Development of novel tools based on immune molecules for antibody detection in fish

Doctoral dissertation summary

Student name: Walissara Jirapongpairoj

Dissertation title: Development of novel tools based on immune molecules for antibody detection in fish

Chapter 1: General introduction

1. Aquaculture

Aquaculture is one of the fast growing food-producing sectors which involves in culture of freshwater and seawater animal such as fish, crustaceans, molluscs and aquatic plants. The food production has been shifted from hunter-gatherer activities to agriculture, aquatic food production since many millennia. Aquacultures of wild range of species and culture types have become faster growing which reached 167.2 million tonnes in 2014 [1]. Due to the geographical features, Japan was the fifth largest capture fishery producer in the world in 2007. Moreover, in 2007, Japan was the second major fishery commodities exporter with USD 13.2 billion [2]. However, in 2012, China ranked the highest aquaculture production in the world with 53.9 million tons which accounted for 59.7% of the world total. Indonesia (10.6%) and India (4.7%) ranked the second and third of aquaculture production respectively. Vietnam ranked fourth at 3.3 million tons (3.7%) and Philippines ranked fifth at 2.5 million tons (2.8%). Japan ranked 11th at 1.1 million tons (1.2%) [3]. In Japan, aquaculture production volume peaked at 1.4 million tons in 1988. However, due to the Great East Japan Earthquake in 2011, the production dropped to 0.9 million tons and it recovered to 1.1 million tons in 2012. By species, the production volume of yellowtail (*Seriola* spp.) has been the highest among fish production in Japan since 2006 which accounts for 56.0% of all production volume. In 2016, the production volume of yellowtail, red seabream (*Pagrus major*) and Pacific bluefin tuna (*Thunnus orientalis*) accounted for 56.0%, 27.0% and 5.4%, respectively (Fig. 1). Aquaculture production value in Japan had declined after peaking at 736.4 billion yen in 1991. However, in 2009 and 2012, the production value recovered to 471.8 and 484.2 billion yen, respectively. In 2012, aquaculture accounted for 34.0% of the total aquaculture production value. By species, yellowtail, ranked the highest production volume, accounted for 50.6% of the total aquaculture

value in 2016 (Fig. 2). Pacific bluefin tuna (18.6%) and red seabream (18.5%) ranked second and third position of aquaculture value in 2016, respectively (Fig. 2) [4].

2. Fish immune systems

The immune system is a biological mechanism that possesses the recognition of wide variety of pathogens such as bacteria, viruses and fungi. The immune system distinguishes the invading pathogens from the organism's own healthy tissue. It is also known as a network of circulating cells and molecules. The immune system possesses layered defense mechanisms by increasing specificity to fight against invading pathogens [5]. In simple terms, physical barriers are the first immune mechanism protecting host from pathogens. The innate immune system is activated and provides an immediate, but non-specific response to pathogens when pathogens breach the barrier. If invading pathogens successfully evade the immune response, the adaptive immune system is activated by innate immune response and response pathogens with more specific than the innate immune system [6, 7]. Fish immune system is composed of innate and adaptive immunity. Innate immune system is the first line of host defense mechanism that is characterized by a set of non-specific responses. Innate immune system plays vital roles in early recognition and regulation of a pro-inflammatory responses to invading pathogens [8]. While adaptive immune system is responsible for elimination of invading pathogens in the late phase of infection and involves in immunological memory generation. The adaptive immunity requires both cell-mediated and humoral immune response for its fully activation. [9-12].

2.1 Immune organs in fish

Fish contains most of the secondary lymphoid organs as present in mammal, except the lymph nodes and the bone marrow [13]. The mucosa-associated lymphoid tissues including the gut, skin and gills. These tissues non-specifically protect fish from both entering of pathogens and from leakage of water, solute and nutrients. Teleost skin is different from mammalian skin because it secretes mucus which is important in immune responses. Moreover, since aquatic environment has pathogenic organisms, skin of aquatic vertebrate is vital as a first line of defense mechanism against invading pathogens [14]. The secreted mucus from skin has evolved to have robust mechanisms such as traps and immobilizes pathogens and prevents the stable colonization of potential infectious microorganisms as well as invasion of metazoan

parasites [15-17]. Teleosts lack of organized gut-associated lymphoid tissue such as the Peyer's patches of mammals. Teleost gut contains leukocytes such as macrophages, lymphocytes and plasma cells. Moreover, leukocytes and plasma cells are also present in the skin and gills. Antigens can be taken up by these epithelia [13]. Because teleosts lack of bone marrow and lymph nodes, thymus is the primary lymphoid tissues. While, spleen, gut-associated lymphoid tissue (GALT) and kidney (anterior and posterior) are secondary lymphoid tissues.

The kidney contains both developing B lymphoid cells and antibody-secreting cells (ASC). In mammals, head/anterior kidney is known to play important roles in hemopoietic functions and is involved in phagocytosis, antigen processing and formation of IgM and immune memory through melanomacrophagic centres [18]. In fish, the primary site for hemopoiesis is an active immune part of kidney, head kidney or pronephros, which is formed by two Y arms and spreads underneath the gills. Teleost head kidney is an important organ which has key regulatory functions and immune-endocrine interactions and neuroimmunoendocrine connections. Moreover, it is the major site of antibody production. In contrast, posterior kidney possesses both renal and immune tissues [19]. Although it has been reported that B cells are present in the posterior kidney, it is still unclear whether it possesses functional roles as observed in the anterior kidney. Thus it has not yet known whether posterior kidney functions as a primary or secondary immune organ [20].

The spleen is considered to be the main secondary lymphoid organ which plays important roles in haematopoiesis, antigen degradation and antibody production processing [5]. Furthermore, because fish lacks lymph nodes, the spleen alone plays an essential role in antigen trapping. Spleen size of fish is widely used as a simple measurable immune parameter for determination of immune responses against pathogens [21]. Activation-induced cytidine deaminase (AID), enzyme for generation of immunoglobulin diversity, has been found in the spleen of teleost, suggesting that the spleen involves in promotion of adaptive humoral immunity [22].

In mammals, the thymus is important for producing T lymphocytes and is involved in stimulation of phagocytosis, allograft rejection and antibody production by B cells in mammal [23]. In teleost, the thymus is located near the gill cavity and is closely associated with the

pharyngeal epithelium. Teleost thymus has also been reported to involve in T cell production. The expression of T cell receptor alpha chain was detected in zebrafish thymus at 4 days post fertilization [24]. In sea bass (*Dicentrarchus labrax*), immunohistrochemical showed the detection of presumptive T cells in thymus 3 days later than the first lymphoblast [25]. Moreover, thymus has been reported to involve in sexual maturation in zebrafish [26].

2.2 Innate immunity

Innate immunity is a non-specific immune response, which is known as the first line of the host defense mechanism against invading pathogens [27]. It also plays a role in activation and regulation of adaptive immune system through activation of both immune cells (such as dendritic cells and macrophages) and immune molecules (such as cytokines and chemokines). Vertebrate innate immunity is divided into cellular and humoral immunity. The important roles of innate immune response to recognize pathogens at early stage of infection are mainly on complement system, phagocytosis and immune activation via pattern-recognition receptors (PRRs). Immune cells such as dendritic cells (DCs) and macrophages utilize PRRs to recognize microbial components known as a recognize pathogen association molecular patterns (PAMPs) [28, 29]. PRRs are classified into a transmembrane receptors and a proteins resided on intracellular compartment (cytoplasmic PRRs).

TLRs are type I transmembrane domain proteins which localize at the cellular or endosomal membranes. TLRs consist of three domains including a leucine-rich repeat (LRR) motif, which is an important domain for ligand recognition, a cytoplasmic signaling Toll/interleukin-1 (IL-1) receptor homology (TIR) domain, which is responsible for signaling transduction, and a transmembrane region with one α-helix [30, 31]. The LRR domains contain 19 to 25 tandem LRR motifs. Each LRR motif is 24 to 29 amino acid residues in length which XLXXLXLXX as well as X*XX*XXXXFXXLX; * is a conserved hydrophobic residue [28]. In vertebrate, TLRs are expressed on surface and intracellular compartment of various immune cells such as macrophages, DCs, B cells, T cells, and also non-immune cells such as fibroblasts and epithelial cells. To date, 11 TLRs including TLR1 to TLR11 have been identified and characterized in mammals. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are localized on the surface of immune cells, whereas TLR3, TLR7, TLR8 and TLR9 localize within the endosome.

