Adaptive Immunity

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Abstract

Two fundamental features characterize adaptive immunity: specific antigen recognition and immunological memory development. Thus, B and T cells form the adaptive immune system that detects and fights infection and disease with specificity. B- and T-cell progenitors rearrange their specific receptors (BCR and TCR, respectively) generating a great variability in the antigen recognition domains. The BCR and its secreted form (antibodies) recognize antigens in their native form and induce humoral immune responses. By contrast, the TCRs bind to antigens processed and presented on major histocompatibility complex (MHC) molecules and develop cellular immune responses. Through the development of both B and T memory cells, subsequent encounters with the antigen induce more efficient and faster immune responses, being this the basis for vaccination. Fish constitute the first animal group in which most basic elements of the adaptive immune system are present. Still, given the great diversity of fish, many fundamental differences exist among different species. Likewise, the fish adaptive immune system shows some unique features. These structural and functional differences with their mammalian counterparts anticipate important differences in the way that fish regulate both humoral and cellular adaptive immune responses. In the present chapter, we provide an overview of the basic organization of the adaptive immune system in fish, highlighting its main specific traits and providing some insights as to how these particularities might condition the response to antigens.

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Abbreviations

AID	activation-induced cytidine deaminase
APC	antigen-presenting cell
APRIL	proliferation-inducing ligand
ASC	antibody-secreting cell
BAFF	B-cell-activating factor
BCR	B-cell receptor
Blimp1	B lymphocyte-induced maturation protein 1
CD40L	CD40 ligand
CDR	complementarity-determining region
CH	constant heavy
CHNV	crucian carp necrosis virus
CL	constant light
CSR	class switch recombination
CTLA-4	cytotoxic T lymphocyte-associated protein 4
EBF1	early B-cell factor 1
EPC	epithelioma papulosum cyprini
FasL	Fas ligand
fDC	follicular dendritic cell
FITC-KLH	fluorescein–keyhole limpet haemocyanin
GALT	gut-associated lymphoid tissue
GC	germinal centres
GIALT	gill-associated lymphoid tissue
HK	head kidney
IEL	intraepithelial lymphocyte
IFN	interferon
Ig	immunoglobulin
Ii	invariant chain
IL	interleukin
ILT	interbranchial lymphoid tissue
IRF4	interferon regulatory factor 4
LN	lymph node
LP	lamina propria
LRR	leucine-rich repeat
MALT	mucosa-associated lymphoid tissue

MBL	mannose-binding lectin
MHC	major histocompatibility complex
MLC	mixed leucocyte culture
MMC	melanomacrophage centre
MZ	marginal zone
NALT	nasopharynx-associated lymphoid tissue
NKEF	natural killer cell enhancement factor
NO	nitric oxide
PALS	periarteriolar lymphoid sheath
PBL	peripheral blood leucocyte
PC	plasma cells
pIgR	polymeric Ig receptor
PKD	proliferative kidney disease
RAG	recombination-activating gene
RSS	recombination signal sequence
RTG2	rainbow trout gonad 2
SALT	skin-associated lymphoid tissue
SHM	somatic hypermutation
Tc	T cytotoxic
TCR	T-cell receptor
TD	thymus-dependent
TdT	terminal deoxynucleotidyl transferase
Tfh	T follicular helper cell
TGF	transforming growth factor
Th	T helper
TI	thymus-independent
TLR	Toll-like receptor
TNF	tumour necrosis factor
TNP-KLH	trinitrophenyl–keyhole limpet haemocyanin
TNP-LPS	trinitrophenyl–lipopolysaccharide
Treg	T-regulatory
V(D)J	variable (diversity) joining
VH	variable heavy
VHSV	viral haemorrhagic septicaemia virus
VL	variable light
VLR	variable lymphocyte receptor
Xbp1	X-box-binding protein 1

3.1 Introduction

The immune system of vertebrates consists of two fundamental components: innate immunity and adaptive immunity. The innate immune system acts as a first line of defence and relies on a set of germline-encoded host receptors that recognize conserved molecular signatures found in microorganisms. These receptors stimulate a broad variety of myeloid and lymphoid cells that can exert rapid microbicidal functions, at the same time initiating the adaptive immune response through the presentation of antigens or through the effect of secreted soluble factors (chemokines and cytokines) that provide co-stimulation to cells of the adaptive immune system (Mogensen 2009).

In contrast, in more complicated or reoccurring scenarios the adaptive immune system has emerged and evolved to provide specific protection against subsequent pathogenic infections. Two fundamental features characterize adaptive immunity: specific antigen recognition and immunological memory development (Gourley et al. 2004). Collectively, B and T cells form the adaptive immune system that detects and fights infection and disease with specificity. Recognition specificity and broad reactivity are enabled via antigen receptors on B and T cells generated by recombination-activating genes (RAG1 and RAG2) (Nishana and Raghavan 2012). B lymphocytes express membrane-bound immunoglobulins (Igs), known as B-cell receptors (BCR), which can recognize specific antigens in their native form. These Igs can be secreted, being then referred to as antibodies. T cells, on the other hand, have their own specific receptor (T-cell receptor, TCR) that recognizes processed antigens presented mostly in the context of major histocompatibility complex (MHC) molecules. T cells provide co-stimulatory signals to promote the activation and differentiation of B cells (helper T cells, Th cells) or mediate cellular immune responses aimed at the destruction of pathogens that are localized inside cells (cytotoxic T cells, Tc cells).

The adaptive immune system is able to recognize millions of many different antigens through the generation of the lymphocyte receptor repertoire. This process can be accomplished with a limited number of available genes owing to the exceptional organization of the genes encoding the BCR and the TCR. These receptor genes contain multiple versions that code for each region of the protein, allowing the lymphocyte to obtain a specific sequence among all the possible combinations and expressing these molecules in the membrane. The repertoire generation occurs randomly and independently from antigen exposition. For this, the BCR and TCR contain variable regions encoded by the variable (V), diversity (D) and joining (J) gene segments. These fragments undergo a process of splicing and somatic recombination as well as the random addition of nucleotides in the junction of VD or DJ segments, increasing the diversity in the potential repertoire of lymphocyte receptors (Nishana and Raghavan 2012). However, this large diversity includes the possibility of generating autoreactive cells that can recognize and attack self-antigens. To avoid this process, these cells undergo a selection process in the thymus and the bone marrow or its equivalent (birthplaces for T and B cells, respectively) that destroys or inactivates lymphocytes with autoreactivity (Strasser et al. 2008).

In mammals, adaptive immunity is the basis of immunological memory as successive encounters with a specific antigen lead to the development of quicker and more vigorous immune responses compared to primary responses. This is achieved via the previous generation of long-lived memory T and B cells during the clonal expansion of antigen-specific lymphocytes in the primary response (Sprent and Surh 2001; McHeyzer-Williams and McHeyzer-Williams 2005). Secondary antibody responses reach higher concentrations and produce Igs with higher affinities for the antigen. The increase in the antibody affinity is called affinity maturation and results from selection events suffered by the BCR throughout the differentiation of B cells (McHeyzer-Williams and McHeyzer-Williams 2005). By contrast, the TCR does not undergo this affinity maturation process, instead memory T cells change their responsive capacity having lower activation thresholds against the antigen (Sprent and Surh 2001).

It has to be taken into account that recent studies have established that the concept of immunological memory is not exclusive to adaptive immunity. Hence, the capacity to respond faster and more efficiently to an antigen re-exposure may also be associated with metabolic and epigenetic reprogramming of innate immune cells (termed trained immunity or innate immune memory), evoked by exogenous or endogenous insults, which lead to an altered response towards a second challenge after the return to a non-activated state (Topfer et al. 2015). In any case, the cognate immunological memory mediated by B and T lymphocytes is the basis for vaccination, the most effective method for preventing infectious diseases. Thus, the artificial induction of immunity against different pathogens constitutes one of the most important applications of immunology to human health. Vaccines pretend to create memory T and B cells that produce specific immune response quicker and of higher magnitude against the antigen to which the individual was immunized (Sallusto et al. 2010). This is achieved modifying the pathogen or its toxins in a way that can induce an immune response without producing disease. The main requirements for an effective vaccine depend on the nature of the microorganism nature. In extracellular infections, antibody responses are essential for the host protection, while activation of CD8 T cells is also required to fight intracellular microorganisms.

Most teleost fish possess an adaptive immune system capable of both humoral and cellular responses, specific antigen recognition and immunological memory development as per their mammalian counterparts. Still, a mosaic of noticeable differences exists among teleost species and with their mammalian counterparts. The goal of the present chapter is to provide an overview of the basic organization of the adaptive immune system in fish, detailing the distinct features that condition fish B- and T-cell responses.

