript levels of *ifnb*, while elevated *ifna1* mRNA levels were detected in B cells from all three tissues (Fig. 7). Together, these results propose that salmon B cells exhibit a type I IFN-response. In line with this, rainbow trout IgM+ PB and spleen cells have been shown to transcribe *ifn1* upon poly I:C and VHSV infection[64] and type I IFN responses have been reported for CpG B treated murine B cells[65]. In general, the basal levels of IFN transcripts in the purified salmon B cells were low (*ifna1* mean Cq 31.8–33.7) or non-detectable for *ifnb* and i*fnc* (Supplementary Table 2). Interestingly, elevated *ifna1* mRNA levels were detected both for CpG alone and for CpG + PAS during the course of time, while *ifnb* levels peaked at 24 hours and then declined to its basal levels at 48 hours. A possible explanation for this is that the initial *ifna1* burst triggers the transcription of interferon regulatory factors, which mediate a positive feedback loop that lead to the induction of a second wave of *ifna1* transcription[66,67]. The cytosolic receptors RIG-I/MDA5 that recognize viral RNA are suggested to be the main receptors to activate pathways leading to *ifna1* expression in salmon[68]. Our results imply that other receptors specific for DNA induce *ifna1* gene transcription in salmon B cells. The inverted (GpC) ODN control was non-stimulatory (Supplementary Fig. S2), proposing that the *ifna1* induction in salmon IgM+ cells is mediated through a CpG-specific TLR. Unlike *ifna1*, *ifnb* is reported to show modest basal expression in most salmon organs, while a rapid and potent induction appear in salmon HK and spleen upon treatment with R848, a ligand for TLR7/TLR8[68]. This observation, together with the rapid increase in *ifnb* levels in the purified HK and spleen B cells upon TLR9 ligation, suggest that signaling through the TLR7 family activates pathways important for *ifnb* expression in salmon leukocytes. The significant increase in *ifnb* expression when combining PAS and CpG, suggest that other signals provided by the supernatant are necessary for optimal *ifnb* production in B cells.

B cells have important protective roles against many viral infections, mainly through the production of neutralizing Abs. In mammals type I IFN promotes B cell activation and Ab responses, including class switch during viral infections[69,70]. It is thus possible that type I IFNs produced by salmon B cells, affect the regulation and functional activities of the B cells themselves, in addition to that of other immune cells. Interestingly, by using a DNA vaccine model, adjuvant effects of Atlantic salmon type I IFNs were demonstrated and shown to increase the virus specific IgM response[71]. These results establish a link between type I IFNs and the adaptive immune system in salmon, although the mechanisms behind are currently unknown. In support, our group has earlier reported that CpG and poly I:C significantly enhance virus-specific humoral responses *in vivo*, where a substantial increase in *ifna1* transcripts in HK and spleen were detected at 12 and 48 hours post injection[26]. In the previous study[26], however, whether the IFNs was produced by B cells or by other cell types was not investigated. The implications of direct interactions between type I IFNs and B cells in salmon has not been elucidated and is a topic for future studies.

Materials and Methods

Fish. Atlantic salmon (*Salmo salar* L.) roe from Aqua Gen (Aqua Gen, Kyrksæterøra, Norway) was hatched and smoltified at Tromsø Aquaculture Research Station (Tromsø, Norway). The fish were kept in seawater at natural temperature and photoperiod and fed with dry commercial feed (Skretting, Stavanger, Norway) before transfer to the research facility of the Arctic University of Norway, Tromsø, where they were kept at similar conditions until sacrifice. Fish were acclimated for at least two weeks before sampling. Two batches of fish were used; 400 g fish for FACS analysis and 1000–1500 g fish for MACS sorting and immune gene expression analysis of IgM$^+$ B cells. We confirm that the experimental protocols used for the live fish experiments were based on the Animal Welfare Act (https://www.regjeringen.no/en/dokumenter/animal-welfare-act/id571188/) and performed in accordance with relevant guidelines and regulations given by the Norwegian Animal Research Authority.

Reagents. Phosphorothioate-modified B class CpG 2006 ODN (5′-T*CG T*CG T*TT T*GT C*GT T*TT G*TC G*T*T-3′, * indicates phosphothioate bonds) and non-CpG 2007 ODN, inverting all CGs to GCs, were purchased from Integrated DNA technologies. The monoclonal Abs IgF1-18 (6-1-18) anti-trout IgM[38] and anti-trout IgT[13] are described earlier. Isotype specific secondary Abs; IgG1-RPE (for IgM) and IgG2a-APC (for IgT) were both from Jackson ImmunoResearch. Fixable Viability Dye 780 (FVD780) was purchased from eBioscience. MACS MS columns and anti-Mouse IgG1 MicroBeads were purchased from Miltenyi Biotec. Anti-actin Ab was purchased from Sigma, while the preparation of MHCIIβ antiserum is described previously[56].