Mammalian TLR1, TLR2, TLR4 and TLR6 are known to recognize bacterial components. The TIR domains of TLR1 and TLR6 are associated with the TIR domain of TLR2 whereas TLR4 mediates singling pathway via the TIR domain itself. TLR3, TLR7, TLR8 and TLR9 recognize nucleic acids. TLR5 recognizes flagellin of flagellated bacteria [29, 32]. However, some TLRs can recognize structurally unrelated ligands such as TLR2 and TLR4. Although TLR2 is known to recognize lipoprotein and peptidoglycan, it was also found to recognize bacterial LPS which is basically recognized by TLR4 [33]. It has been revealed that pathogenic LPS can be recognized by association of TLR4 with a PAMPs-binding molecule, MD-2. Moreover, MD-2 is also found to be important for TLR2 in response to Gram-negative and Gram-positive bacterial LPS [34].

TLRs are also present in many fish families such as cyprinid, salmonid, perciform, pleuronectiform and gadiform. To date, at least 17 TLRs have been identified in teleosts. Among TLRs, TLR14, TLR19, TLR20, TLR21, TLR22 and TLR23 are non-mammalian TLRs and TLR5S is a unique TLR5 in fish [35]. The interleukin-1/TLR family was firstly identified in rainbow trout (*Oncorhyncus mykiss*) [36]. Then, several TLRs have been identified in many teleost species including pufferfish (*Tetraodon nigroviridis*), common carp (*Cyprinus carpio*), orange-spotted grouper (*Epinephelus coioides*), catfish (*Ictalurus punctatus*) and yellow croaker (*Pseudosciaena crocea*) [37-41]. The genomic organizations of mammalian and teleost TLRs genes are not quite conserved. For example, phylogenetic tree analysis revealed that pufferfish TLR1 was grouped with other fish TLRs on a separated branch, excluded from mammalian and avian TLR1s. However, the TLR2 gene has one and two introns in human and murine, respectively, but pufferfish has ten introns [37]. TLR22, a novel member of the teleost TLRs family, has similar function to mammalian TLR3 which recognizes viral dsRNA [42]. Moreover, a recent study reported that not just dsRNA and DNA viruses but parasite (*Amyloodinium ocellatum*) also could induce the expression of TLR22 in yellowtail (*S. lalandi*). This indicates a possible role of fish TLR22 in responding to broad ranges of pathogens [43].

Vertebrate TLR signaling pathway requires recruitment of several adaptor molecules including Myeloid differentiation primary response 88 (MyD88), TIR domain-containing

adaptor inducing IFN-beta (TRIF) and tumor necrosis factor (TNF) receptor-associated factor (TRAF). MyD88 is a critical adaptor molecule in TLR signaling pathway except for TLR3. Stimulation of several adaptor molecules leads to the activation of TLR downstream signaling pathway via phosphorylation, ubiquitination, or protein-protein interactions to activate the transcription of pro-inflammatory cytokine and chemokine genes [28, 31, 44]. Additionally, NF-kB is also important for TLR signaling pathway and inflammatory cytokine genes expression. Upon ligand stimulation, interferon-regulatory factor (IRF)-5 stimulates IkB-like molecule, which is also critical for TLR signaling activation, and subsequently induces cytokine genes transcription by association with NF-kB p50 [45]. However, TLR3 and TLR4 activate the downstream signaling pathway without MyD88. TRIF adaptor molecule is a critical adaptor molecule in MyD88-independent pathway. TRIF initially interacts with receptor-interacting protein 1 (RIP1) and TRAF-family-member-associated NF-kB activator (TANK) binding kinase 1 (TBK1) resulting in the activation of NF-kB and type I interferon (IFN) signaling pathways [46, 47]. Furthermore, type I IFN signaling pathway can also be activated through TLR7 and TLR9 stimulation. TLR7 and TLR9 mediate type I IFN via MyD88-dependent pathway by sensing ssRNA and CpG DNA, respectively. During TLR7 and TLR ligands recognition, several molecules are recruited including MyD88, interleukin-1 receptor-associated kinase 4 (IRAK-4), TRAF and IRF-7 mediating to type I IFN signaling pathway activation [48].

As TLRs are expressed at either the surface of immune cells or endosome membranes, they seem not to be able to recognize intracellular pathogens invading cytosolic compartments and their components such as viral ssRNA, dsRNA and DNA. PAMPs can also be detected by the other cytoplasmic PRRs which are retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [49, 50]. RLRs are a family of DExD/H box RNA helicases that recognize viral RNA. The downstream signaling pathway of RLRs activates type I IFN gene transcription to control viral infection. To date, three RLRs have been identified in both mammals and teleosts including retinoic acid-inducible gene I (RIG-I), melanoma differentiation associated factor 5 (MDA5) and laboratory of genetics and physiology 2 and a homolog of mouse D11lgp1 (LGP2) which recognizes viral

RNA in the cytoplasm [51]. RIG-1 is composed of tandem caspase recruitment domain (CARD)-like regions at the N-terminus and C-terminal repressor domain (RD). The CARD domain is important for interacting with the other CARD-containing proteins, whereas RD plays crucial roles in binding to RNA. RIG-I is an inactivated monomer in resting cells. During viral infection, the conformation of CARD domain changes to promote its interaction to downstream signaling molecules [44]. The MDA5 also contains tandem CARD-like region and a DExD/H helicase domain. The RIG-I recognizes 5'-phosphorylated blunt ends of viral genomic dsRNA, whereas MDA5 binds internally to long dsRNA (>1,000 bp) with no end specificity [52, 53]. The interaction of CARD domain with the interferon promoter-stimulating factor 1 (IPS-1) activates the transcription of several transcriptional factors including IRF3, IRF7 and NF-kB. IRF-3 and IRF-7 control the expression of type I IFNs, whereas production of inflammatory cytokines are regulated by NF-kB [54].

In addition to TLRs and RLRs, cytokines and chemokines are known as important molecules in the vertebrate immune system. Cytokines and chemokines play crucial roles in both innate and adaptive immunity by inducing or inhibiting the activation, differentiation and/or proliferation of various immune cells [55, 56]. Upon pathogen infection, cytokines are secreted from various immune cells in response to pathogens infection. Expressions of cytokine genes have been broadly detected in many studies to study vertebrate immune responses and also to evaluate the vaccine efficacy [57, 58]. The activation of pro-inflammatory responses in innate immunity induced by PRRs regulates innate immune response and also play roles in activation, maturation and regulation of the adaptive immune response [59].

2.3 Adaptive immunity

The adaptive or acquired immune system is activated with immunological memory to recognize and cope with the encountered antigens. Adaptive immunity of jawed vertebrates is based on antigen-recognizing receptors known as B cell receptors (BCRs), T cell receptors (TCRs) as well as antigen-presenting molecules known as major histocompatibility complex (MHC), including MHC class I and II. Two types of lymphocytes play important in regulation of adaptive immune system including T cells and B cells. T cells are major components in cell-mediated (cellular) immunity, whereas B cells produce immunoglobulins (Igs) or antibodies

which are key components of humoral immunity [60]. Both T and B cells express antigen-specific receptor on their cell surface for binding to the distinct forms of antigen [61]. Teleost immune system contains almost all of the elements of the innate and adaptive immune system as presented in mammals.

In vertebrate, immature T cells are activated by interacting of TCRs with the antigenic peptides presented on MHC molecules expressed on the surface of antigen-presenting cells. TCRs including α and β chains are associated with the cluster differentiation 3 (CD3) molecules (γ, δ, ε and ζ chains) which bind specifically to peptides presented on MHC molecules. Moreover, TCRs are also connected to CD4/CD8 molecules, which stabilize the interaction of TCR-peptide-MHC complexes by binding to non-polymorphic regions of MHC molecules [62, 63]. The interaction of these molecules results in close proximity of their cytosolic domains and activation of downstream signaling pathway including activation of Src family protein kinase, recruitment of adaptor molecules (ζ-associated protein, 70 kDa (ZAP-70)) through immunoreceptor tyrosine-based activation motif (ITAM) as well as phosphorylation and/or dephosphorylation of phosphotyrosine residues in Src family kinases. ZAP-70 has also been identified and reported in teleosts that its phosphorylation sites which is important for T-cell antigen receptor signaling in mammalian were conserved [64]. These signaling pathways lead to increase of intracellular calcium (Ca^{2+}) flux and activation of transcriptional factor molecules such as nuclear factor κB (NF-κB). Furthermore, activation of T cells mediates the differentiation of T cell effector subsets [65]. T cells can be differentiated into two types: cytotoxic T cells (CD8+) and helper T cells (CD4+) [66]. The helper T cells provide signals such as cytokines for regulation of both T and B cell responses, whereas the cytotoxic T cells mainly play direct roles in elimination of pathogens.

