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REVIEW

Innate Immune Responses in Fish: Antigen Presenting Cells and Professional Phagocytes

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Abstract

Similar to higher vertebrates, the immune system of fish is composed of two major components, innate (non-specific) and adaptive (specific) immune responses. However, the innate immune system in fish has a fundamental importance in preventing pathogen entry as the adaptive immune responses are less efficient compared to mammals. The components of the innate immune system in fish are commonly divided into three compartments: physical parameters, humoral parameters, and cellular factors.

Recently, the fish professional APCs received more attention resulting in the increased numbers of studies on their morphology and function. Following a lengthy gap, in the last decades, considerable progress has been made in the mechanistic understanding of fish APC-dependent immune responses. Dendritic cells (DCs), the universal APCs and the major players in bridging and shaping both innate and adaptive immune responses have been characterized in several teleost fish based on their morphology and function. In addition to innate immunity, macrophages have been demonstrated as essential in initiation of adaptive immunity as another professional APCs in teleost fish. Like in mammals, teleost B cells were characterized as important APCs that activate naïve T cells and initiate adaptive immunity.

In this study, we provide an overview of innate immune responses in teleost fish and discuss the current status of the field of teleost fish DCs, macrophages and B cells as professional APCs.

Keywords: Dendritic cells, macrophages, B cells, pattern recognition receptors, innate immune memory.

Introduction

The vertebrate immune system is a large, complex network that includes multiple tissues, organs, cells and molecules focusing on host defense (Zimmerman, Vogel, & Bowden, 2010). The cells and molecules of the immune system are equipped to recognize and destroy foreign substances, in particular, pathogens, thus protecting organisms against diseases and maintaining homeostasis (Chaplin, 2010). Fish are a highly diverse group of organisms representing the earliest vertebrates, including the Agnatha (hagfish and lamprey), Chondrichthyes fish (sharks and rays), and Osteichthyes (teleost or bony fish) (Bolis, Piccolella, Dalla Valle, & Rankin, 2001). Similar to higher vertebrates, the immune system of fish is composed of two major components, innate (non-specific) and adaptive (specific) immune responses. However, the innate immune system in fish has a fundamental importance in preventing pathogen entry as the adaptive immune responses are less efficient compared to mammals. Therefore, in this review, we aim to highlight recent knowledge, including the data obtained in our laboratory, on the innate immune mechanisms mediated and controlled by professional antigen presenting cells (APCs) in teleost fish.

Fish Immune System

The fish immune system is composed of innate (non-specific) and adaptive (specific) immune mechanisms. Innate immunity components respond to pathogenic organisms by recognizing pathogen-associated molecular patterns (PAMPs), which are not expressed in host cells. Namely, lipopolysaccharides (LPS) in Gram-negative bacterial cell wall, lipoteichoic acid (LTA) in Gram-positive bacteria, phospholipomannan, beta-glucan, and chitin in fungi, hemagglutinin in viruses are among the most common PAMPs recognized by the innate immune receptors (Mogensen, 2009; Silva-Gomes, Decout, & Nigou, 2015; Taghavi, Khosravi, Mortaz, Nikaein, & Athari, 2017). Following pathogen recognition, one of the

effector mechanisms of innate immunity is the destruction of pathogens by phagocytosis. In contrast, adaptive immunity components recognize pathogens by highly specific receptors generated by V(D)J recombination and somatic hypermutation, resulting in proliferation and differentiation of specific B and T-cells clones (Bonilla, 2010).

General Properties of Fish Innate Immunity

Similarly to mammals, the innate immune system in fish is the first line of defense that reacts to pathogens within a very short time and does not provide a long-lasting protection (Turvey & Broide, 2010). However, unlike in mammals, the innate immune system of fish is a fundamental component in preventing pathogen entry due to the inefficiency of the adaptive immune response (Whyte, 2007). The evolutionary position and poikilothermic nature of fish affect the efficiency of adaptive immunity due to slow lymphocytes proliferation and maturation, memory formation and limited antibody repertoire (Magnadóttir, 2006). Fish contain most of the primary and secondary lymphoid organs present in mammals except bone marrow and lymph nodes. However, the structure of these organs in fish lacks the complexity compared to the mammalian counterparts leading to potential limitation and delay in the generation of fully functional adaptive immune responses (Firdaus-Nawi & Zamri-Saad, 2016; Tort L, 2003).