3.2 Appearance of Adaptive Immune Responses in Fish

To date, most studies have suggested that adaptive immunity appeared during the early stages of vertebrate evolution, most likely in the disappeared placoderms (Cooper and Alder 2006). Accordingly, the genes that define adaptive immune systems such as Igs,

TCR, MHC I and MHC II, and RAG1 and RAG2 are present in gnathostomes (jawed vertebrates) including cartilaginous fish such as sharks (the most ancient jawed fish) and teleost fish. While these basic components of adaptive immunity are present, it must be taken into account that the adaptive branch of the immune system continued to evolve in tetrapods, reaching further degrees of specialization and sophistication in mammals.

Agnathans (lampreys and hagfish), on the other hand, are jawless vertebrates that lack these cognate adaptive molecules, as well as the classical primary and secondary lymphoid organs found in gnathostomes. However, agnathans possess cells with lymphocyte morphology and much of the lymphocyte molecular machinery (Mayer et al. 2002; Uinuk-Ool et al. 2002; Rothenberg and Pant 2004). In lampreys, a TCR-like gene containing only one copy of V- and J-like sequences, thus impairing the possibility of rearrangeable events, has been identified (Pancer et al. 2004). Nevertheless, leucine-rich repeat (LRR) proteins were identified in activated lamprey lymphocyte-like cells, and these seem to carry out similar functions to BCR and TCR genes (Pancer et al. 2004). These proteins known as variable lymphocyte receptors (VLRs) contain a high number of variable LRR units placed between less variable amino- and carboxyl-terminal LRR units. Additionally, VLRs contain a stalk-like region of invariant sequence that is bound to the plasma membrane through a glycosylphosphatidylinositol (GPI) anchor that permits its secretion. The germline configuration of the VLR gene appears incomplete and contains non-coding sequences that lack typical RNA splicing signals or recombination signal sequences. However, the flanking regions of the VLR gene are formed by a large number of cassettes that encode LRR units, which are randomly incorporated into the VLR gene by a multistep joining process (Alder et al. 2005). This mechanism for receptor variability generation is capable of generating a large VLR repertoire, similar to the repertoire for BCRs and TCRs. Subsequent studies have shown that lampreys contain one B-like and two T-like lymphocyte populations, according to the expression of an exclusive VLR isotype (VLRB in B-like cells and VLRA or C in T-like cells) (Kasamatsu et al. 2010; Hirano et al. 2013). Although agnathans are basal to gnathostomes, VLR features seem to be too different to the BCR/TCR recombination system to be its precursor. Furthermore, LRR proteins are present in a variety of unicellular organisms, plants and animals (especially cephalochordates) exerting different actions, including host defence responses (Pancer and Cooper 2006). Hence, the most accepted theory regarding the appearance of the adaptive immune system in jawed vertebrates established that RAG1 and RAG2 genes were once components of a transposable element (Agrawal et al. 1998).

3.3 Immune Organs Involved in Adaptive Immune Responses

Immune organs can be classified as primary and secondary lymphoid organs. Primary immune organs are sites for lymphocyte generation and maturation and in mammals include the thymus, foetal liver and bone marrow, whereas secondary organs are the sites

where mature lymphocytes interact with antigens and become activated and include the spleen, lymph nodes (LNs) and the mucosa-associated lymphoid tissues (MALTs).

The thymus is where T cells mature, while the foetal liver and bone marrow constitute the B-cell maturation organs in mammals. In the absence of bone marrow, teleost fish primary lymphoid organs include the thymus, the head kidney (HK) and the foetal liver. Recently, a structural analogue to the bursa of Fabricius, a primary immune organ present in birds, has been identified in Atlantic salmon (Løken et al. 2020). However, the expression of RAG1 and the absence of RAG2 transcripts suggest secondary lymphoid organ functions for this structure, in contrast to the primary immune function of its avian analogue.

In most teleosts, the thymus is placed near the gill cavity associated with the pharyngeal epithelium (Zapata 1981). The thymus structure can be divided into the medulla (inner zone) and the cortex (outer zone). The cortex is mostly composed of thymocytes (immature T cells) supported by a network of epithelial cells and it is where TCR rearrangements and positive selection take place. While the medulla contains fewer lymphoid cells and the network of epithelial cells is less organized than in the cortex, this part of the thymus is specialized in the negative selection of autoreactive T cells. In teleost fish, the structure of the thymus can be very variable depending on the species, but generally there is not a clear differentiation between the thymic medulla and cortex as that of mammals (Zapata 1981).

As mentioned before, the haematopoietic function of the mammalian bone marrow is substituted in fish by the HK (Zapata 1979). This organ is formed by two arms that penetrate under the gills and it is composed of a reticuloendothelial stroma with sinusoidal cells and reticular cells, similarly to the mammalian bone marrow. In addition to the lymphopoietic progenitors and mature lymphocytes, the HK contains active and inactive plasma cells (PCs). Similarly to the mammalian bone marrow that constitutes a survival niche for long-term PCs, it seems that plasmablasts and PCs in fish migrate back from secondary lymphoid organs to the HK where they are stored for long time periods (Bromage et al. 2004; Zwollo et al. 2005). Together with its haematopoietic and immune role, the fish HK is an endocrine organ homologous to mammalian adrenal glands. On the other hand, the posterior kidney is the renal organ in teleost fish but seems to also function as a secondary immune site in which many B and T cells are also found (Zapata 1979; Zwollo et al. 2005, 2010).

Once mature, B and T cells migrate into peripheral secondary organs where antigens are recognized and the immune response was triggered. In mammals, during thymus-dependent (TD) immune responses, activated B cells form structures called germinal centres (GCs) in the follicles of secondary lymphoid organs (Victora and Nussenzweig 2012). These GCs are the sites where antigen-specific B cells, T follicular helper cells (Tfh) and follicular dendritic cells (fDCs) interact to activate the differentiation of B cells by TD antigens. Activated B cells proliferate massively with some of them differentiating into PCs and/or memory B cells depending on the signals they receive in the GC. Alternative to this TD response, mammals have additional thymus-independent (TI) pathways to elicit faster antibody responses that are mainly orchestrated by innate B-cell subsets, mainly B1 cells or

marginal zone (MZ) B cells. These TI responses do not require T-cell cooperation, but are activated by BCR stimulation usually in combination with a direct recognition of pathogen motifs by innate receptors also expressed on B cells (Cerutti et al. 2011). Generally, the B-cell differentiation processes that take place outside the GC are designated as extrafollicular immune responses.

Teleost fish secondary immune organs include the spleen, the posterior kidney and the MALTs; however, fish lack lymph nodes and lymphoid follicles and conventional GCs have never been identified in these species. Interestingly, melanomacrophage centres (MMCs), which are aggregates of highly pigmented phagocytes, found mainly in the spleen and the posterior kidney of fish have been proposed as primitive GCs (Steinel and Bolnick 2017). In MMCs, B and T cells are found in association with MMs, which have been shown to retain antigens for long time periods. Despite this, much more evidence should be gathered to unequivocally establish them as primitive GCs (Steinel and Bolnick 2017). Nevertheless, the teleost spleen is the site where mature B cells encounter blood-borne antigens and become activated resulting in differentiated plasmablasts and PCs. In mammals, the spleen can be anatomically divided into white and red pulp. Most of the spleen is composed of red pulp where damaged erythrocytes are destroyed. The white pulp, in turn, constitutes the lymphoid tissue, and it is constituted by different areas: the periarteriolar lymphoid sheaths (PALS); the lymph follicles; and the MZ. In fish, the difference between red and white pulp is less notable than in superior vertebrates and the white pulp appears to be less developed.

Regarding the MALTs, four main mucosal immune tissues have been identified in fish: (1) the gut-associated lymphoid tissue (GALT), (2) the skin-associated lymphoid tissue (SALT), (3) the gill-associated lymphoid tissue (GIALT) and the nasopharynx-associated lymphoid tissue (NALT).

Although the mammalian GALT contains some isolated lymphoid follicles scattered along the lamina propria (LP) and some intraepithelial lymphocytes (IELs), it is mainly formed by Peyer's patches, which are aggregations of lymphoid follicles where B cells, T cells, macrophages and DCs interact and GCs are formed. However, fish do not contain Peyer's patches nor do they organize GCs in the GALT. In fish, B and T cells are dispersed through the mucosa, both within the LP or as IELs. Thus, these B- and T-cell responses might best resemble mammalian extrafollicular responses, but how these cells detect antigens and become activated still remains mostly unknown. This diffuse organization of B and T cells throughout the mucosa has also been described in SALT, GIALT and NALT (Salinas 2015). In the case of the GIALT, an organized lymphoid structure with high numbers of T cells has been detected on the caudal edge of the interbranchial septum in salmonids, designated as interbranchial lymphoid tissue (ILT) (Haugarvoll et al. 2008; Koppang et al. 2010). Yet, the exact role of this tissue in the immune response remains unsolved.