In vertebrate, CD8+ T cells is a major circulating T cells which is important for eliminating and removing the cells harboring intracellular pathogens especially virus. Recognition of antigenic peptides by naïve CD8+ T cells via MHC class I and TCR molecules results in differentiation of CD8+ T cells to cytotoxic T lymphocytes (CTLs). CTLs play important roles in elimination of virus-infected cell by releasing of cytotoxins such as perforin and granzymes. The cytotoxins enter the target cells through plasma membrane leading to cell

apoptosis [67, 68]. To date, perforin genes have been identified in Japanese flounder (*Paralichthys olivaceus*) [69], channel catfish (*Ictalurus punctatus*) [70], rainbow trout (*Oncorhynchus mykiss*) [71] and crucian carp (*Carassius auratus langsdorfii*) [72]. Several studies have reported the effects of cytotoxic on T cells in teleost. mRNA expression of perforin is mainly detected in CD8+ lymphocytes of crucian carp and PHA, a mitogen that induces cytotoxic functions in mammals. PHA stimulation also showed expression of the three CTL effector genes (perforin, granzyme and IFN-γ) in CD8+ lymphocytes [73]. Furthermore, a rainbow trout macrophage cell line RTS-11 showed significant increase in mRNA expression of perforin and granzyme genes after VHSV infection [74]. Moreover, CTLs can eliminate the target cell by Fas ligand pathway which is independent of cytotoxic enzymes. TCR activation induces the expression of Fas ligand on the CTLs. Ligation of Fas ligand to Fas (CD95) on the target cell membrane leads to activation of caspases which induce apoptosis in the target cell [62, 75]. In mammals, Fas ligand can be expressed as membrane bound or soluble cytosolic forms. In teleosts, Fas ligand has been found expressing in soluble cytosolic form in channel catfish [76] and gilthead seabream (*Sparus aurata* L.) [77]. Furthermore, teleost Fas ligand has similar roles in activation of cell apoptotic [76, 78].

CD4+ T cells are known as T helper cells which can be differentiated into Th1, Th2 and Th17 cells. Th1 responses result in an activation of CTLs, whereas Th2 responses lead to B cell activation and antibody production. Th17 responses are characterized by the recruitment of neutrophils and important for autoimmune disease [79]. Cytokines are also involved in mediation of T cell differentiation. Signature cytokines of Th1 response including interferon (IFN)-γ and IL-12 are produced during Th1 development. IFN-γ is produced by transcriptional factor signal transducers and activators of transcription (STAT) 1, STAT4 and T-bet, whereas IL-12 is mainly produced by antigen presenting cells (APCs) such as dendritic cells and macrophages. These cytokines promote the development of cytotoxic responses, activate cell-mediated immunity against intracellular pathogen and interfere the Th2 driven immune responses. In contrast, IL-4, IL5, IL-13 and transcriptional factors STAT6 and GATA3 are involved in activation of Th2 immune response development [79]. Th2 cells regulate humoral immune response by activation of antibody production against extracellular pathogens. T cell

responses have also been well described in teleost. In Atlantic halibut (*Hippoglossus hippoglossus* L.), mRNA levels of Th1 cytokine genes were increased in anterior kidney leukocytes stimulated with imiquimod (a chemical that mimics the effects of viral RNA), suggesting a Th1 skewed environment [80]. IL-4 and IL-13 are strongly expressed by Th2 cells. IL-4 promotes Th2 development and also suppresses the development of Th1 and Th17 cells. Moreover, the transcriptional factor GATA3 is important for enhancing the transcription of the Th2 cytokine genes including IL-4 , IL-5 and IL-13 [81]. Recently, the Th2 immune responses have been studied in fish where a molecule having relative of both IL-4 and IL-13 is present, known as IL-4/13. A possible Th2 inflammatory process has been described in coho salmon (*Oncorhynchus kisutch*) [82]. Furthermore, several studies have produced recombinant IL-4/13 proteins for investigation of Th2 regulatory mechanisms in fish [83-85]. In carp (*Cyprinus carpio*), the recombinant IL-4/13B protein induced *in vitro* proliferation of IgM^+ B cells [83]. The Th17 cells are mainly involved in mucosal immune responses against pathogens by secreting pro-inflammatory cytokines IL-17 and IL-22 which subsequently stimulate intestinal and skin epithelia to secrete chemokine and also activate neutrophils. Five forms of IL-17, a hallmark cytokine of Th17 cells, and other cytokines such as IL-21, IL-23 and IL-26 have been identified in zebrafish [86]. Moreover, nuclear RAR-related orphan receptor gamma (RORc), a regulator of Th17 differentiation, is also conserved in zebrafish [87]. Furthermore, Th17 is known to be involved in mucosal immune response of zebrafish after bath-vaccination with live attenuated *Vibrio anguillarum* [88].

In vertebrate, antigens can also be recognized by B cells through B cell receptor (BCR). After recognition, B cells are activated, differentiated and secreted a soluble form of the BCR called as antibody or Ig. This immune mechanism is known as the adaptive humoral immune response which is mediated by specific antibodies-produced by plasma cells. Igs play important roles in recognition of antigen and activation of vertebrate adaptive immune response. The structure of both mammalian and teleosts Ig consists of two identical heavy chains (H) and two identical light chains (L) which are held together by disulfide bridge. Antigen-binding sites are located on the variable region of IgH and IgL chains [89]. Moreover, both IgH and IgL chains contain variable (V) and constant (C) domains which are responsible for antigen recognition

and mediation of signaling pathway, respectively [90]. IgL chains contain one C domain, whereas IgH chains contain either three or four C domains. Igs are categorized into specific isotypes based on the structure of constant region of heavy chain. IgH and IgL chains are encoded by separate genomic loci and their V and C domains are encoded by independent elements including the variable (V), diversity (D) and joining (J) gene segments for the V domain and individual constant (C) gene segments for the C domains. The V domain of IgL chains is encoded by V and J gene segment which is in the absence of D gene segment [91]. Igs are composed of fragment antigen-binding (Fab) and fragment crystallizable (Fc) regions. The Fab contains complete L chain in its entirely, paired with one V and C domains of IgH (Fig. 3). The Fc region mediates the effector function of Ig by binding to the Fc receptor (FcR) on effector cells or activating other immune mediators such as complement [89]. Fab region contains the sites responsible for binding to antigen. V domains are further divided into three hypervariable parts known as complementarity-determining regions (CDRs) and four framework regions (FRs) (Fig. 4). CDR1 and CDR2 are encoded by V gene alone, whereas CDR3 is encoded by the V-J or V-D-J rearrangement junction and thus this is the most diverse CDR [89]. Teleost IgM is mainly secreted by plasmablasts and plasma-like cells that reside in the head kidney [92].

One of the hallmarks of adaptive immunity is the variation of Igs and TCRs which allows them to recognize wide variety of antigens. Several antibody affinity maturation mechanisms are responsible for generation of this variability in mammals such as V(D)J recombination, somatic hypermutation (SHM), class switch recombination (CSR) and gene conversion (GC) [93]. The exon encoding Ig and TCR variable regions are assembled from germline V(D)J gene segments in developing B or T lymphocytes. The V(D)J recombination is a site-specific recombination process for generation of diverse antigen receptor repertoires. The recombination activating genes (RAGs) are required for the initiation of V(D)J recombination process [94]. To date, RAG genes have been identified and characterized in zebrafish [95], European sea bass (*D. labrax*) [96], haddock *Melanogrammus aeglefinus*, L. [97] and rainbow trout [98]. These studies indicated that RAG enzyme is required for the V(D)J recombination process in teleost as in mammals. SHM is a process in which point mutations

occur in the V-region of the heavy and light chains. This process is triggered when the Ig on the B cell surface engages antigen. The mutagenized antibodies will have a higher affinity for the antigen, and higher affinity antibodies-harboring cells proliferate and survive preferentially [99]. CSR is a process for alteration of constant region genes in the IgH locus to switch from expressing one class of Ig to another class by naïve B cells. The CSR occurs at the switch (S) region locating upstream of each of the C_H region [100]. GC is an antibody diversification mechanism that generates templated changes in the sequence of the V-region of Ig [101]. AID is known as an essential enzyme for initiation of SHM, CSR and GC. Although, teleost have been reported to lack of class switch recombination process, some studies reported the expression of AID and the presence of antibody SHM. Moreover, it has been revealed that zebrafish AID could catalyze the class switch recombination in AID-deficient mouse B cells indicating that the functional domains of AID enzyme required for CSR were all intact [102]. Therefore, the rate-limiting step for CSR was the evolution of appropriate DNA switch regions which might miss in fish Ig heavy chain locus [103].