The components of the innate immune system in fish are commonly divided into three compartments: physical parameters, humoral parameters, and cellular factors (Uribe, Folch, Enriquez, & Moran, 2011) (Figure 1). The physical barriers include fish scales, a mucus layer, and epithelial cells, which line the skin, gills, and alimentary tract, providing a crucial role in combating infection (Ellis, 2001; Magnadottir, 2010). Goblet cells produce a mucus layer in which antimicrobial substances trap pathogens and inhibit the spread of infection (Alexander & Ingram, 1992; Harris & Hunt, 1975). Fish mucus contains a wide range of immune substances, in particular, lectins, pentraxins, lysozyme, complement proteins, antibacterial peptides and IgM), which are capable of inhibiting pathogen entry or elimination (Aranishi & Nakane, 1997; Boshra, Li, & Sunyer, 2006; Rombout, Taverne, van de Kamp, & Taverne-Thiele, 1993; Saurabh & Sahoo, 2008). In addition to the mucus barrier, the epidermis, composed of epithelial cells, prevents the entry of foreign materials and is populated with effector cells, such as macrophages and lymphocytes (Esteban, 2012; Fischer *et al*., 2006). Both mucus and epithelial cells act as physical and chemical barriers against microorganisms and foreign agents (Firdaus-Nawi & Zamri-Saad, 2016).

Among the humoral immune parameters that include cell receptors or soluble molecules, teleost fish contain a large number of non-specific defense substances, such as transferrin, antimicrobial peptides, lysozyme, lectins, natural antibodies, cytokines, and complement components, which are able to kill microorganisms or inhibit their growth (García-Fernández, Sánchez, & Blanco, 2011; Magnadóttir,

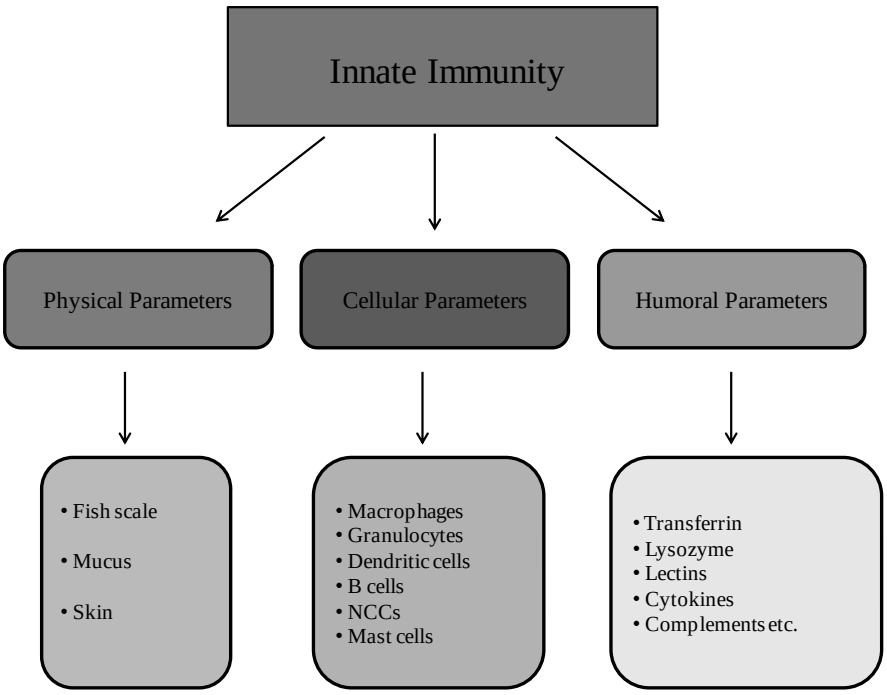

Figure 1. The components of the fish innate immune system. The fish innate immune system is composed of three main parameters: 1) Physical parameters, 2) Cellular parameters, and 3) Humoral parameters.

2006). The function of these humoral factors was reviewed and discussed in multiple studies previously (Firdaus-Nawi & Zamri-Saad, 2016; Uribe *et al*., 2011). In this review, we will focus on the role of cellular parameters and receptors of innate immunity.