3.4 B cells

Humoral adaptive immune responses are mediated by B cells. B cells have the capacity to recognize soluble antigens and synthesize specific antibodies owing to their membrane antigen receptor or BCR. The BCR complex is composed of an Ig (slightly modified to be tethered to the membrane) and by two invariant chains, CD79α and CD79β (or Igα and β). In this complex, the Ig is the one that recognizes the antigen and the CD79 proteins mediate the signal transduction to the cellular nucleus.

In addition to producing antibodies, B cells in both mammals and fish are also involved in antigen presentation. The antigen presentation requires a first interaction of the BCR with the native antigen and its subsequent internalization and processing to expose the antigenic fragments in membrane MHC class II molecules (Rodriguez-Pinto 2005). The presentation of this specific antigen through the BCR occurs with very high efficiency and is associated with B-cell activation, resulting in the activation of cognate T cells. In mammals, however, the presentation of non-specific antigens by B cells is dissociated from B-cell activation generally leading to the inactivation of T cells (Rodriguez-Pinto 2005).

In contrast to conventional mammalian B cells, teleost B cells have a strong phagocytic capacity (Li et al. 2006). This additional capacity to internalize particulate antigens is thought to increase the potential of teleost B cells as antigen-presenting cells (APCs) (Zhu et al. 2014), especially in what concerns particulate antigens. Furthermore, this phagocytic capacity is also related to a microbicidal role, demonstrated in rainbow trout (*Oncorhynchus mykiss*) (Zhang et al. 2017). Hence, it seems that teleost B cells also play an important role in innate responses and early clearance of pathogens, similarly to mammalian B1 cells. In mammals, B1 cells arise early during the ontogeny and are considered components of the innate immune system. They are found primarily in the peritoneal cavity, mucosal surfaces, and spleen where they account for 2% of the spleen lymphocyte population. These cells are responsible for an early recognition of pathogens and production of natural IgMs that help clear pathogens until a specific conventional B-cell response is mounted (Martin and Kearney 2001). This first wave of antibodies is produced by B1 cells independently of T cells outside GCs (extrafollicularly) (Martin et al. 2001). In this context, recent studies have reported many functional and phenotypical resemblances between teleost B cells and mammalian B1 cells, in addition to the strong phagocytic capacity also identified in mammalian B1 cells (Gao et al. 2012; Parra et al. 2012a). These include the transcription of a broad range of innate receptors (Abos et al. 2013); a low IgD and high IgM surface expression; extended survival in cell culture; lack of proliferation upon BCR engagement (Abos et al. 2018a); and expression of B1-specific markers, such as CD9 and CD5 (Castro et al. 2015; Abos et al. 2018a). Collectively, these lines of evidence point to the hypothesis that mammalian B1 lineage arose from ectotherm IgM^+ B cells and that B2 cells emerged later in evolution as a more efficient population specialized in adaptive immune responses (Sunyer 2012; Zhang et al. 2017). These shared

characteristics between fish B cells and mammalian B1 cells will surely condition the way in which fish B-cell responses are orchestrated.

3.4.1 B-cell Development

In mammals, B-cell generation starts in the foetal liver from multipotential stem cells. In early stages of the development, the liver is replaced by the bone marrow that becomes the main B-cell production site (Kantor and Herzenberg 1993). From the bone marrow, B cells are generated during all the lifespan decreasing progressively over time. Early precursor cells in the bone marrow have not started to rearrange their Ig genes yet, having them in the germline configuration. B-cell differentiation requires the presence of stromal cells in the bone marrow (adipocytes, fibroblasts, reticulocytes, endothelial cells) that provide direct contacts and soluble factors to induce B-cell maturation. This maturation process produces important changes in the phenotype of B cells with the expression of specific proteins in each stage (summarized in Fig. 3.1). The Ig gene rearrangement is accomplished during late pro-B- and pre-B-cell differentiation phases, and it is possible to see IgM heavy chains in the membrane of pre-B cells, conforming the pre-BCR. Pre-B cells become immature B cells that express membrane IgM molecules, but it is only when B cells mature that they exhibit IgM and IgD molecules at the same time in the membrane. Throughout the maturation process, immature B cells that interact with self-antigens abundant in the bone marrow are eliminated or inactivated in a negative selection process to avoid autoreactive immune responses (Kantor and Herzenberg 1993). In teleosts, given the lack of bone marrow, this B-cell development occurs in the HK. Accordingly, the rainbow trout HK has been shown to express genes encoding RAG-1/2, terminal deoxynucleotidyl transferase (TdT) (enzyme that adds N-nucleotides to the V, D and J exons) and Ikaros (a transcription factor that regulates lymphocyte differentiation/proliferation) and to contain B cells in multiple maturation stages (Zwollo et al. 2005, 2010). Despite this, to date, a full phenotypical characterization of the developmental stages of B cells has not yet been undertaken in fish.

3.4.2 Immunoglobulins and B-cell Subsets

Igs are composed typically of two identical heavy chains (IgH) and two identical light chains (IgL) bound by disulphide bonds. Each chain contains one N-terminal variable domain (VH and VL) and one or more C-terminal constant domains (CH and CL). The variable regions from both, the heavy and the light chains, are assembled via somatic gene rearrangement from an array of multiple V, D (not present in light chains) and J segments during B-cell development. The variable domains contain three hyper-variable regions named complementarity-determining regions (CDR) that are crucial for antigen specificity. The constant region on the other hand mediates the effector functions of the different Igs.

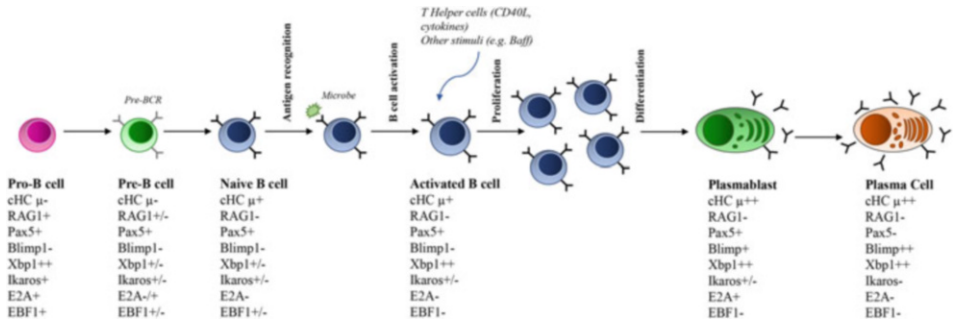

Fig. 3.1 Stages of B-cell development and differentiation. The levels of expression of the cytoplasmic Ig heavy chain μ, the recombinase RAG1 and the transcription factors Pax5, Blimp1, Xbp1, Ikaros, E2A and EBF1 are indicated in each stage. Adapted from Zwollo (2011)

Three types of Igs have been identified in teleost fish: IgM, IgD and IgZ/T since no homologues to mammalian IgA, IgG or IgE are present. As studied in zebrafish (*Danio rerio*) and rainbow trout, the genes in the IgH chains show a particular organization, with upstream VH-DJCμCδ, elements for the H chains of IgM (μ) and IgD (δ) and an exclusive VH-DJCτ for the H chain of IgT (τ) (Danilova et al. 2005; Hansen et al. 2005). Thus, alternative splicing between the recombined VDJ region and Cμ (IgM) or Cδ (IgD) occurs to generate IgM or IgD, rendering IgM and IgD of the same specificity expressed on a single naïve B cell. On the contrary, fish B cells express either IgM or IgT as V segments rearrange either to DJCτ or to DJCμ (Zhang et al. 2011). Therefore, IgT cells seem to constitute an independent B-cell linage in which IgM and IgD are not expressed (Zhang et al. 2010).

IgM was the first Ig identified in fish and is the most abundant Ig in plasma (Flajnik 2002). It can be found as a transmembrane surface protein or secreted as an antibody. Surface IgM is one domain shorter than the secreted form, lacking the Cμ4 domain due to an alternative splicing (Ross et al. 1998). IgM forms tetramers in both serum and mucus, although some studies have reported monomers of IgM in species such as margate (Clem 1975), sheepshead (*Archosargus probatocephalus*) (Lobb and Clem 1981) or rainbow trout (Elcombe et al. 1985). As in higher vertebrates, the teleost IgM isotype achieves multiple effector functions in the humoral immune responses. Fish IgM can activate the complement (Cooper 1985; Boshra et al. 2004), induce agglutination, bind mannose-binding lectin (MBL) motifs (Arnold et al. 2006) and mediate cellular cytotoxicity (Ohlander and Perlmann 1982; Stafford et al. 2006). Furthermore, teleost IgM has been shown to increase the phagocytic capacity of macrophages through antigen-specific opsonization (Soto et al. 2011a, b).