2.3.1 Fish immunoglobulins

Cartilaginous fish (sharks and rays) are the oldest vertebrate species containing bona-fide immunoglobulin-producing B cells. Three types of Ig produced by cartilaginous fish B cells are IgW, IgM and IgNAR (immunoglobulin new antigen receptor) [104]. IgW was phylogenetically related to teleost IgD, whereas IgNAR is known as a shark-specific Ig that is composed of only disulfide-linked heavy chains. Shark B cells are morphologically similar to that of teleost fish and higher vertebrates. However, shark IgNAR and IgM are expressed by different B cell subsets and this species are devoid of B cells-expressing both B cell receptors [105]. It has been reported that teleost leukocytes are morphologically and functionally similar to mammalian macrophages, dendritic cells, neutrophils, monocytes, T cells and plasma cells. Although there are studies reporting cells having similar morphology and functions with eosinophils, mast cells and basophils, these cells are not certain in teleosts [106]. In teleost, development of B cells at early stages varies in different species. Several studies have been reported about development of B cells [93].

Teleost contains three Ig isotypes including IgM, IgD and IgT/IgZ. IgM is the main Ig isotype in teleosts that is found as a tetrameric form in serum. It can be expressed on the surface of B cells as BCRs or secreted as antibodies. IgM concentration in teleost serum has been found between 800 and 9000 μg/ml [107]. In teleost, IgM is secreted by plasmablasts and plasma-like cells. The majority of IgM-secreted cells are localized in the head kidney [108]. However, teleost lacks of Ig class-switch mechanism and they are known to have low affinity maturation for the IgM responses in comparison to mammalian species. Furthermore, teleost requires longer time for generating a significant antigen-specific response than mammals[108, 109]. IgM is involved in wide varieties of immune mechanisms such as mediation of agglutination for phagocytosis, cellular cytotoxicity and modulation of complement system [107]. To date, serum IgD has been reported in channel catfish, rainbow trout, Atlantic salmon and Japanese flounder [110-112]. Two variants of the secreted IgD at about 130 and 180 kDa were found in channel catfish serum. In Japanese flounder, IgD-positive B lymphocytes are mainly present in peripheral blood leukocytes (PBLs) [111]. Channel catfish has been reported to express three different types of B cells: single positive IgM^{+}/IgD^{-} B cells, double positive IgM^{+}/IgD^{+} B cells and IgD-only B cells [113]. Teleost IgT/IgZ is a recent teleost Ig isotype that was firstly identified in rainbow trout (as a IgT) [114] and in zebrafish (as a IgZ) [115]. To date, IgT/IgZ has been cloned and characterized in various fish species [116-118]. Expression analyses of teleost IgT/IgZ reported that it is expressed in both primary and secondary lymphoid organs. Moreover, at early developmental stages of IgT/IgZ, it can be expressed as early as 4 days post fertilization of teleost fish and its expression is more rapidly than that of IgM. In adult fish, IgM is the dominant isotype in serum, whereas IgT being more highly expressed in mucosal organs. These findings suggest that IgM plays vital role in protection of adult fish through systemic immune response, while IgT may be specialized in mucosal immunity [119].

3. Antibody detections and applications

Due to the high affinity and high binding specificity of antibodies, they have become one of the most effective tools for immunological studies and immunodiagnostic of numerous diseases [120]. Detections of antibody in the circulation or in tissues have become useful analytical tool for immunodiagnostic of diseases, therapeutics purposes and to study

invertebrate and vertebrate immune systems. Several immunological techniques are available for antibody detection such as immunosorbent assay, immunoprecipitation, immunoblotting and immunocytochemistry [121]. The immunoassays are based on the reaction of antibody-target antigen binding. Antibodies specific to a desired antigen are basically required for the immunoassays. Antibodies can be produced by immunization of animals such as rabbit, mouse, sheep and goat with desired antigens. High molecular weight compounds such as proteins can be used as antigens for immunization of animal to produce the specific antibodies [122]. However, in the absence of native proteins and if the amino acid sequence of desired antigens is known, antibody can be produced by immunization of animal with low molecular weight proteins such as synthetic peptide [123]. Synthetic peptides are widely used for antibody production since they are easily synthesized with high purity. Nevertheless, since low molecular weight compounds have low immunogenicity, it needs to be conjugated with carrier proteins such as bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) to increase its immunogenicity [123]. Antibodies produced by immunization of animal with synthetic coupled to a carrier protein are known as anti-peptide antibodies. Peptide antibody is one of powerful tools in experimental studies. The main advantages of using peptide antibody are that since antibody is raised against selected region, the epitope of antigen recognized by peptide antibody is well defined. Moreover, peptide antibody is easily produced against isoform or modified proteins [124]. However, since synthetic peptide lacks the normal three-dimensional or folding structure of the desired protein, it might not be able to recognize native forms of the protein [125]. On the other hand, serum collected from the animal after immunization with the desired antigens and booster infections without purification process is known as polyclonal antibody, whereas antibody produced by screening of the antigen-specific B cell (hybridoma) is known as monoclonal antibody. The major differences of polyclonal and monoclonal antibody are polyclonal antibody recognizes multiple epitopes on any one antigen, whereas monoclonal antibody recognizes only one epitope on an antigen. Therefore, monoclonal antibody has higher specificity and affinity to the desired antigen than polyclonal antibody [121]. In teleost, several studies have reported the detection antibody responses specific to the desired antigen to evaluate the vaccine efficacy [126-128]. To date, antibody

responses have been broadly detected in various fish species to study their specific immune responses by immunoassays. Moreover, both monoclonal and polyclonal antibodies specific to fish Ig have been widely developed under laboratory condition in various fish species. For example, antibodies specific to recombinant proteins of Japanese flounder IgM heavy chain constant regions were produced and antibody specificity to fish IgM was determined. The antibodies are useful for IgM detection in Japanese flounder by Western blot and they are also applicable for immunohistochemical analysis [129, 130]. Moreover, also the antibody specific to serum Ig of black rockfish (*Sebastes schlogeli*) was produced by commercial affinity column. The antibody was also applicable for fish IgM detection by immunoblotting [131].

Research objective

Detection of antibody has become a useful analytical tool in immunological studies and immunodiagnostic of various diseases. Detection of antibody responses by immunoassays requires the Ig-specific antibody. To date, IgM-specific antibodies have been available as commercial products and have been developed under laboratory condition in many fish species. However, there are many fish species from both fishery and aquaculture productions in Japan that have been threatened from numerous infectious diseases. Furthermore, their specific immune responses have been broadly studied by using Ig-specific antibodies. Development of IgM-specific antibody for each fish species would be expensive and time-consuming. Therefore, the detection tools are developed based on immune molecules for antibody detection in multiple fish species

References

[1] FAO, The state of world fisheries and aquaculture. Contributing to food security and nutrition for all, Rome, 2016, p. 200.

[2] FAO, Fishery and aquaculture country profiles. Japan. Country profile fact sheets, FAO fisheries and aquaculture department, Rome, 2009.

[3] UJNR, Annual report on aquaculture in Japan, 2015.

[4] e-Stat, Portal of official statistics of Japan. (https://www.e-stat.go.jp/en).

[5] P.R. Rauta, R. Nayak, S. Das, Immune system and immune responses in fish and their role in comparative immunity study: a model for higher organisms, Immunol. Lett. 148 (2012) 23-33.

[6] A. Bruce, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walters, Molecule biology of the cell (4th ed.), Garland Science, New york and London, 2002.