Cell isolation. Leukocytes from HK, PK, spleen and PB were isolated on Percoll (GE Healthcare) gradients[72,73]. Briefly, HK and spleen were sampled aseptically and kept on ice-cold transport medium (L-15 medium with 10 U/ml penicillin, 10 μg/ml streptomycin, 2% fetal bovine serum (FBS), 20 U/ml heparin) until homogenization on 100-μm cells strainers (Falcon). The resulting cell suspensions were layered on 25/54% discontinuous Percoll gradients and centrifuged at 400 × *g* for 40 min at 4 °C. Cells at the interface were collected and washed twice in L-15 medium. PB was collected from the caudal vein using heparinized vacutainer tube and diluted immediately at least two fold in ice-cold transport medium. The resulting suspension was layered on a 54% Percoll gradient, centrifuged, and harvested as above. Cells were counted using an automatic cell counter (NucleoCounter, YC-100). The isolation of HK monocytes/macrophages were performed as described elsewhere[56] and the adherent cells were cultivated in L-15$^+$ (L-15 supplemented with 5% FBS and penicillin/streptomycin) in 24 well plates (Nunclon Delta Surface, Thermo Scientific) for 3 days before being analyzed.

Flow cytometry analyses. For the Ig-frequency analysis, 3×10^5 HK, PK, PB and spleen leukocytes from twelve individuals were each pelleted at 500 × *g* directly after isolation, further washed in PBS$^+$ (PBS with 0.5% BSA) and stained with anti-trout IgM (1:200 dilution) and anti-trout IgT (2 μg/mL) mAbs for 30 minutes. After washing with PBS$^+$, the samples were incubated with isotype specific secondary Abs; IgG1-RPE and IgG2a-APC (1:400 dilution), respectively, and FVD780 (1 μl/ml) in PBS$^+$ for 20 minutes. All staining and centrifugation steps were done at 4 °C in 96-well U-bottom plates (Nunclon Delta Surface). Ig-frequencies were analyzed on Aria II flow cytometer (Becton Dickinson), while data processing was done in FlowJo (Tree Star). Dead cells (FVD780$^+$) and doublets (SSC-A vs SSC-H) were excluded before the frequency of IgM and IgT were determined

Magnetic activated cell sorting (MACS) of IgM$^+$ B cells. The sorting procedure was performed as per the company's recommendation (Miltenyi Biotec). All washes and incubations were done using MACS sorting buffer (SB; PBS with 0.5% bovine serum albumin and 2 mM EDTA) and spun at 450 × *g* 4 °C for 5 minutes. Briefly, after one wash with SB, $7–8 \times 10^7$ cells were incubated with a 1:200 dilution of anti-trout IgM mAb for 30 minutes at 4 °C. After two washes, cells were incubated in 80 μl SB and 20 μl anti-mouse IgG1 microbeads for 15 minutes at 4 °C. Sorted IgM$^+$ B cells were pelleted immediately and resuspended in L-15$^+$. Zero time-point samples were obtained before initiation of stimulations. A time course study of viability of cultured IgM$^+$ MACS purified cells from blood was performed by flow cytometry using the FVD780 stain as described above (n = 2). The cells were seeded and stimulated with CpG (2 μM) as described below. Purity of sorted IgM$^+$ B cells was determined by flow cytometry and RT-qPCR of *cd4–2* (T cell marker), macrophage colony stimulating factor receptor (*csf-1r*) and the scavenger receptor MARCO genes transcripts. For comparison, the expression of the same genes was measured in HK-derived untreated MLC and FACS sorted IgM$^+$ splenic cells, where MLC were excluded by gating.

Stimulation of sorted IgM$^+$ B cells for gene expression study. MACS sorted IgM$^+$ B cells, 1×10^6, were seeded in 100 μl L-15$^+$ in 96-well U-bottom plates (Nunclon Delta Surface, Thermo Scientific). For stimulation, either PAS or CAS (see below) in a 1:2 dilution or 2 μM CpG B ODN was applied to the cells. In addition, some cells were stimulated with PAS (diluted 1:2) combined with CpG (2 μM). Controls received only L-15$^+$. A

non-CpG control (2 μM) was also included. Cells were incubated at 14 °C for 12, 24 or 48 hours before harvested for RNA isolation. Due to variations in cell yield at each isolation, the number of individuals for each treatment varied between 3 to 6.