Innate Immune Receptors in Teleost Fish

The innate immune response to non-self or self-antigens relies on the germline-encoded pattern recognition receptors (PRRs) that recognize PAMPs or danger-associated molecular patterns (DAMPs) (Santoni *et al*., 2015). Non-self or exogenous antigens are not expressed in the host, and PAMPs are derived from diverse pathogens. In contrast, DAMPs are intracellular molecules which are secreted to the extracellular environment from apoptotic cells or damaged extracellular matrices, such as host DNA and RNA, high-mobility group box 1 (HMGB1) protein, S100 proteins, and heat-shock proteins (HSP) (Land, 2015; Rosin & Okusa, 2011).

In mammals, PRRs can be divided into five groups based on their protein domain homology as follows: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding domain (NOD), leucine-rich repeat (LRR)-containing (NOD-like) receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), and the absent in melanoma-2 (AIM-2)-like receptors (ALRs) (Brubaker, Bonham, Zanoni, & Kagan, 2015). These receptors can be localized at the different compartments of cells; for example, TLRs and CLRs are found at the cell surface or endocytic compartment whereas NLRs, RLRs, and ALRs are located in the cytoplasm (Takeuchi & Akira, 2010; Thompson, Kaminski, Kurt-Jones, & Fitzgerald, 2011). The cell surface classes of PRRs sense the extracellular environment for the presence of pathogen ligands, while the cytoplasmic PRRs survey the presence of intracellular pathogens. Importantly, endosomal types of PRRs detect pathogens which are engulfed into the phagolysosome (Kawai & Akira, 2009).

Following the recognition of PAMPs or DAMPs, PRRs induce signaling cascades, which lead to the NF-κ and interferon (IFN) response factor (IRF) transcription factors activation, thus triggering the production of pro-inflammatory cytokines, chemotactic cytokines, and interferons (Li, Li, Cao, Jin, & Jin, 2017). Furthermore, activation of PRRs also lead to the nontranscriptional innate immune responses, such as phagocytosis, autophagy, cell death, and cytokine processing (Deretic, Saitoh, & Akira, 2013; Drummond & Brown, 2011; Lamkanfi & Dixit, 2014).

Similar to the mammalian innate immune system, teleost fish have multiple PRRs to recognize non-self and self-antigens. Surprisingly, fish possess higher numbers of different TLRs compared to mammals (Takano *et al*., 2011). Currently, at least 17 or 18 TLRs have been identified in teleost fish, and the following TLRs are conserved in both mammals and fish: TLR1, TLR2, TLR3, TLR5, TLR7, TLR8, and TLR9, whereas mammalian TLR4 has been detected only in zebrafish (Takano *et al*., 2011). Several novel TLR genes, such as TLR14, TLRs 21-23 have been identified in fugu, while TLRs18-22 have been identified in zebrafish (Jault, Pichon, & Chluba, 2004; Meijer *et al*., 2004; Oshiumi *et al*., 2003; Roach *et al*., 2005). Furthermore, 20 different TLRs have been described in channel catfish, including the unique TLRs such as TLR25 and TLR26, which were not found in other vertebrates, and TLR26 was found to be a channel catfish-specific (Quiniou, Boudinot, & Bengtén, 2013).

Multiple CLR genes have been identified in fish, namely, mannose receptor in seabream and grass carp, and CLR-like protein A, B, and C genes in Atlantic salmon (Rodríguez, Esteban, & Meseguer, 2003; Soanes, Figuereido, Richards, Mattatall, & Ewart, 2004; Wang *et al*., 2014). Furthermore, a lectin-like receptor gene was described in zebrafish, and a CLR-like protein gene was detected in ayu (Chen, Lu, Yang, & Shi, 2010; Panagos *et al*., 2006). Recently, a novel CLR gene has been identified and tentatively named PaCD209L in ayu (Yang, Lu, Chen, & Chen, 2015).