[7] G.W. Litman, J.P. Cannon, L.J. Dishaw, Reconstructing immune phylogeny: new perspectives, Nat. Rev. Immunol. 5 (2005) 866-879.

[8] R. Medzhitov, C.J. Janeway, Innate immunity, N. Engl. J. Med 343 (2000) 338-344.

[9] L.M. Zimmerman, L.A. Vogel, R.M. Bowden, Understanding the vertebrate immune system: insights from the reptilian perspective, J. Exp. Biol. 213 (2010) 661-671.

[10] R. Coico, G. Sunshine, E. Benjamini, Immunology, 5th ed., Wiley-Liss, Hoboken, New Jersey, 2003.

[11] S. Reyes-Cerpa, K. Maisey, F. Reyes-Lpez, D. Toro-Ascuy, A. Mara, M. Imarai, Fish cytokines and immune response, In: Turker et al (eds) New advances and contributions of fish biology. INTECH, Turkey, 2012.

[12] T. Boehm, Evolution of vertebrate immunity, Curr. Biol. 22 (2012) R722-R732.

[13] C.M. Press, O. Evensen, The morphology of the immune system in teleost fishes, Fish Shellfish Immunol. 9 (1999) 309-318.

[14] M.A. Esteban, An overview of the immunological defenses in fish skin, ISRN Immunol. 2012 (2012) 29.

[15] R.A. Cone, Barrier properties of mucus, Adv. Drug Deliv. Rev. 61 (2009) 75-85.

[16] L. Mayer, Mucosal immunity, Pediatrics 111 (2003) 1595-1600.

[17] Y. Nagashima, N. Kikuchi, K. Shimakura, K. Shiomi, Purification and characterization of an antibacterial protein in the skin secretion of rockfish *Sebastes schlegeli*, Comp. Biochem. Phys C 136 (2003) 63-71.

[18] J. Meseguer, A. Lopez-Ruiz, A. Garcia-Ayala, Reticulo-endothelial stroma of the head-kidney from the seawater teleost gilthead seabream (*Sparus aurata* L.): an ultrastructural and cytochemical study, Anal. Rec. 241 (1995) 303-309.

[19] A.G. Zapata, E.L. Cooper, The immune system: comparative histophysiology, John Wiley and Sons, Chichester, 1990.

[20] A.G. Zapata, M. Torroba, A. Vicente, A. Varas, R. Sacedon, E. Jimenez, The relevance of cell microenvironments for the appearance of lympho-haemopoietic tissues in primitive vertebrates, Histol. Histopathol. 10 (1995) 761-778.

[21] F. Lefebvre, B. Mounaix, G. Poizat, A.J. Crivelli, Impacts of the swim bladder nematode *Anguillicola crassus* on *Anguilla anguilla*: variations in liver and spleen masses, J. Fish Biol. 64 (2004) 435-447.

[22] H.R. Neely, M.F. Flajnik, Emergence and evolution of secondary lymphoid organs, Annu. Rev. Cell Dev. Biol. 32 (2016) 693-711.

[23] T.J. Bowden, P. Cook, J.H. Rombout, Development and function of the thymus in teleosts, Fish Shellfish Immunol. 19 (2005) 413-427.

[24] N. Danilova, V.S. Hohman, F. Sacher, T. Ota, C.E. Willett, L.A. Steiner, T cells and the thymus in developing zebrafish, Dev. Comp. Immunol. 28 (2004) 755-767.

[25] G. Scapigliati, N. Romano, F. Buonocore, S. Picchietti, M.R. Baldassini, D. Prugnoli, The immune system of sea bass, *Dicentrarchus labrax*, reared in aquaculture, Dev. Comp. Immunol. 26 (2002) 151-160.

[26] S.H. Lam, H.L. Chua, Z. Gong, Z. Wen, T.J. Lam, Y.M. Sin, Morphologic transformation of the thymus in developing zebrafish, Dev. Dyn. 225 (2002) 87-94.

[27] A. Iwasaki, R. Medzhitov, Control of adaptive immunity by the innate immune system, Nat. Immunol. 16 (2015) 343-353.

[28] S. Akira, S. Uematsu, O. Takeuchi, Pathogen recognition and innate immunity, Cell 124 (2006) 783-801.

[29] T.H. Mogensen, Pathogen recognition and inflammatory signaling in innate immune defenses, Clin. Microbiol. Rev. 22 (2009) 240-273.

[30] L.A. O'Neill, A.G. Bowie, The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling, Nat. Rev. Immunol. 7 (2007) 353-364.

[31] L.Y. Zhu, L. Nie, G. Zhu, L.X. Xiang, J.Z. Shao, Advances in research of fish immune-relevant genes: A comparative overview of innate and adaptive immunity in teleosts, Dev. Comp. Immunol. 39 (2013) 39-62.

[32] S. Janssens, R. Beyaert, Role of Toll-like receptor in pathgen recognition, Clin. Microbiol. Rev. 16 (2003) 637-646.

[33] R. Barbalat, L. Lau, R.M. Locksley, G.M. Barton, Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands, Nat. Immunol. 10 (2009) 1200-1207.

[34] R. Dziarski, D. Gupta, Role of MD-2 in TLR2- and TLR4-mediated recognition of Gram-negative and Gram-positive bacteria and activaton of chemokine genes, J. Endotoxin Res. 6 (2000) 401-405.

[35] Y. Palti, Toll-like receptors in bony fish: from genomics to function, Dev. Comp. Immunol. 35 (2011) 1263-1272.

[36] A. Sangrador-Vegas, S.A. Martin, P.G. O'Dea, T.J. Smith, Cloning and characterization of the rainbow trout (*Oncorhynchus mykiss*) type II interleukin-1 receptor cDNA, Eur. J. Biochem. 267 (2000) 7031-7037.

[37] X.Y. Wu, L.X. Xiang, L. Huang, Y. Jin, J.Z. Shao, Characterization, expression and evolution analysis of Toll-like receptor 1 gene in pufferfish (*Tetraodon nigroviridis*), Int. J. Immunogenet. 35 (2008) 215-225.

[38] C. Yang, J. Su, Molecular identification and expression analysis of Toll-like receptor 3 in common carp *Cyprinus carpio*, J. Fish Biol. 76 (2010) 1926-1939.

[39] Y.C. Wei, T.S. Pan, M.X. Chang, B. Huang, Z. Xu, T.R. Luo, P. Nie, Cloning and expression of Toll-like receptors 1 and 2 from a teleost fish, the orange-spotted grouper *Epinephelus coioides*, Vet. Immunol. Immunopathol. 141 (2011) 173-182.

[40] P. Baoprasertkul, P. Xu, E. Peatman, H. Kucuktas, Z. Liu, Divergent Toll-like receptors in catfish (*Ictalurus punctatus*): TLR5S, TLR20, TLR21, Fish Shellfish Immunol. 23 (2007) 1218-1230.

[41] X. Xiao, Q. Qin, X. Chen, Molecular characterization of a Toll-like receptor 22 homologue in large yellow croaker (*Pseudosciaena crocea*) and promoter activity analysis of its 5′-flanking sequence, Fish Shellfish Immunol. 30 (2011) 224-233.

[42] A. Matsuo, H. Oshiumi, T. Tsujita, H. Mitani, H. Kasai, M. Yoshimizu, M. Matsumoto, T. Seya, Teleost TLR22 recognizes RNA duplex to induce IFN and protect cells from birnaviruses, J. Immunol. 181 (2008) 3473-3485.

[43] M. Reyes-Becerril, F. Ascencio-Valle, E. Alamillo, I. Hirono, H. Kondo, W. Jirapongpairoj, C. Angulo, Molecular cloning and comparative responses of Toll-like receptor 22 following ligands stimulation and parasitic infection in yellowtail (*Seriola lalandi*), Fish Shellfish Immunol. 46 (2015) 323-333.

[44] T. Kawai, S. Akira, The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors, Nat. Immunol. 11 (2010) 373-384.

[45] M. Yamamoto, S. Yamazaki, S. Uematsu, S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Kuwata, O. Takeuchi, K. Takeshige, T. Saitoh, S. Yamaoka, N. Yamamoto, S. Yamamoto, T. Muta, K. Takeda, S. Akira, Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaBzeta, Nature 430 (2004) 218-222.