Preparation of conditioned supernatant. Six million HKLs were pulsed with 2 μM CpG 2006 for 8 hours (pulse adherent cell supernatant; PAS) or left unstimulated (control adherent cell supernatant; CAS) at 14 °C. After the pulse treatment, culture media and non-adherent cells were removed, while the adherent cells were washed and further incubated at 14 °C for 72 hours. After centrifugation at $450 \times g$ at 4 °C for five minutes, PAS and CAS, respectively, were harvested, pooled (three individuals) and stored at −80 °C until used.

RNA extraction and transcript analyses. Total RNA from sorted IgM$^+$ B cells and HK MLC was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer's recommendation. On-column DNase digestion was performed using RNase-free DNase set (Qiagen), and RNA was quantified using NanoDrop (ND 1000 Spectrophotometer). Twenty-five microliter cDNA reactions with 150 ng total RNA were synthesized using TaqMan reverse transcription reagents (Applied Biosystems) under the following conditions: 25 °C for 10 min, 48 °C for 30 min and 95 °C for 5 min. cDNA samples were diluted 1:5 and stored at −20 °C until use.

qPCR was run as 20 μl duplicate reactions with 6 ng cDNA/reaction on a 7500 Fast Real-Time PCR Systems (Applied Biosystems) according to their standard protocol. Primer and probe sequences are listed in Supplementary Table 1. A negative control (no template) reaction was performed for each primer pair. The Cq-threshold was set to 0.20 for both reference gene EF1aB and target genes. A melt curve analysis was also performed to ensure that a single product had been amplified. For the stimulated cells, fold change was calculated using the non-treated cells from each tissue and time point as a control[74]. Relative expression (zero hour samples) of *tlr3*, 22, 9, 21 and 8a1 were calculated using the $2^{-\Delta Cq}$ method[75] where ΔCq was calculated by subtracting the EF1aB Cq value from the target gene Cq value.

Western blot analysis of MHC II and secreted IgM. Supernatants and cell lysates from non-treated or CpG (2 μM) treated HK (n = 6) and spleen (n = 6) sorted IgM$^+$ B cells were harvested after 72 hours culture at 14 °C. Supernatants were up-concentrated by 0.5 ml centrifugal filter columns (Millipore) and then diluted 32-fold before denaturation in 2× LDS buffer at 70 °C for 10 minutes. Cells were sampled in 2× LDS buffer and diluted to 1.62×10^5 cells/sample before denaturation. All samples were run on precasted 4–12% gradient NuPAGE Novex Bis-Tris gels and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 1× MOPS buffer (Invitrogen) for 50 min at 200 V and 120 mA. MagicMark™ XP and SeeBlue Plus 2 pre-stained (both from Invitrogen), were loaded for molecular weight estimation. The proteins were blotted onto a polyvinylidene difluoride membrane, blocked with 5% BSA (Sigma) and incubated overnight with either anti-trout IgM Ab (1:200 dilution), anti-salmon MHCII Ab (1:1000)[56] or anti-actin rabbit Ab (1:600). The respective blots were incubated for 1 hour with goat anti-mouse-HRP Ab or goat anti-rabbit-HRP Ab (1:8000 dilution; Santa Cruz Biotechnology) in 5% BSA. The blots were developed using SuperSignal West Femto Trial Kit (Thermo) and a KODAK Image Station 4000 MM Digital Imaging System. For IgM, band intensities (arbitrary units, AU) were determined by subtracting the background noise from the visualized bands, while the relative expression of MHCII was calculated by:

$$MHCII(Relative\ expression) = \frac{MHCII(AU\ IgM) - Noise(AU)}{Actine\ (AU) - Noise\ (AU)}$$

Statistical analysis. RT-qPCR data were based on duplicate measurements of three to six individuals and were analyzed in GraphPad Prism 5.04 or SPSS version 24. Statistical evaluation were performed using two-tailed non-parametric Mann-Whitney U or Dunn's multiple comparison test following a significant Kruskal-Wallis test on the fold change and relative expression of the transcript data, respectively. The IgM secretion and MHCII protein expression data were analyzed by Mann-Whitney U. For all analyses, differences were considered significant at $p < 0.05$.

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Author Contributions

S.J. and H.L.T. processed the samples used in this study and analyzed the data. S.J. performed cell stimulation, MACS sorting and RT-qPCR while H.L.T. performed FACS and Western blot analysis. S.J., H.L.T., I.J. and J.B.J. prepared and reviewed the manuscript. O.S. and K.S. provided the anti-trout IgT and IgM Abs, respectively, and reviewed the manuscript. I.J. and J.B.J. designed and supervised the experiments, collaborated in obtaining funding and J.B.J. directed the project.

Additional Information

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Paper II

Paper III