Teleost fish also contain the NLR family of PRRs. For example, the members of NLR family, NOD1, NOD2, and NOD3 that are conserved between zebrafish and mammals have been identified in zebrafish (van der Vaart, Spaink, & Meijer, 2012). NOD1 was also detected in rainbow trout, channel catfish, and goldfish, and its expression was increased in goldfish infected with *Aeromonas salmonicida* or *Mycobacterium marinum* (Jang, Kim, Kim, & Cho, 2016; Li *et al*., 2012; Xie, Hodgkinson, Katzenback, Kovacevic, & Belosevic, 2013). Moreover, the mammalian analog of the NLR subfamily member NALP was detected in zebrafish (Laing, Purcell, Winton, & Hansen, 2008; Stein, Caccamo, Laird, & Leptin, 2007). On the other hand, no counterparts of members of ALR family have been detected in teleost fish species yet (Aoki, Takano, & Hikima, 2015).

Innate Immunity and Immunological Memory

According to the well-accepted definition of innate immunity, the innate immune cells are thought to respond rapidly in a non-specific manner to pathogens and to be short-lived (Sun, Ugolini, & Vivier, 2014). However, a new paradigm has emerged in the past decade suggesting that natural killer (NK) cells can acquire memory from their first encounter with the pathogen and exhibit more robust and fast responses during the subsequent encounter with the antigen. Also, recent studies demonstrate that NK cells have specific adaptive immune features like specificity and memory against numerous pathogens

and foreign chemicals (Cooper & Yokoyama, 2010; Marcus & Raulet, 2013; O'Sullivan, Sun, & Lanier, 2015). Furthermore, certain infections or vaccinations, which are mediated by prototypical cells of innate immunity, such as NK cells and monocytes/macrophages, can trigger the enhanced features of innate immunity (Netea, 2014). The following mechanisms were suggested to mediate innate immune memory or also termed "trained immunity": changes at the level of membrane receptors of NK cells and epigenetic reprogramming of monocytes/macrophages through histone modifications (Quintin *et al*., 2012; Sun, Beilke, & Lanier, 2009). Trained immunity offers protection upon second encounter of the same antigen in a T and B cell-independent manner and may result in increased resistance to re-infection, and NK cells and monocytes/macrophages are vital players in training immunity (Netea, Quintin, & van der Meer, 2011; Quintin *et al*., 2012).

In mammals, β-glucans are known to be potent inducers of trained immunity. Similar to mammals, β-glucans can stimulate the trained immunity of teleost fish species, such as zebrafish, Atlantic salmon, rainbow trout, and sea bass (Petit & Wiegertjes, 2016). Preliminary evidence for the presence of trained immunity is also documented in fish. For example, vaccination of Japanese flounder with Bacillus Calmette–Guérin (BCG) triggered the upregulation of pro-inflammatory cytokines and provided a more robust protection against *Mycobacterium* sp. (Kato, Kondo, Aoki, & Hirono, 2010). Like Japanese flounder, vaccination of amberjack with BCG resulted in increased protection against challenge with *Mycobacterium* sp. (Kato *et al*., 2011).

Antigen Presenting Cells

In mammals, there are three professional antigen presenting cells (APCs) described, that have crucial roles in recognition of pathogens by PRRs and activation of adaptive immunity by the presentation of antigens to naïve T cells. As professional APCs, dendritic cells (DCs), monocyte/macrophages and B cells, engulf pathogens and process antigen to peptides. Following antigen processing, APCs present peptides by major histocompatibility complex (MHC) molecules (Drutman & Trombetta, 2010). For example, intracellular antigens are presented by MHC class I molecules to cytotoxic T cells (CD8+ T cells), while extracellular antigens are presented by MHC class I molecules to helper T cells (CD4+ T cells) (Keech, Pang, McCluskey, & Chen, 2010; Vyas, Van der Veen, & Ploegh, 2008) (Figure 2). Upon activation, another critical feature of APCs is the expression of co-stimulatory molecules, such as B7-1 (CD80), and B7-2 (CD86), which are necessary to prime naïve T cells and induce the differentiation of T cells by producing cytokines (Chen & Flies, 2013). Recently, the fish professional APCs received more attention resulting in the increased numbers of studies on their morphology and function. In this review, we discuss the data obtained in our and other studies on the important functions of teleost fish APCs to uptake and process antigens and provide three crucial signals (antigen-specific, co-stimulatory, and cytokine) to naïve T cells.