[46] K. Honda, H. Yanai, H. Negishi, M. Asagiri, M. Sato, T. Mizutani, N. Shimada, Y. Ohba, A. Takaoka, N. Yoshida, T. Taniguchi, IRF-7 is the master regulator of type-I interferon-dependent immune responses, Nature 434 (2005) 772-777.

[47] H. Hacker, V. Redecke, B. Blagoev, I. Kratchmarova, L.C. Hsu, G.G. Wang, M.P. Kamps, E. Raz, H. Wagner, G. Hacker, M. Mann, M. Karin, Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6, Nature 439 (2006) 204-207.

[48] K. Honda, H. Yanai, T. Mizutani, H. Negishi, N. Shimada, N. Suzuki, Y. Ohba, A. Takaoka, W.C. Yeh, T. Taniguchi, Role of a transductional-transcriptional processor

complex involving MyD88 and IRF-7 in Toll-like receptor signaling, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 15416-15421.

[49] T.D. Kanneganti, M. Lamkanfi, G. Nunez, Intracellular NOD-like receptors in host defense and disease, Immunity 27 (2007) 549-559.

[50] M. Yoneyama, M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, T. Fujita, The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses, Nat. Immunol. 5 (2004) 730-737.

[51] Y.M. Loo, M. Gale, Immune Signaling by RIG-I-like Receptors, Immunity 34 (2011) 680-692.

[52] M. Yoneyama, T. Fujita, Structural mechanism of RNA recognition by the RIG-I-like receptors, Immunity 29 (2008) 178-81.

[53] J.P. Ting, R.C. Lovering, E.S. Alnemri, J. Bertin, J.M. Boss, B.K. Davis, R.A. Flavell, S.E. Girardin, A. Godzik, J.A. Harton, H.M. Hoffman, J.P. Hugot, N. Inohara, A. Mackenzie, L.J. Maltais, G. Nunez, Y. Ogura, L.A. Otten, D. Philpott, J.C. Reed, W. Reith, S. Schreiber, V. Steimle, P.A. Ward, The NLR gene family: a standard nomenclature, Immunity 28 (2008) 285-287.

[54] T. Kawai, K. Takahashi, S. Sato, C. Coban, H. Kumar, H. Kato, K.J. Ishii, O. Takeuchi, S. Akira, IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction, Nat. Immunol. 6 (2005) 981-988.

[55] T. Wang, C.J. Secombes, The cytokine networks of adaptive immunity in fish, Fish Shellfish Immunol. 35 (2013) 1703-1718.

[56] R. Savan, M. Sakai, Genomics of fish cytokines, Comp. Biochem. Physiol. Part D Genomics Proteomics (2006) 89-101.

[57] M. Arnemo, A. Kavaliauskis, T. Gjøen, Effects of TLR agonists and viral infection on cytokine and TLR expression in Atlantic salmon (*Salmo salar*), Devel. Comp. Immunol. 46 (2014) 139-145.

[58] Y. Sun, M. Zhang, C.S. Liu, R. Qiu, L. Sun, A divalent DNA vaccine based on Sia10 and OmpU induces cross protection against *Streptococcus iniae* and *Vibrio anguillarum* in Japanese flounder, Fish Shellfish Immunol. 32 (2012) 1216-1222.

[59] A. Iwasaki, R. Medzhitov, Toll-like receptor control of the adaptive immune responses, Nat. Immunol. 5 (2004) 987-995.

[60] S. Mashoof, M.F. Criscitiello, Fish immunoglobulins, Biology (Basel) 5 (2016) 45-68.

[61] J.O. Sunyer, Fishing for mammalian paradigms in the teleost immune system, Nat. Immunol. 14 (2013) 320-326.

[62] F.A. Bonilla, H.C. Oettgen, Adaptive immunity, J. Allergy Clin. Immunol. 125(2, Supplement 2) (2010) S33-S40.

[63] M.L. Dustin, The cellular context of T cell signaling, Immunity 30 (2009) 482-492.

[64] A.C. Overgard, A.H. Nerland, S. Patel, Cloning, characterization, and expression pattern of Atlantic halibut (*Hippoglossus hippoglossus* L.) CD4-2, Lck, and ZAP-70, Fish Shellfish Immunol. 29 (2010) 987-997.

[65] J.E. Smith-Garvin, G.A. Koretzky, M.S. Jordan, T cell activation, Annu. Rev. Immunol. 27 (2009) 591-619.

[66] N.D. Pennock, J.T. White, E.W. Cross, E.E. Cheney, B.A. Tamburini, R.M. Kedl, T cell responses: naive to memory and everything in between, Adv. Physiol. Educ. 37 (2013) 273-283.

[67] R.K. Bakshi, M.A. Cox, A.J. Zajac, Cytotoxic T lymphocytes, in: I.R. Mackay, N.R. Rose, B. Diamond, A. Davidson, (Eds.), Encyclopedia of medical immunology, Springer, New York, 2014.

[68] S.S. Metkar, B. Wang, M. Aguilar-Santelises, S.M. Raja, L.U. Hansen, E. Podack, J.A. Trapani, C. Froelich, Cytotoxic cell granule-mediated apoptosis: perforin delivers granzyme B-serglycin complexes into target cells without plasma membrane pore formation, Immunity 16 (2002) 417-428.

[69] J.Y. Hwang, T. Ohira, I. Hirono, T. Aoki, A pore-forming protein, perforin, from a non-mammalian organism, Japanese flounder, *Paralichthys olivaceus*, Immunogenetics 56 (2004) 360-367.

[70] K. Praveen, J.H. Leady III, D.L. Evans, L. Jaso-Friedmann, Nonspecific cytotoxic cells of teleosts are armed with multiple granzymes and other components of the granule exocytosis pathway, Mol. Immunol. 43 (2006) 1152-1162.

[71] S. Athanasopoulou, D. Marioli, A. Mikrou, A.D. Papanastasiou, I.K. Zarkadis, Cloning and characterization of the trout perforin, Fish Shellfish Immunol. 26 (2009) 908-912.

[72] H. Toda, K. Araki, T. Moritomo, T. Nakanishi, Perforin-dependent cytotoxic mechanism in killing by CD8 positive T cells in ginbuna crucian carp, *Carassius auratus langsdorfii*, Dev. Comp. Immunol. 35 (2011) 88-93.

[73] F. Takizawa, J.M. Dijkstra, P. Kotterba, T. Korytář, H. Kock, B. Köllner, B. Jaureguiberry, T. Nakanishi, U. Fischer, The expression of CD8α discriminates distinct T cell subsets in teleost fish, Dev. Comp. Immunol. 35 (2011) 752-763.

[74] M.C. Ordas, A. Cuesta, L. Mercado, N.C. Bols, C. Tafalla, Viral hemorrhagic septicaemia virus (VHSV) up-regulates the cytotoxic activity and the perforin/granzyme pathway in the rainbow trout RTS11 cell line, Fish Shellfish Immunol. 31 (2011) 252-259.

[75] C.A. Jr. Janeway, P. Travers, M. Walport, Immunobiology: The Immune System in Health and Disease. 5th edition, Chapter 3, Antigen recognition by B-cell and T-cell receptors, Garland Science, New York, 2001.

[76] H. Kaur, L. Jaso-Friedmann, D.L. Evans, Single base oligodeoxyguanosin upregulates Fas ligand release by nonspecific cytotoxic cells, Dev. Comp. Immunol. 28 (2004) 571-579.

[77] A. Cuesta, M.A. Esteban, J. Meseguer, Identification of a FasL-like molecule in leucocytes of the teleost fish gilthead seabream (*Sparus aurata* L.), Dev. Comp. Immunol. 27 (2003) 21-27.

[78] P.M. Eimon, E. Kratz, E. Varfolomeev, S.G. Hymowitz, H. Stern, J. Zha, A. Ashkenazi, Delineation of the cell-extrinsic apoptosis pathway in the zebrafish, Cell Death Differ. 13 (2006) 1619-1630.

[79] U. Fischer, E.O. Koppang, T. Nakanishi, Teleost T and NK cell immunity, Fish Shellfish Immunol. 35 (2013) 197-206.

[80] A.C. Overgard, I. Nepstad, A.H. Nerland, S. Patel, Characterisation and expression analysis of the Atlantic halibut (*Hippoglossus hippoglossus* L.) cytokines: IL-1b, IL-6, IL-11, IL-12b and IFNc, Mol. Biol. Rep. 39 (2012) 2201-2213.