IgD has been described in many vertebrate classes, including teleost (Ohta and Flajnik 2006), but its precise role remains an enigma. Interestingly, orthology between IgD and IgW (also known as IgX, IgNARC or IgR), an Ig-like molecule present in cartilaginous fish, lungfish and coelacanths, suggested that IgD may be as ancient as IgM (Ohta and

Flajnik 2006; Saha et al. 2014). This implies that IgD may play an important but still elusive role in adaptive immunity (Gutzeit et al. 2018). Teleost IgD is characterized by a Cμ1 domain followed by a different number of Cδ domains that can range from 2 to 16, depending on the species. Thus, the fish IgH δ locus is composed of a rearranged VDJ spliced to Cμ1, a variable number of C domains and a transmembrane tail (Bengten et al. 2006; Stenvik and Jorgensen 2000; Srisapoome et al. 2004; Xiao et al. 2010). Both membrane and secreted IgD have been found in different species of teleost fish including channel catfish (*Ictalurus punctatus*), rainbow trout (*O. mykiss*), Atlantic salmon (*Salmo salar*), Atlantic cod (*Gadus morhua*) and grass carp (*Ctenopharyngodon idella*) (Bengten et al. 2006; Ramirez-Gomez et al. 2012).

IgT is an exclusive Ig of bony fish discovered in 2005 in rainbow trout (IgT) and zebrafish (where it was designated as IgZ) (Danilova et al. 2005; Hansen et al. 2005). The majority of teleost IgT contains 4 C domains, although this can differ in some species (Salinas et al. 2011). IgT has been reported to be specialized in mucosal responses, since it was found that the ratio of IgT:IgM was much higher in gut mucus than in the serum of rainbow trout (Zhang et al. 2010). Similarly, the ratio of IgT^+ B cells to IgM^+ B cells was also higher in mucosal tissues than in systemic compartments (Zhang et al. 2010). Furthermore, in response to some parasitic infections, IgT production was increased in the mucosal surfaces such as the gut (Zhang et al. 2010), gills (Xu et al. 2016) or skin (Xu et al. 2013), while the changes in IgM were limited to systemic compartments. IgT is a monomer in rainbow trout serum and a tetramer in the mucus (Zhang et al. 2010), where it has the capacity to pass through the mucosal epithelium owing to the polymeric Ig receptor (pIgR). Despite these results, it should be noted that systemic IgT responses have also been reported (Castro et al. 2013, 2014b; Abos et al. 2018b), along with mucosal IgM and IgD responses; therefore, the precise role of IgT in the adaptive immune response of fish remains largely unexplored.

In addition to IgT^+ B cells, which constitute an independent B-cell linage from IgM- and IgD-bearing cells (Zhang et al. 2010), different B-cell subsets can be distinguished in fish according to the pattern of surface IgM and IgD expression. As in mammals, mature IgM^+IgD^+ B cells constitute the main B-cell subset in lymphoid tissues and peripheral blood (Tafalla et al. 2017; Simon et al. 2019). Upon activation, these cells lose surface IgD to become IgM^+IgD^- B cells with a plasmablast profile (Tafalla et al. 2017; Granja and Tafalla 2019). Moreover, through some still unknown mechanisms, certain cells lose surface IgM and become IgD^+IgM^- B cells. These cells have been identified in catfish blood (Edholm et al. 2010) and in rainbow trout gills (Castro et al. 2014a) and gut (Perdiguero et al. 2019). In rainbow trout, these cells were shown to secrete IgD, which was found bound to intestinal bacteria (Perdiguero et al. 2019), as previously shown for IgT and IgM (Zhang et al. 2010). Interestingly, the intestinal bacteria were found to regulate IgD transcription in the gut (Perdiguero et al. 2019). This suggests a two-way interaction between IgD and the intestinal microbiota that points to an important role of IgD in intestinal homeostasis, maybe complementing the functions of IgM and IgT in this mucosa. In humans, a small subset of mammalian B cells has also been shown to undergo an

unconventional form of class switch recombination (CSR) from IgM to IgD and thereafter differentiate into IgD^+IgM^- plasmablasts specifically secreting IgD (Arpin et al. 1998; Koelsch et al. 2007; Chen et al. 2009; Rouaud et al. 2014; Choi et al. 2017). Although also detected in the general circulation, these IgD class-switched cells mostly inhabit the organized lymphoid tissue from nasopharyngeal cavities, including human tonsils (Arpin et al. 1998; Koelsch et al. 2007; Chen et al. 2009), but have never been identified in the human gut. Secreted IgD in humans has been shown to bind to basophils through a calcium-mobilizing receptor, inducing the release of antimicrobial peptides and pro-inflammatory cytokines (IL-1, IL-4) (Arpin et al. 1998; Chen et al. 2009; Koelsch et al. 2007). Whether secreted IgD plays a similar regulatory role in fish is still unknown.

3.4.3 B-cell Differentiation

In mammals, naïve mature B lymphocytes developed in the bone marrow recirculate throughout secondary lymphoid organs where they can encounter specific antigens. Antigen recognition together with the adequate co-stimulatory signals (provided by Th cells in the case of TD antigens or by innate signals in the case of TI antigens) activates B cells inducing their proliferation and differentiation. B cells have the capacity to endocytose the complex Ig/antigen, allowing the antigen to be processed and presented by their MHC II molecules to Th cells. For this, both B and T cells must be specific for the same antigen but not necessarily the same epitope. When Th lymphocytes recognize the specific peptides, they proliferate and differentiate to further activate antigen-specific B lymphocytes (Lanzavecchia 1985). In this interaction, the recognition of CD40 ligand (CD40L) expressed on the surface of Th cells by CD40, expressed on the surface of B cells, is essential for the activation of B cells. In addition, the secretion of cytokines by Th cells further conditions B-cell activation. For example, interleukin 4 (IL-4) secreted by Th2 cells induces B-cell clonal expansion in synergy with CD40L. In rainbow trout, the effect of cytokines commonly produced by T cells, namely IL-2, IL-4/13B (homologous to both IL-4 and IL-13), IL-10 and IL-21, on splenic IgM^+ B cells alone or in combination with CD40L was recently studied (Abos et al. 2020). The authors reported that the cytokines had significant effects on IgM^+ B-cell survival, proliferation and IgM secretion either individually or in cooperation with CD40L. Additionally, factors secreted by innate cells such as DCs or macrophages are known to condition B-cell activation both within the GCs and in extrafollicular responses. Among these cytokines, additional members of the tumour necrosis factor (TNF) family such as BAFF (B-cell-activating factor) (Tafalla et al. 2017) or APRIL (a proliferation-inducing ligand) (Soleto et al. 2018b) have been shown to play an important role in B-cell survival and differentiation also in fish.

Within GCs, mammalian B cells undergo profound changes in the structure of their Igs by means of two different processes: CSR and somatic hypermutation (SHM). Through CSR, the constant region of IgM is replaced by that of other Igs with higher affinity and different effector functions. This process is mediated by the activation-induced cytidine

deaminase (AID) that replaces the constant region of IgM by that of IgG, IgA or IgE (Nera et al. 2015). Concerning IgD, it is generally accepted that the δ gene is co-expressed with μ by differential transcription and alternative splicing, both sharing the same antigen-binding variable region (Enders et al. 2014). However, as mentioned above, a possible unconventional form of CSR has been suggested in the case of IgD-secreting plasmablasts (Rouaud et al. 2014). Throughout the differentiation process that takes place within the GC, the Igs undergo affinity maturation as a consequence of SHM. In this context, point mutations are introduced in the Ig variable regions through the action of AID. Subsequently, B cells with high-affinity receptors are rescued in a positive selection, while those with less affinity than the B cell they derive from will be eliminated. These positively selected B cells will then differentiate to long-lived PCs or to memory B cells according to the signals they receive in the GC. Memory B-cell differentiation relies on CD40/CD40L new contacts with Tfh cells and memory B cells are characterized by a slow division capacity and non-Ig secretion. Meanwhile, plasmablast/PC differentiation appears to depend on the CD23-expressing fDC and the co-receptors CD19/CD21/CD81 in the B cell. Plasmablasts have an increased capacity to secrete Igs, but they are still dividing and share many of the characteristics of activated B cells that allow them to interact with T cells. After several days, plasmablasts stop their proliferation and either die or differentiate to PCs. Hence, PCs have a greater capacity to secrete Igs but lose their dividing capacity. The terminal differentiation of B cells is controlled in mammals by a complex regulatory network in which the transcription factor Blimp1 (B lymphocyte-induced maturation protein 1) acts as a key regulator. Blimp1 acts as a transcriptional repressor of relevant genes involved in the functionality of undifferentiated B cells, along with other molecules such as X-box-binding protein 1 (Xbp1) and interferon regulatory factor 4 (IRF4), also known to be critical to acquire a plasma cell phenotype (Calame 2001; Shapiro-Shelef and Calame 2005). Blimp1 also represses the promoters of genes involved in functions of mature naïve B cells such as Pax5 (Lin et al. 2002), Bach2 (Ochiai et al. 2006) or Bcl6 (Shaffer et al. 2002). Eventually, PCs migrate to the bone marrow where they receive signals from the stromal cells that allow them to survive for a long time maintaining high-affinity antibodies in the serum (Slifka et al. 1995).