Dendritic Cells

Dendritic cells (DCs) are the universal and powerful APCs that are critical players in bridging and shaping both innate and adaptive immune responses in vertebrates (Mildner & Jung, 2014). They are present in most tissues and sense the environment for foreign materials and self-antigens, and are able to capture pathogens or antigens and travel to the secondary lymphoid tissues for presentation to T cells to regulate adaptive immune responses (Alvarez, Vollmann, & von Andrian, 2008; Summerfield, Auray, & Ricklin, 2015). Recently,

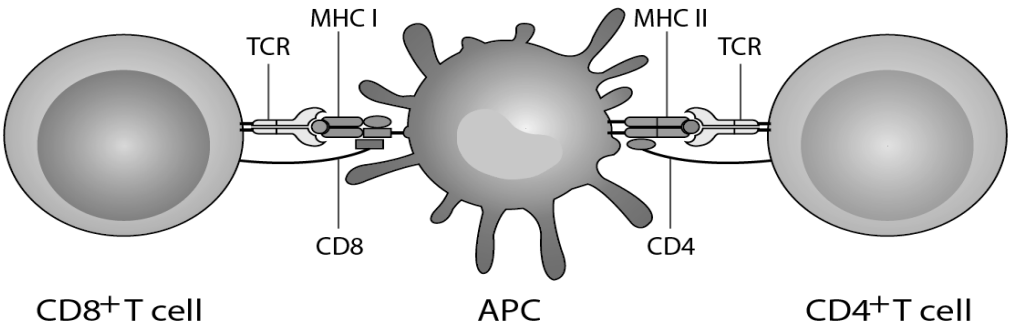

Figure 2. APC-T cell interactions. Intracellular antigens are presented by MHC class I molecules to $CD8^+$ T cells. The TCRs on $CD8^+$ T cells recognize the antigens, and CD8 molecules interact with MHC I molecules. On the other hand, extracellular antigens are presented by MHC class II molecules to $CD4^+$ T cells. TCRs recognize the antigens, and CD4 molecules interact with MHC class II molecules.

DCs have been characterized in several teleost fish based on their morphology and function. For example, DC-like cells with mammalian DC morphology were identified in cell cultures from whole kidney marrow of zebrafish, and T-cell stimulatory capability of these cells have been documented in zebrafish (Lugo-Villarino *et al*., 2010; Wittamer, Bertrand, Gutschow, & Traver, 2011). Moreover, non-adherent cells with dendritic morphology in rainbow trout were identified in hematopoietic cultures including anterior kidney (AK), the anterior portion of trunk kidney, and spleen (Bassity & Clark, 2012). In addition to morphology, DCs in rainbow trout showed active motility, and their migration *in vivo* to mucosal and lymphoid tissues were determined by flow cytometry (Bassity & Clark, 2012). Also, the same study reported that DCs in rainbow trout could phagocyte fluorescent beads, and these cells are activated by TLR-ligands (imiquimod, Poly I:C, ssRNA, and flagellin) and showed aggregation (Bassity & Clark, 2012). Furthermore, DCs in barramundi were identified in hematopoietic cell cultures, and these cells have with many large dendrites which extend from the cell body (Zoccola, Delamare-Debouteville, & Barnes, 2015). Also, migration and phagocytic abilities of DCs in barramundi have been demonstrated by the same study. After exposure to TLR ligands (LPS and PTG) and two strains of barramundi primary bacterial pathogens, *Streptococcus iniae (*strains QMA0076 and QMA0248), DCs actively migrated towards PTG and both *S. iniae* strains (Zoccola *et al*., 2015). Barramundi DCs were also able to uptake micro-beads and two strains of *S. iniae after two hours incubation, and the proliferation assay showed that DCs in barramundi capable to stimulate the proliferation of T cells (Zoccola et al., 2015). Another study reported DCs with* long cytoplasmic extensions and an eccentric nucleus in medaka (Aghaallaei, Bajoghli, Schwarz, Schorpp, & Boehm, 2010).