[81] K.M. Ansel, I. Djuretic, B. Tanasa, A. Rao, Regulation of Th2 differentiation and IL4 locus accessibility, Annu. Rev. Immunol. 24 (2006) 607-656.

[82] L.M. Braden, B.F. Koop, S.R. Jones, Signatures of resistance to Lepeophtheirus salmonis include a TH2-type response at the louse-salmon interface, Dev. Comp. Immunol. 48 (2015) 178-191.

[83] T. Yamaguchi, S. Mityata, F. Katakura, T. Nagasawa, Y. Shibasaki, T. Yabu, U. Fischer, C. Nakayasu, T. Nakanishi, T. Moritomo, Recombinant carp IL-4/13B stimulates in vitro proliferation of carp IgM(+) B cells, Fish Shellfish Immunol. 49 (2016) 225-229.

[84] T. Wang, P. Johansson, B. Abos, A. Holt, C. Tafalla, Y. Jiang, A. Wang, Q. Xu, Z. Qi, W. Huang, M.M. Costa, P. Diaz-Rosales, J.W. Holland, C.J. Secombes, First in-depth analysis of the novel Th2-type cytokines in salmonid fish reveals distinct patterns of expression and modulation but overlapping bioactivities, Oncotarget 7 (2016) 10917-10946.

[85] L.Y. Zhu, P.P. Pan, W. Fang, J.Z. Shao, L.X. Xiang, Essential role of IL-4 and IL-4Rα interaction in adaptive immunity of zebrafish: insight into the origin of Th2-like regulatory mechanism in ancient vertebrates, J. Immunol. 188 (2012) 5571-5584.

[86] I. Gunimaladevi, R. Savan, M. Sakai, Identification, cloning and characterization of interleukin-17 and its family from zebrafish, Fish Shellfish Immunol. 21 (2006) 393-403.

[87] M.V. Flores, C. Hall, A. Jury, K. Crosier, P. Crosier, The zebrafish retinoid-related orphan receptor (ror) gene family, Gene Expr. Patterns 7 (2007) 535-543.

[88] H. Zhang, C. Fei, H. Wu, M. Yang, Q. Liu, Q. Wang, Y. Zhang, Transcriptome profiling reveals Th17-like immune responses induced in zebrafish bath-vaccinated with live attenuated *Vibrio anguillarum*, PLoS ONE 8 (2013) e73871.

[89] H.W. Schroeder, L. Cavacini, Structure and function of immunoglobulins, J. Allergy Clin. Immunol. 125 (2010) S41-S52.

[90] A. Janda, A. Bowen, N.S. Greenspan, A. Casaadevall, Ig constant region effects on variable region structure and function, Front. Microbiol. 7 (2016) 22.

[91] Y.A. Zhang, I. Salinas, J.O. Sunyer, Recent findings of the structure and function of teleost IgT, Fish Shellfish Immunol. 31 (2011) 627-634.

[92] J. Ye, I. Kaattari, S. Kaattari, Plasmablasts and plasma cells: reconsidering teleost immune system organization, Dev. Comp. Immunol. 35 (2011) 1273-1281.

[93] D. Parra, F. Takizawa, J.O. Sunyer, Evolution of B cell immunity, Annu. Rev. Anim. Biosci. 1 (2013) 65-97.

[94] C.H. Bassing, W. Swat, F.W. Alt, The mechanism and regulation of chromosomal V(D)J recombination, Cell 109 (2002) S45-S55.

[95] C.E. Willett, J.J. Cherry, L.A. Steiner, Characterization and expression of the recombination activating genes (rag1 and rag2) of zebrafish, Immunogenetics 45 (1997) 394-404.

[96] I. Boschi, E. Randelli, F. Buonocore, D. Casani, C. Bernini, A.M. Fausto, G. Scapigliati, Transcription of T cell-related genes in teleost fish, and the European sea bass (*Dicentratchus labrax*) as a model, Fish Shellfish Immunol. 31 (2011) 655-662.

[97] Y. Corripio-Miyar, S. Bird, J.W. Treasurer, C.J. Secombes, RAG-1 and IgM genes, markers for early development of the immune system in the gadoid haddock, *Melanogrammus aeglefinus*, L., Fish Shellfish Immunol. 23 (2007) 71-85.

[98] J.D. Hansen, Characterization of rainbow trout terminal deoxynucleotidyl transferase structure and expression. TdT and RAG1 co-expression define the trout primary lymphoid tissues, Immunogenetics 46 (1997) 367-375.

[99] A. Martin, R. Chahwan, J.Y. Parsa, M.D. Scharff, Chapter 20-Somatic hypermutation: The molecular mechanisms underlying the production of effective high-affinity antibodies, Molecular Biology of B cells (second edition) 2015, 363-388.

[100] J. Stavnezer, J.E.J. Guikema, C.E. Schrader, Mechanism and regulation of class switch recombination, Annu. Rev. Immunol. 26 (2008) 261-292.

[101] E.S. Tang, A. Martin, Immunoglobulin gene conversion: Synthesizing antibody diversification and DNA repair, DNA Repair 6 (2007) 1557-1571.

[102] K. Wakae, B.G. Magor, H. Saunders, H. Nagaoka, A. Kawamura, K. Kinoshita, T. Honjo, M. Muramatsu, Evolution of class switch recombination function in fish activation-induced cytidine deaminase, AID, Int. Immunol. 18 (2006) 41-47.

[103] H.L.V. Epps, Evolution of class switching, J. Exp. Med. 202 (2005) 724.

[104] M.F. Flajnik, M. Kasahara, Origin and evolution of the adaptive immune system: genetic events and selective pressures, Nat. Rev. Genet. 11 (2010) 47-59.

[105] M.F. Flajnik, Comparative analyses of immunoglobulin genes: surprises and portents, Nat. Rev. Immunol. 2 (2002) 688-698.

[106] O.B. Reite, O. Evensen, Inflammatory cells of teleostean fish: a review focusing on mast cells/eosinophilic granule cells and rodlet cells, Fish Shellfish Immunol. 20 (2006) 192-208.

[107] J. Ye, I.M. Kaattari, C. Ma, S. Kaattari, The teleost humoral immune response, Fish Shellfish Immunol. 35 (2013) 1719-1728.

[108] P. Zwollo, S. Cole, E. Bromage, S. Kaattari, B cell heterogeneity in the teleost kidney: evidence for a maturation gradiet from anterioe to posterior kidney, J. Immunol. 174 (2005) 6609-6616.

[109] J.O. Sunyer, Evolutionary and functional relationships of B cells from fish and mammals: insights into their novel roles in phagocytosis and presentation of particulate antigen, Infect. Disord. Drug Targets 12 (2012) 200-212.

[110] D. Parra, T. Korytář, F. Takizawa, J.O. Sunyer, B cells and their role in the teleost gut, Dev. Comp. Immunol. 64 (2016) 150-166.

[111] I. Hirono, B.H. Nam, J. Enomoto, K. Uchino, T. Aoki, Cloning and characterisation of a cDNA encoding Japanese flounder *Paralichthys olivaceus* IgD, Fish Shellfish Immunol. 15 (2003) 63-70.

[112] I. Hordvik, Immunoglobulin isotypes in Atlantic salmon, *Salmo salar*, Biomolecules 5 (2015) 166-177.

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

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

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

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

[117] R. Savan, A. Aman, M. Nakao, H. Watanuki, M. Sakai, Discovery of a novel immunoglobulin heavy chain gene chimera from common carp (*Cyprinus carpio* L.), Immunogenetics 57 (2005) 458-463.

[118] F.S. Xiao, Y.P. Wang, W. Yan, M.X. Chang, W.J. Yao, Q.Q. Xu, Ig heavy chain genes and their locus in grass carp *Ctenopharyngodon idella*, Fish Shellfish Immunol. 29 (2010) 594-599.

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

[120] G. Buyukkoroglu, B. Senel, Chapter 16 - Engineering Monoclonal Antibodies: Production and Applications, in: V. Azevedo (Eds.), Omics Technologies and Bio-Engineering, Academic Press 2018, 353-389.

[121] F.P. Nijkamp, M.J. Parnham, Principles of immunopharmacology, illustrated 3th edition, Springer Science & Business Media 2005.

[122] G. Kohler, C. Milstein, Continuous cultures of fused cells secreting antibody of predefined specificity, Nature 256 (1975) 495-497.