Affinity maturation in fish and other ectotherms has been shown to be much less efficient than in mammals (Wilson and Warr 1992; Warr 1995) even though the expression of AID has been reported in many fish species, with this teleost AID maintaining the capacity to induce point mutations and even mediating class switch in murine B cells (Barreto et al. 2005; Quinlan et al. 2017). For instance, rainbow trout immunized with a TD antigen such as TNP-KLH along with an adjuvant developed IgM antibodies that after a few months reached a 100-fold increase in antigen affinity when compared to antibodies produced the first 5 weeks post-immunization (Ye et al. 2011). However, this increase in antigen affinity is much lower than that reported in mammals. Nevertheless, the presence of plasmablasts and PCs has been demonstrated in some fish species, especially rainbow trout, where studies characterizing these two cell subsets were performed (Bromage et al. 2004; Zwollo et al. 2005; Zwollo 2011).

Despite all the innate-like features of fish B cells and the lack of organized structures where B and T cells can meet, a recent study performed in rainbow trout demonstrated that fish B cells respond to CD40L in a similar way to mammalian B2 cells, upregulating IgM^+ B-cell survival and proliferation and inducing PC differentiation (Granja et al. 2019). It should be taken into consideration, however, that some innate cells such as DCs also have the capacity to produce CD40L, as demonstrated in both mammals (Tough 2009) and fish (Granja et al. 2019); thus, it may be possible that under some circumstances innate cells can provide B cells with these 'TD' stimulatory signals.

In the absence of additional adjuvants, rainbow trout B cells were much more responsive to TI antigens (TNP-LPS) than to TD antigens (TNP-KLH) (Granja et al. 2019). TI antigens, opposed to TD antigens, provide B cells with co-stimulatory signals through innate receptors in addition to the BCR (Cerutti et al. 2011). Therefore, these results suggest the importance of innate activation to induce the differentiation of fish B cells. In this sense, fish B cells have been shown to be equipped with a wide range of innate receptors such as Toll-like receptors (TLRs) (Abos et al. 2013) and to be highly responsive to TLR ligands such as LPS (Abos et al. 2016) and CpGs (Simon et al. 2019) that on their own have the capacity to induce the proliferation and antibody secretion of rainbow trout IgM^+ B cells. Along this line, a recent study by Soleto and collaborators revealed that TLR signalling is essential for the activation of IgM^+ B cells by the bacterial pathogen *Aeromonas salmonicida* (Soleto et al. 2020).

Regarding the characterization of the B-cell differentiation process in fish, already in 2011, Zwollo and collaborators established different phenotypes associated with the expression of different transcription factors such as EBF (early B-cell factor 1), Pax5 and Xbp-1 (X-box-binding protein 1), being able to discriminate between early B-cell progenitors, late developing B cells, resting B cells, activated B cells, plasmablasts and PCs (Zwollo 2011). Additionally, Blimp1 transcription was shown to increase upon activation of B cells with IL-6 (Abos et al. 2016). Interestingly, other stimuli able to activate B-cell proliferation and IgM secretion did not imply an upregulation of Blimp1 transcription. This is the case for BAFF (Tafalla et al. 2017), APRIL (Soleto et al. 2018b) or LPS (Abos et al. 2016). Finally, a recent work provided insights on how the Blimp1 gene family evolved in fish, demonstrating that four homologues of Blimp1 are present in salmonids, all of them with a yet not well-defined role in B-cell activation (Perdiguero et al. 2020). Therefore, it might be possible that different Blimp1 genes are involved in the terminal differentiation of different B-cell subsets or that they are involved in different steps. Alternatively, B cells could be activated to a plasmablast state in response to some stimuli that might not require the expression of any of these Blimp1 homologues.

3.4.4 Antibody Responses

The production of specific IgM antibodies has been demonstrated against viruses (Lorenzen and LaPatra 1999), bacteria (Ellis 1999) and parasites (Alvarez-Pellitero 2008;

Sitja-Bobadilla et al. 2016). In the case of viral agents, the capacity of IgM to neutralize viral replication has also been revealed in some conditions. For example, neutralizing serum IgMs produced against fish rhabdoviruses in rainbow trout have been broadly characterized (Winton et al. 1988; Lorenzen and LaPatra 1999). Neutralizing IgM antibodies with the capacity to limit pathogen progression have also been reported against several bacteria and parasites such as *Aeromonas salmonicida* (Hirst and Ellis 1999), *Francisella asiatica* (Soto et al. 2011b), *Ceratomyxa shasta* (Zhang et al. 2010) or *Cryptobia salmositica* (Ardelli and Woo 1997). In some cases, the capacity of these specific antibodies to provide protection against the pathogen has been demonstrated in passive immunization experiments (LaPatra et al. 2010; Hershberger et al. 2011; Soto et al. 2011b). Still, the precise contribution of pathogen-specific serum IgMs to pathogen clearance in natural infections in fish is still not clear, as in many cases specific IgM levels peak weeks after the mortality terminates (Bernard et al. 1983; Olesen and Verstergaard Jørgensen 1986).

Most studies aimed at investigating antibody production in response to different pathogens are focused on IgM and very few on other isotypes such as IgD or IgT. Although IgD is known to be secreted in many teleost fish species (Ramirez-Gomez et al. 2012), the production of antigen-specific IgD has never been demonstrated in fish. However, a few studies have revealed changes in total IgD levels, suggesting a role of this Ig isotype in the response to pathogens. In rainbow trout, for example, immunization with an attenuated *Flavobacterium psychrophilum* significantly increased the levels of secreted IgD in gills and gut (Makesh et al. 2015). The levels of transcription of IgD and the presence of IgD^+ B cells also increased in the skin of rainbow trout infected with *F. psychrophilum* (Munoz-Atienza et al. 2019). Finally, an upregulation of IgD transcription in response to viral, bacterial or parasitic antigens has been reported in freshwater carp (*Catla catla*) (Banerjee et al. 2017) and rohu (*Labeo rohita*) (Basu et al. 2016).

As with IgD, only a few studies have demonstrated the production of antigen-specific IgT. In rainbow trout infected with the intestinal parasite *Ceratomyxa shasta,* specific IgMs to the parasite were found in serum, whereas specific IgT was exclusively found in the gut mucus (Zhang et al. 2010). Other studies have further pointed to the *preferential production* of IgT in mucosal secretions when compared to systemic compartments, but analysing total Ig levels and not antigen-specific Igs (Piazzon et al. 2016; Buonocore et al. 2017; Tongsri et al. 2020). On the other hand, several studies have demonstrated significant IgT responses outside the mucosal compartments that point to the presence of low but significant IgT production in systemic compartments in response to some stimuli/pathogens. For example, IgT responses were evident in the spleen after intramuscular injection with inactivated VHSV (viral haemorrhagic septicaemia virus) (Castro et al. 2013), in the muscle of fish vaccinated with a DNA vaccine against VHSV (Castro et al. 2014b) or in the kidney of rainbow trout affected by proliferative kidney disease (PKD) (Abos et al. 2018b).

Interestingly, some teleost species such as the Atlantic cod lack MHC II genes, CD4 and the invariant chain (Ii) that facilitates peptide loading onto MHC II (Landsverk et al. 2009).

This loss is accompanied by an elevated number of MHC I genes (Persson et al. 1999), but it is unclear if this expansion is related to the loss of the MHC II pathway (Malmstrom et al. 2013). As a consequence, the production of specific antibodies seems impaired in this species, although there is some controversy. Some studies have reported a poor antibody response of cod (Pilstrom et al. 2005), whereas other studies obtained a significant specific IgM response in this species to different bacteria such as *Vibrio anguillarum, Aeromonas salmonicida* and *Francisella* sp. (Schroder et al. 2009) or to antigens of the anisakis nematode *Contracaecum osculatum* (Mohamed et al. 2020).