Professional APCs in mammals express co-stimulatory molecules which play a crucial role in priming naïve T-cells (Chen & Flies, 2013). A previous study reported the presence of the major co-stimulatory molecules (e.g., CD80/CD86 and CD83) in zebrafish (Lin *et al*., 2009). Furthermore, a recent study showed that the surface molecules of zebrafish DCs (CD80/86/83/CD209$^+$) could promote CD4$^+$ naïve T cell stimulation as in mammals (Shao *et al*., 2015). Moreover, the expression of CD83 in rainbow trout DCs significantly increased after the TLR-ligands treatment, and also, MHC II expression on their surface was higher than macrophages and B cells (Bassity & Clark, 2012). Furthermore, DC-SCRIPT, a specific molecular marker for DCs, and MHC II were expressed by barramundi DCs (Zoccola *et al*., 2015). Also, PTG and LPS were injected to barramundi, and DC-SCRIPT expression increased in spleen and AK at the beginning of infection, and then its expression decreased in both organs after 3 days post injection, but the expression of DC-SCRIPT increased again in spleen at 7 days post-injection (Zoccola *et al*., 2015). This study showed that immature DCs migrated from AK and spleen to the site of infection, and migrated back the spleen for antigen presentation (Zoccola *et al*., 2015).

Dendritic cells in mammals have different subsets, and the Langerhans cell (LC) is a unique subset of DCs present in the epidermis of the skin. This unique location provides LCs with the ability to recognize pathogens, foreign chemicals, and self-antigens soon after invasion (Igyártó & Kaplan, 2013). Langerhans cells are able to engulf antigens and migrate to the secondary lymphoid tissues to present the antigen to T cells, thus initiating adaptive immune responses (Sugita *et al*., 2007). Langerhans cells are characterized by the presence of Birbeck granules (BGs), which are rod-shaped organelles consisting of superimposed and zippered membrane (Mc Dermott *et al*., 2002). Langerin is a type II transmembrane C-type lectin and specific marker for LCs, also associated with the formation of BGs (Lau, Chu, & Weiss, 2008; Valladeau *et al*., 2000). The antigen capture function of Langerin triggers the induction of BGs by allowing routine antigens into BGs, thus providing non-classical antigen processing pathway and cross-presentation (Fehres *et al*., 2015; Valladeau *et al*., 2000).

Several studies identified cells with mammalian LCs morphology in teleost fish. In particular, Langerin/CD207$^+$ (L/CD207$^+$) cells were described in the AK and spleen of Atlantic salmon and rainbow trout (Lovy, Wright, & Speare, 2008). Also, L/CD207$^+$ cells have been identified in the gills of Chinook salmon during *Loma salmonae* infection (Lovy, Wright, & Speare, 2006). Furthermore, BG-like granules were observed in the lymphoid organs (spleen and AK) of Atlantic salmon and rainbow trout (Lovy *et al*., 2008). In addition, BG-like granules in the cytoplasm of DCs were observed in the skin of zebrafish (Lugo-Villarino *et al*., 2010). Recently, our group identified L/CD207$^+$ cells in the AK, spleen, and gill of channel catfish by immunohistochemistry (Figure 3) (Kordon *et al*., 2016). Additionally, BG-like granules in our study were observed in the DC-like cells of the spleen, anterior and posterior kidneys, and gill by transmission electron microscopy of channel catfish (Kordon *et al*., 2016).

Macrophages

Macrophages are large mononuclear cells present in virtually all animal tissues. Until quite recently, according to the mononuclear phagocytes system theory, peripheral blood monocytes in vertebrates were considered the progenitors for the tissue macrophages (van Furth *et al*., 1972). However, recent evidence demonstrates that self-maintaining resident populations of tissue macrophages derived from different sources, such as yolk sac and fetal liver during embryonic development of mammals

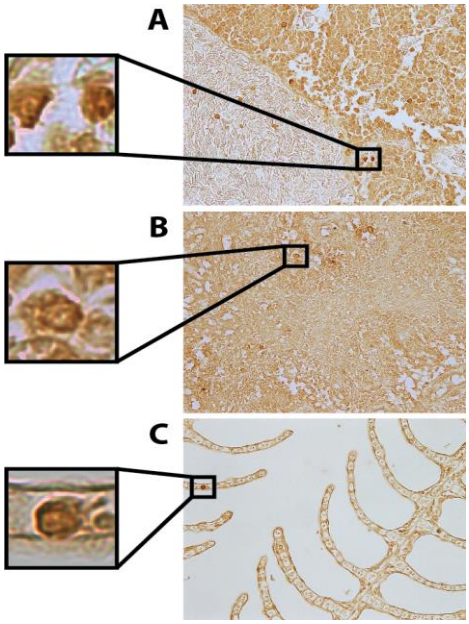

Figure 3. Langerin$^+$ cells in channel catfish, *Ictalurus punctatus.* A) Anterior kidney contains more Langerin$^+$ cells than spleen (B). Langerin$^+$ cells were scarce in the gill of channel catfish (C).