[123] N.H. Trier, P.R. Hansen, G. Houen, Production and characterization of peptide antibodies, Methods 56 (2012) 136-144.

[124] H.L. Niman, R.A. Houghten, L.E. Walker, R.A. Reisfeld, I.A. Wilson, J.M. Hogle, R.A. Lerner, Generation of protein-reactive antibodies by short peptides is an event of high frequency: implications for the structural basis of immune recognition, Proc. Natl. Acad. Sci. U.S.A. 80 (1983) 4949-4953.

[125] J.A. Ramos-Vara, Technical aspects of immunohistrochemistry, Vet. Pathol. 42 (2005) 405-426.

[126] G. Kato, K. Yamashita, H. Kondo, I. Hirono, Protective efficacy and immune responses induced by a DNA vaccine encoding codon-optimized PPA1 against *Photobacterium damselae* subsp. *piscicida* in Japanese flounder, Vaccine 33 (2015) 1040-1045.

[127] C.F. Lin, H.K. Jiang, N.C. Chen, T.Y. Wang, T.Y. Chen, Novel subunit vaccine with linear array epitope protect giant grouper against nervous necrosis virus infection, Fish Shellfish Immunol. 74 (2018) 551-558.

[128] P.Y. Ho, Y.C. Chen, S. Maekawa, H.H. Hu, A.W. Tsai, Y.F. Chan, P.C. Wang, S.C. Chen, Efficacy of recombinant protein vaccines for protection against *Nocardia seriolae* infection in the largemouth bass *Micropterus salmoides*, Fish Shellfish Immunol. 78 (2018) 35-41.

[129] M.D. Santos, T. Saito-Taki, T. Takano, H. Kondo, I. Hirono, T. Aoki, Characterization of polyclonal antibodies against Japanese flounder IgM derived from recombinant IgM constant region proteins, Fish Shellfish Immunol. 27 (2009) 374-378.

[130] Q. Li, W. Zhan, J. Xing, X. Sheng, Production, characterisation and applicability of monoclonal antibodies to immunoglobulin of Japanese flounder (*Paralichthys olivaceus*), Fish Shellfish Immunol. 23 (2007) 982-990.

[131] G. Shin, H. Lee, K.J. Palaksha, Y. Kim, E. Lee, Y. Shin, E. Lee, K. Park, T. Jung, Production of monoclonal antibodies against serum immunoglobulins of black rockfish (*Sebastes schlegeli Higendorf*), J. Vet. Sci. 7 (2006) 293-295.

Chapter 2: Development and evaluation of fish IgM heavy chain polyclonal antisera for fish IgM detection

Materials and methods

Two synthetic peptides (IgH-1 and IgH-2) were designed based on the conserved sequence of fish IgH including Japanese flounder *Paralichthys olivaceus*, seabream *Pagrus major*, yellowtail *Seriola quinqueradiata*, carp *Cyprinus carpio* L., rainbow trout *Oncorhynchus mykiss*, hybrid sturgeon *Huso huso* x *Acipenser ruthenus* and banded houndshark *Triakis scyllium*. The synthetic peptides were used for immunization of rabbit to produce peptide polyclonal antisera (anti-IgH-1 and anti-IgH-2). The specificity of peptide antisera was determined by Western blot and enzyme-linked immunosorbent assay (ELISA).

Results and discussion

Anti-IgH-1 antiserum showed reactivity to IgMs of Japanese flounder, seabream, yellowtail, carp, rainbow trout and hybrid sturgeon under reducing and non-reducing condition of Western blot. Anti-IgH-2 antiserum reacted to IgMs of seabream, yellowtail and rainbow trout under reducing condition. However, under non-reducing condition, anti-IgH-2 antiserum solely reacted to IgM of rainbow trout. Attempts to use the antisera to measure fish antibody titer by ELISA were unsuccessful.

Conclusion

These results demonstrate that anti-fish IgH peptide polyclonal antisera are potentially applicable tool for detecting immunoglobulins in various fish species by Western blotting.

Chapter 3: Development and evaluation of fish IgM light chain polyclonal antisera for fish IgM detection

Materials and methods

Recombinant proteins of three isotypes of Japanese flounder IgL (L1, L2 and L3) were produced and used for immunization of rabbit to produce specific antiserum. The cross-reactivities of three antisera to IgLs of seven fish species including Japanese flounder *Paralichthys olivaceus*, seabream *Pagrus major*, yellowtail *Seriola quinqueradiata*, carp *Cyprinus carpio* L., rainbow trout *Oncorhynchus mykiss*, hybrid sturgeon *Huso huso* x *Acipenser ruthenus* and banded houndshark *Triakis scyllium*. were determined by Western blot (under reducing and non-reducing conditions) and ELISA.

Results and discussion

Anti-JF IgL1 and L2 antisera could react to Japanese flounder, seabream and yellowtail IgLs under reducing condition. However, fish IgLs were not clearly detected by anti-JF IgL3 antiserum under reducing condition. Unexpectedly, measurements of fish antibody titer by ELISA using the polyclonal antisera were unsuccessful. These results demonstrate the cross-reactivities of anti-JF IgL antisera to fish IgLs.

Conclusion

Although anti-JF IgL antisera did not have reactivity to fish IgM determined by ELISA, they showed cross-reactivities to red seabream and yellowtail IgLs under reducing condition by Western blot. These antisera are useful for detection of IgMs in Japanese flounder, red seabream and yellowtail which are economically important fish species in Japan.

Chapter 4: Development of fish IgM detection tools based on Japanese flounder Fc receptor-like proteins

Materials and methods

FcR-like proteins 1, -2, -3 and -4 were identified and characterized in Japanese flounder. JF-FcR-like protein mRNAs were examined in each tissue of healthy fish by reverse-transcription PCR (RT-PCR). The mRNA levels of JF-FcR-like protein were also detected after formalin-killed cells (FKCs) of *Edwardsiella tarda* and *Streptococcus iniae* stimulations by quantitative PCR (qPCR). Recombinant proteins of FcR were produced for determination of their binding specificity to fish IgMs and bacterial components by enzyme-linked immunosorbent assay (ELISA).

Results and discussion

Their open reading frames of JF-FcR-like protein 1, 2, 3 and 4 encoded 358, 255, 519 and 441 amino acid residues, respectively. JF-FcR-like protein mRNAs were mainly detected in kidney and spleen of healthy fish. Injection of formalin-killed cells (FKCs) of *E. tarda* significantly increased the spleen mRNA levels of JF-FcR-like protein 1 but not the other JF-FcR-like proteins. Injection of FKC of *S. iniae* did not significantly affect any of the JF-FcR-like protein mRNAs. ELISA reveals that recombinant JF-FcR-like proteins have different binding affinities for the serum proteins. Interestingly, recombinant JF-FcR-like proteins also bind to formalin-killed cells of *E. tarda* and *S. iniae*. Therefore, attempts to use the recombinant protein as tool for antigen-specific antibody detection were unsuccessful.

Conclusion

These results indicate that all FcR-like proteins are involved in regulation of immune response through binding not only to the Japanese flounder IgM molecules but also to pathogenic bacteria.

Chapter 5: Development of single chain variable fragment antibodies from phage display library for fish IgM detection

Materials and methods

The phage library was generated from variable regions of rabbit spleen B cells-immunized with tuna IgM. Fish IgM-specific phage library was enriched by two rounds of bio-panning with purified yellowtail IgM and further two rounds of bio-panning with purified red seabream IgM. Then the single clonal phage library was isolated and their nucleotide and amino acid sequences were analyzed. The recombinant single chain variable fragment (scFv) proteins were produced for determination of their binding specificity to native form and denatured form of fish IgM by enzyme-linked immunosorbent assay (ELISA) and Western blot, respectively.

Results and discussion

The enriched phages library with yellowtail and red seabream IgMs demonstrated increase in binding specificity to the purified tuna, yellowtail, red seabream and rock bream IgMs compared with an unpanned library. The recombinant proteins had binding properties to fish IgMs determined by ELISA. Western blot revealed that a clone of recombinant scFv protein had binding specificity to both native and denatured forms of fish IgM. However, the rest of recombinant proteins bound to only native forms of fish IgM.

Conclusion

These results indicate that recombinant scFv proteins might potentially be applicable tools for detecting immunoglobulins in various fish species by ELISA.