3.4.5 B-cell Memory Responses

Very few studies have explored whether immune memory is established in teleost fish. One of the first studies that described B-cell memory was performed in rainbow trout. In that study, when fish were immunized with TNP-KLH along with an adjuvant, a more potent response was found in the secondary immune response in comparison with the primary response (Arkoosh and Kaattari 1991). Nevertheless, most reports sustain that affinity maturation of serum IgM after immunization is weak (Cain et al. 2002; Kaattari et al. 2002; Ye et al. 2011). For example, Kaattari et al. showed a two- to threefold increase in affinity in IgMs obtained from rainbow trout immunized with FITC-KLH from week 4 to week 12 post-immunization (Kaattari et al. 2002). As mentioned before, other studies reported increases in affinity of up to 100-fold (Ye et al. 2011), although this increase is still much lower than that observed in mammals.

Primary and secondary antibody responses in teleost show a similar pattern to mammalian responses regarding the distribution of antibody-secreting cells (ASCs) (Ma et al. 2013). After the intraperitoneal immunization of rainbow trout with either TNP-LPS or TNP-KLH, the spleen was the tissue that contained the highest number of ASCs during the primary response, while the HK was the predominant organ during the secondary immune response, showing the strongest PC activity (Ma et al. 2013). Interestingly, the numbers of ASCs reached in response to TNP-LPS during the secondary response were higher than those obtained in response to the TD antigen TNP-KLH (Ma et al. 2013). Similarly, when rainbow trout were anally immunized with TNP-LPS or TNP-KLH, the spleen was the site where most TNP-specific ASCs were found at early time points, while it was the kidney where these cells were mostly found at later time points (Martin-Martin et al. 2020). In this case, in the absence of additional adjuvants, the response to TNP-LPS was much higher than that observed in response to TNP-KLH.

3.5 Adaptive Cellular Responses: T Cells

Cellular adaptive immune responses are mediated by T lymphocytes. Unlike B cells that can directly recognize antigens, T cells generally react to antigens that have been processed by APCs and are exposed on the cell surface in the context of MHC I or MHC II molecules. This recognition is performed throughout their antigen receptor or TCR that always remains membrane-bound. Attending to the nature of their TCR, T cells can be classified into αβ T cells (formed by an α and a β chain) and γδ T cells (formed by a γ and a δ chain). In mammals, αβ T cells are the most abundant T cells in peripheral blood and lymphoid organs, whereas γδ T cells are less common and predominantly found in mucosal tissues (Bonneville et al. 2010). Interestingly, most γδ T cells are activated by antigens in an MHC-independent manner.

From a functional point of view, T cells can be divided into two general populations: cytotoxic T cells (Tc) that express the CD8 co-receptor that interacts with MHC class I and helper T (Th) cells that express CD4 molecules that interact with MHC class II. Both co-receptors stabilize the interaction between the TCR and the MHC molecules. $CD8^+$ T cells are cytotoxic T cells responsible for directly destroying target cells, whereas $CD4^+$ T cells are T helper cells that contribute to the activation of other immune cells. Among these effects, the activation of B cells by $CD4^+$ T cells after the recognition of specific antigens in the context of MHC II on the surface of the B cell delivers the needed activating signals for B cells to mount efficient TD responses as already explained.

3.5.1 T-cell Development

T cells develop in the thymus from common lymphoid progenitors coming from the bone marrow or foetal liver. Thymic stromal cells that include epithelial cells, DCs and macrophages interact with the thymocytes through their MHC I and MHC II molecules, accessory molecules and cytokines in order to orchestrate the maturation of T cells (Takahama 2006). The most immature thymocytes lack TCR, CD4 and CD8 molecules, but then quickly divide and express a pre-TCR that precedes the definitive αβ receptor. This pre-TCR contains a rearranged TCRβ chain together with an invariant protein called the pre-Tα. Later, the thymocyte starts the rearrangement of its TCRα chain that will replace the pre-Tα, interrupting any further recombination process to guarantee allelic exclusion (ensuring that only one specific TCR is expressed in each T lymphocyte) (von Boehmer et al. 1998). At this stage, thymocytes often express CD4 and CD8 simultaneously and suffer a process of positive selection in the thymic cortex by which only the thymocytes that recognize self-MHC molecules will survive (Fowlkes and Schweighoffer 1995). Later, autoreactive thymocytes are negatively selected in the thymic medulla by APCs. The result of this process is a population of mature naïve αβ T cells that will abandon the thymus and recirculate between the blood and lymphoid organs searching for a specific antigen through its TCR (Takahama 2006).

Cartilaginous and bony fish are the most primitive vertebrates with a histologically distinguishable thymus (Chilmonczyk 1983). All evidence suggests that the primary T-cell differentiation and maturation occur in the thymus similarly as in mammals. Interestingly, experiments performed in adult fish from different teleost fish species have confirmed a high presence of T cells in mucosal surfaces such as gills (Koppang et al. 2010) or intestine (Rombout et al. 1998; Romano et al. 2007). This together with the identification of RAG1 expression in these tissues (Huttenhuis et al. 2006; Picchietti et al. 2011) suggests the possibility of an extra-thymic development of T cells in some specific mucosal surfaces.

3.5.2 T-cell Receptors (TCR) and Co-receptors

Similarly to the BCR, the TCR undergoes RAG-mediated V(D)J rearrangement to create a wide range of repertoires. The structure of the TCR is well conserved in most vertebrates, containing a heterodimer of type I transmembrane glycoproteins (α and β chains or γ and δ chains) with extracellular V and C Ig domains and a short cytoplasmic tail. The constant region is proximal to the cell membrane, while the variable region binds to the peptide/MHC complex. The TCR is attached to the signalling CD3 complex and recognizes co-stimulatory and co-inhibitory molecules to regulate the activation/inhibition state of the T cell (Castro et al. 2011).

The TCR genes are TRA, TRB, TRG and TRD encoding for the TCR-α, β, γ, and δ chains, respectively. The TCR can be formed by a heterodimer of α and β chains (αβ-TCR) or a heterodimer of γ and δ chains (γδ-TCR). TRA and TRG undergo VJ recombination, whereas TRB and TRD go through VDJ recombination. All these TCR genes are well conserved in evolution and have been described in many fish species (Castro et al. 2011). Similarly to mammals, the TCR gene segments in fish are organized in a translocon arrangement (Criscitiello et al. 2010; Castro et al. 2011). The variable domain of the α and β chains has 3 CDRs from which CDR3 is the main responsible for recognizing the processed antigen. The constant domain of the TCR consists of short connecting sequences in which a cysteine residue forms disulphide bonds, which link the two chains. Interestingly, a new TCR chain (TCRμ) was discovered in 2007 in marsupials and monotremes with high similarity to a TCRδ isoform in sharks, probably arising from an ancient receptor system (Parra et al. 2012b).

$CD4^+$ T cells recognize peptides presented via MHC II molecules. Once activated, they release cytokines that can activate and regulate the immune responses. The CD4 molecule is a monomer with four extracellular Ig-like domains and a cytoplasmic tail that contains a CxC motif. This motif binds to the tyrosine kinase Lck, activating the intracellular signalling (Gratton et al. 2000). Even though fish CD4 protein sequences do not show a high similarity with those of higher vertebrates, the gene organization and the crucial residues are well conserved. In teleost, CD4 has been identified in several species, including fugu, rainbow trout, channel catfish, sea bass (*Dicentrarchus labrax*), Atlantic halibut (*Hippoglossus hippoglossus*) and Atlantic salmon [reviewed in Ashfaq et al.

(2019)]. Two different types of CD4 molecules have been described in bony fish: CD4-1 containing four Ig domains, as in tetrapods, and CD4-2, which contains either two or three Ig domains, depending on the species (Laing et al. 2006). CD4-2 might be the primitive CD4 molecule from which CD4-1, with its 4 Ig domains, would have emerged following duplication of the two Ig domains (Laing et al. 2006). Interestingly, CD4-2 was expressed in a Treg-like subset with a CD25-like^{+}Foxp3-like^{+} phenotype from a pufferfish model, suggesting different functional roles between the two CD4 populations (Wen et al. 2011). Finally, salmonids contain two CD4-2 molecules: CD4-2a, also known as CD4-related; and CD4-2b (Moore et al. 2009).

CD8^{+} T cells recognize peptides presented via MHC-I molecules and release the cytotoxins perforin, granzymes and granulysin. As a result of the perforin action, granzymes enter the cytoplasm of the target cell and activate apoptosis. The CD8 molecules may appear as homodimers composed of two α chains (CD8αα) or heterodimers formed from one α chain and one β chain (CD8αβ) (Cole and Gao 2004). In mammals, mature Tc cells containing αβ-TCRs mostly contain CD8αβ molecules. In contrast, most γδ T cells do not express CD8 (Kabelitz et al. 2000). Interestingly, a minor population of intestinal epithelial T cells (both αβ and γδ T cells) express CD8αα (Jarry et al. 1990). In teleost fish, both CD8 chains have been well characterized in different species and the structure of the CD8 genes seems to be conserved throughout evolution. Interestingly, CD4 and CD8 molecules are expressed not only on T cells but also in other cell types as reported in mammalian DC subsets (Vremec et al. 2000). For example, Takizawa et al. identified a CD4^{+} monocyte/macrophage population in rainbow trout (Takizawa et al. 2016), while CD8α is also expressed in a DC-like population described in different rainbow trout mucosal surfaces (Granja et al. 2015; Soleto et al. 2018a, 2019).

T-cell activation requires the CD3 protein complex associated with the TCR to generate the activation signal within the cell. CD3 is composed of four distinct chains: a CD3γ chain, a CD3δ chain, two CD3ε chains and two CD3ζ chains. The extracellular domains of CD3 ε, γ and δ contain an Ig-like domain, and the cytoplasmic tail contains a single ITAM (immunoreceptor tyrosine activation motif). CD3ζ chains, in turn, possess three ITAMs in their cytoplasmic domains. CD3 in teleost fish seems to have a conserved structure with that of mammalian CD3 (Shang et al. 2008; Overgard et al. 2009).

Finally, additional co-stimulatory signals are required for the complete activation of naïve T cells. This further stimulation is given by the T-cell receptor CD28, when it binds to their ligands: B7.1 (CD80) and B7.2 (CD86) expressed on the APCs. On the other hand, CTLA-4 (cytotoxic T lymphocyte-associated protein 4) is expressed after T-cell activation and transmits inhibitory signals to T cells. This protein also binds to CD80 and CD86 molecules but with higher affinity than CD28. Both CD28 and CLTA-4, as well as orthologues of CD80 and CD86 ligands, have been found in different teleost species (Bernard et al. 2006; Hansen et al. 2009; Sugamata et al. 2009; Zhang et al. 2009, 2018). The binding sites for CD80 and CD86 are evolutionarily conserved in teleost CD28 and CTLA4 molecules (Bernard et al. 2006). Similarly to mammals, rainbow trout increases

CTLA-4 expression after a viral infection, while CD28 remains constitutively expressed, suggesting similar roles to their mammalian orthologs (Bernard et al. 2006).

3.5.3 T-cell Populations

In comparison with conventional αβ T cells, γδ T cells can directly recognize unconventional antigens such as phosphorylated microbial metabolites and lipid antigens (Hayday 2000). The recognition of this kind of unprocessed antigens seems to be similar to that of pattern recognition receptors. CD4 or CD8 expression is absent in the majority of γδ T cells in agreement with the non-MHC I and MHC II restriction. In mammals, low percentages of γδ T cells are found in the blood (1–10% of all T cells), while higher proportions have been identified in the intestine, lungs, the reproductive tract or the skin, suggesting an essential role of γδ T cells in mucosal immunity (Sheridan et al. 2013). This evidence points to γδ T cells as primitive lymphocytes that seem to be implicated in both innate and adaptive immune responses (Hayday 2000). Regarding teleost fish, γδ T cells have been described in zebrafish, accounting for 8–20% of total lymphocytes in different tissues. These cells were shown to phagocytose soluble and particulate antigens and to initiate antigen-specific $CD4^+$ T-cell proliferation and B-cell activation. They have also been shown to be essential for the production of antigen-specific IgZ secretion in the intestinal mucus (Wan et al. 2016).

Regarding Tc cells, two mechanisms by which these cells kill target cells have been described in mammals: the secretory and the non-secretory pathway. The secretory pathway secretes perforin and granzymes that induce cell apoptosis, whereas the non-secretory pathway uses target cell death receptors, such as Fas, that induce caspase-dependent apoptosis when FasL expressed on activated Tc cells attaches to it (Elmore 2007). In relation to the secretory pathway, perforin-like molecules have been described in different fish species such as Japanese flounder (*Paralichthys olivaceus*) (Hwang et al. 2004), rainbow trout (Athanasopoulou et al. 2009) or zebrafish (Varela et al. 2016). Studies performed in ginbuna crucian carp (*Carassius langsdorfii*) revealed that the mode of action of fish perforin was similar to its mammalian homologue (Toda et al. 2011a). Similarly, fish granzyme has also been found to have a similar primary structure than that of mammals (Praveen et al. 2006; Matsuura et al. 2014). On the other hand, the FasL protein involved in the non-secretory pathway has been identified in gilthead sea bream (*Sparus aurata*), channel catfish and Nile tilapia (*Oreochromis niloticus*) (Cuesta et al. 2003; Long et al. 2004; Ma et al. 2014). Moreover, in Japanese flounder, addition of recombinant FasL in a flounder cell line induced apoptosis, showing a similar mechanism to that of mammals (Kurobe et al. 2007).

As stated above, Th cells play an important role in cooperating with B cells, while they also regulate the immune responses of B cells and other cells through the secretion of different cytokines. After activation, naïve Th cells can differentiate into specific subpopulations characterized by specific cytokine secretion patterns and therefore with

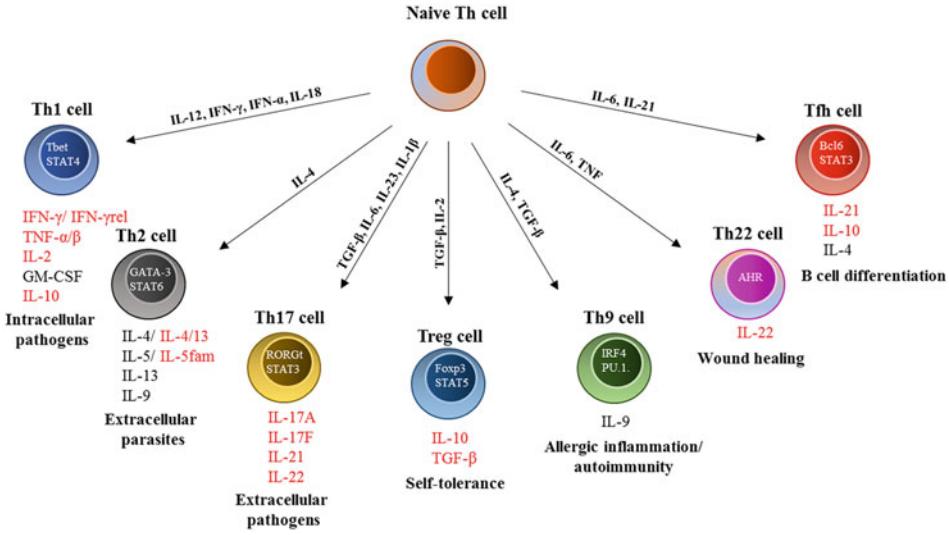

Fig. 3.2 Differentiation of naïve T helper (Th) cells to distinct Th subsets in response to a variety of cytokines. These Th cell subsets express specific transcription factors (indicated inside the cells), secrete specific cytokines (indicated below the cells) and are involved in the immune response against different types of pathogens or immune disorders. Those cytokines identified in teleost fish are highlighted in red

differentiated actions in the immune responses. These Th subsets mainly include Th1, Th2, Th17 and inducible T-regulatory (Treg) cells, although additional subsets are sometimes described in mammals such as Th9, Th22 or Tfh (Geginat et al. 2013). Figure 3.2 shows the main Th cell subsets generated from naïve T cells.

Th1 cells are known to promote cell-mediated immune responses against intracellular viral and bacterial pathogens. The main cytokines produced by Th1 cells upon activation include IFN-γ, IL-2, IL-10 and TNF-α/β. These cytokines induce macrophage activation, nitric oxide (NO) production and the proliferation of Tc cells. The differentiation process to Th1 response is promoted by IL-12, IL-18, IFN-α and IFN-γ, and controlled by the transcription factors T-bet, STAT1 and STAT4. All these molecules have been identified in fish. Two forms of IFN-γ (IFN-γ and IFN-γ-related) have been identified in some species of teleost fish, including Atlantic salmon, rainbow trout and ginbuna crucian carp, while only one form has been found in fugu [reviewed in Pereiro et al. (2019)].

Th2 cells play an important role in the immune response against extracellular pathogens. These cells mainly secrete IL-4, IL-5, IL-9, IL-13 and IL-17E/IL-25. IL-4 regulates clonal expansion of Th2 cells, and together with IL-13, promotes B-cell antibody secretion and alternative macrophage activation. IL-5 stimulates eosinophil activation and survival, and IL-9 promotes mast cell activation. In salmonids, genes with shared homology with IL-4 and IL-13 designated as IL-4/13 have been identified in variable number depending on the fish species (Ohtani et al. 2008; Wang and Secombes 2015; Yang et al. 2016). In rainbow

trout, recombinant IL-4/13A was shown to modulate the expression of several Th2 genes (Wang et al. 2016). Furthermore, GATA3 and STAT6, the transcription factors responsible for Th2 differentiation, have been identified in several fish genomes [reviewed in Nakanishi et al. (2015)].

Th17 cells are involved in immune responses against specific fungi and extracellular bacteria. These cells secrete IL-17A, IL-17F, IL-21 and IL-22 that stimulate chemokine secretion by resident cells leading to the recruitment of neutrophils and macrophages to the sites of inflammation. In teleost, the IL-17 cytokine family has various forms (A-F) of structurally related cytokines to the orthologous proteins in mammals (Kono et al. 2011). The ROR family of transcription factors that control Th17 differentiation have also been identified in fish (Nakanishi et al. 2015).

The main function of Treg cells is to maintain the immune homeostasis, through mechanisms that include the suppression of immune responses and the control of self *versus* non-self recognition. Treg cells are characterized by the expression of the transcription factor FoxP3 and by the secretion of IL-10 and TGF-β. Most of the teleost species studied possess one copy of the IL-10 gene, while two copies have been found in rainbow trout and European common carp (Harun et al. 2011; Piazzon et al. 2017). As per the other Th subsets, the presence of true Treg subsets in fish requires additional investigations to generate a greater understanding and characterization of their response.

On the other hand, Th22 and Th9 subsets have been more recently described in humans and mice. Th9 cells are involved in allergic asthma and autoimmunity and depend on the transcription factor PU.1 and IRF4 (Chen et al. 2019). In turn, Th22 cells express the transcription factor AHR that drives to IL-22 secretion (Jia and Wu 2014). Finally, fTh cells have been sometimes identified as a distinctive Th subtype as they are regulated through an exclusive array of transcription factors. These cells are specialized in inducing B-cell proliferation and differentiation to ASCs through the expression of CD40, ICOSL and the secretion of IL-21, IL-10 or IL-4 (Chtanova et al. 2004).

3.5.4 T-cell Response and Immunity

3.5.4.1 Helper Activity of $CD4^+$ T Cells

The helper function of $CD4^+$ T cells in teleost fish has been assessed in several studies using mixed leucocyte culture (MLC) assays. In these MLC assays, Th responder cells proliferate in response to allogeneic MHC II-expressing APCs. This proliferative capacity has been demonstrated in different fish species after in vitro incubation with allogeneic leucocytes (Meloni et al. 2006). In channel catfish, for example, Ig^- lymphocytes were found to be the responder cells, collaborating with B cells and macrophages in the MLC (Miller et al. 1985). $CD4^+$ and $CD8^+$ T cells both proliferated after the allogeneic stimulation with distinct types of APCs; however, it was after the pre-sensitization of the fish with ovalbumin that an antigen-specific proliferation of $CD4^+$ T cells was observed (Toda et al. 2011b).

When naive ginbuna crucian carp were transplanted with crucian carp necrosis virus (CHNV)-sensitized donor cells, a significantly increased antibody response was reported relative to that observed in fish that received non-sensitized donor cells, or cells in which $CD4^+$ cells had been depleted. This same pattern was observed when cell-mediated cytotoxicity was measured (Somamoto et al. 2014). In rainbow trout, two independent studies have addressed the characterization of $CD4^+$ Th cells. In 2016, Maisey et al. combined antibodies specific for CD3 and CD4-1 to functionally characterize Th cells. They demonstrated that upon stimulation with an antigen, this population proliferated and secreted IFN-γ1, IL-4/13A, IL-15, IL-17D, IL-10 and TGF-β1, suggesting a differentiation to different Th subsets (Maisey et al. 2016). That same year, Takizawa et al. identified two $CD4^+$ Th populations combining monoclonal antibodies against CD4-1 and CD4-2. They found a predominant population, which co-expressed surface CD4-1 and CD4-2 along with a minor subset expressing only CD4-2 (Takizawa et al. 2016). Although both subsets produced equivalent levels of Th1, Th17 and regulatory T-cell cytokines upon bacterial infection, cells expressing only CD4-2 were less proliferative and displayed a more restricted TCRβ repertoire. These data led the authors to hypothesize that these cells represented a functionally distinct population (Takizawa et al. 2016). Similar studies have been undertaken in zebrafish, where an antibody against CD4-1 was combined with an anti-ZAP70 (a T-cell-specific transcription factor) to identify Th cells that increased the expression of cytokines and master transcription factors relevant to Th1/Th2-type responses in response to antigenic stimulation (Yoon et al. 2015).

3.5.4.2 Cytotoxic Activity of $CD8^+$ T Cells

Somamoto and collaborators described Tc virus-specific activity for the first time in fish, generating CHNV-specific ginbuna crucian carp leucocytes after infection with CHNV. This effect was virus-specific and MHC-restricted as in mammals (Somamoto et al. 2004). In addition, the production of virus-specific cytotoxic T cells in MLC has been shown in ginbuna crucian carp. This response seems to be antigen-specific since the effector cells lysed CHNV-infected syngeneic cells but not CHNV-infected allogeneic cells or non-infected syngeneic cells (Somamoto et al. 2009).

Moreover, when PBLs from viral haemorrhagic septicaemia virus (VHSV)-infected rainbow trout were cultured with MHC I-matched VHSV-infected cells, these were efficiently lysed (Utke et al. 2007). This activity went along with an increased expression of CD8 and the natural killer cell enhancement factor (NKEF)-like gene in the leucocyte cultures, suggesting an important role of NK and Tc cells in VHSV protection. Further investigations in rainbow trout responses against VHSV showed that PBLs from fish immunized with a DNA vaccine coding for the VHSV G protein killed both RTG2 (VHSV-infected MHC I-matched target) and EPC (VHSV-infected xenogeneic target) cell lines. Interestingly, the PBLs obtained from fish immunized with a DNA vaccine coding for the VHSV N protein only lysed VHSV-infected RTG-2 cells and not EPC cells. This phenomenon suggests that the G protein has the capacity to activate both adaptive and

NK cell-mediated responses, whereas the N protein would not activate innate cells (Utke et al. 2008).

Regarding bacterial infections, specific Tc cells were shown to have an important role in protection against the intracellular bacteria *Edwarsiella tarda* in ginbuna crucian carp (Yamasaki et al. 2013). Moreover, adoptive transfer of sensitized lymphocytes against *E. tarda* to isogenic naïve ginbuna carp showed that both $CD4^+$ and $CD8^+$ T cells conferred protection against infection. Sensitized $CD8\alpha^+$ cells increased the transcription of IFN-γ and perforin, highlighting the crucial role of cell-mediated cytotoxicity and IFN-γ in this response (Yamasaki et al. 2014). In addition to their specific cytotoxic activity, mammalian Tc cells were shown to have the capacity of directly killing extracellular pathogens through what seems to be an MHC-independent process. In teleost fish, $CD8\alpha^+$, $CD4^+$ T cells and surface IgM^+ cells were shown to all have the capacity to directly destroy bacteria. In both sensitized and non-sensitized ginbuna carp, $CD8\alpha^+$, $CD4^+$ T cells and $sIgM^+$ cells showed antibacterial activity against extracellular and cell-associated bacteria, with lower killing activity in non-immunized fish (Nayak and Nakanishi 2013).

3.6 Conclusion

Fish and mammals appear to share many components of the adaptive immune system. Still, pronounced differences exist concerning the sophistication of this arm of the immune system, in which mammals have possibly reached a greater degree of evolution than that of fish. Collectively, the knowledge reviewed within this chapter has deepened our understanding of the two key processes of adaptive immunity: specific antigen recognition and immunological memory development. These processes depend on both the humoral and cell-mediated arms of the adaptive immune system via the production of effector cells and memory cells.

Although vaccines are available for some fish diseases, often the efficacy is not ideal, while for other diseases no vaccine is available. As adaptive immunity is the immunological baseline for vaccination, a greater elucidation of the features and mechanisms of the adaptive immune system are needed to refine the design of these preventive tools. In this context, recent biotechnology advancements in omics have improved the ability to precisely analyse the molecules involved in the immune response. In particular, transcriptomics are becoming more widely applied in fish immunology as they afford a robust, cost-effective method of quantifying many genes within species. However, reliance of a singular approach cannot suffice for providing a complete understanding of an immune response and this is something that requires complementary approaches in terms of the development of new tools and assays, such as specific antibodies against the different lymphocyte subpopulations. Still, there is great potential to expand our knowledge of the adaptive immune response and to improve our universal understanding of the evolution of the immune system.

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