(Ginhoux & Jung, 2014; Perdiguero & Geissmann, 2016). In addition, blood monocytes contribute to tissue-resident macrophage populations during inflammatory conditions and the depletion of resident macrophages in their environment (Hashimoto *et al*., 2013; Serbina, Jia, Hohl, & Pamer, 2008; Varol, Mildner, & Jung, 2015). The similar developmental pattern has also been determined in teleost fish. In zebrafish, macrophages are produced for the first time from lateral plate mesoderm during primitive hematopoiesis at the 12–24 h post-fertilization period (Herbomel, Thisse, & Thisse, 1999; Rombout, Huttenhuis, Picchietti, & Scapigliati, 2005). Furthermore, another study showed that during the absence of definitive hematopoiesis, tissue macrophages had been observed in zebrafish lacking *c-myb*, which is the transcription factor and the key regulator of definitive hematopoiesis in mice (Soza-Ried, Hess, Netuschil, Schorpp, & Boehm, 2010). Like mammals, monocytes give rise to macrophages in fish during inflammation (Hodgkinson, Grayfer, & Belosevic, 2015).

Macrophages are professional phagocytes and possess germline-encoded PRRs which recognize the PAMPs, such as LPS from the Gram-negative bacterial cell wall, peptidoglycan, and LTA from the gram-positive bacterial cell wall (Akira, Uematsu, & Takeuchi, 2006; Gordon, 2007; Silva & Correia-Neves, 2012). Several PRRs that are described in fish are (TLRs), RIG-I-like receptors (RLRs), Fc receptors, complement components, and scavenger receptors which recognize different PAMPs (Meng, Zhang, Guo, Xiang, & Shao, 2012; Poynter, Lisser, Monjo, & DeWitte-Orr, 2015; Smith *et al*., 2011). Following the recognition of pathogens via different PRRs, macrophages attach and ingest pathogens into vesicles known as phagosomes that fuse with lysosomes to form phagolysosomes (Esteban, Cuesta, Chaves-Pozo, & Meseguer, 2015). Macrophages produce antibacterial substances, such as reactive oxygen and nitrogen species which kill and destroy the pathogen in the phagolysosomes (Esteban *et al*., 2015; Sharp & Secombes, 1993) (Figure 4). Therefore, macrophages have a crucial role in innate immunity for the clearance of pathogens and infections.

Multiple studies reported that AK macrophages in teleost fish showed robust phagocytic capability and bactericidal activity against intracellular pathogens, such as parasites, yeast, and bacteria (Bennani, Schmid-Alliana, & Lafaurie, 1995; Dieter & Katharina, 1997; Esteban, Mulero, Munoz, & Meseguer, 1998; Muñoz, Álvarez-Pellitero, & Sitjà-Bobadilla, 2000; Qiu *et al*., 2016). In addition to AK macrophages, peritoneal macrophages separation method has been established for *in vitro* immunologic studies in catfish (Jenkins & Klesius, 1998). Seabass peritoneal macrophages showed significantly higher phagocytic activity against *Escherichia coli and Salmonella typhimurium* than monocytes and macrophages which were obtained from blood and AK (Esteban & Meseguer, 1997). Moreover, phagocytosis of *Yersinia ruckeri* and *Photobacterium damselae piscicida* by peritoneal macrophages was significantly greater than other phagocytic cells in the peritoneal cavity of sea bass and rainbow trout (António, Susana, Joana, Anthony, & Manuel, 1998; Do Vale, Afonso, & Silva, 2002). Also, the phagocytic and microbicidal activity of peritoneal macrophages were described in many fish species,

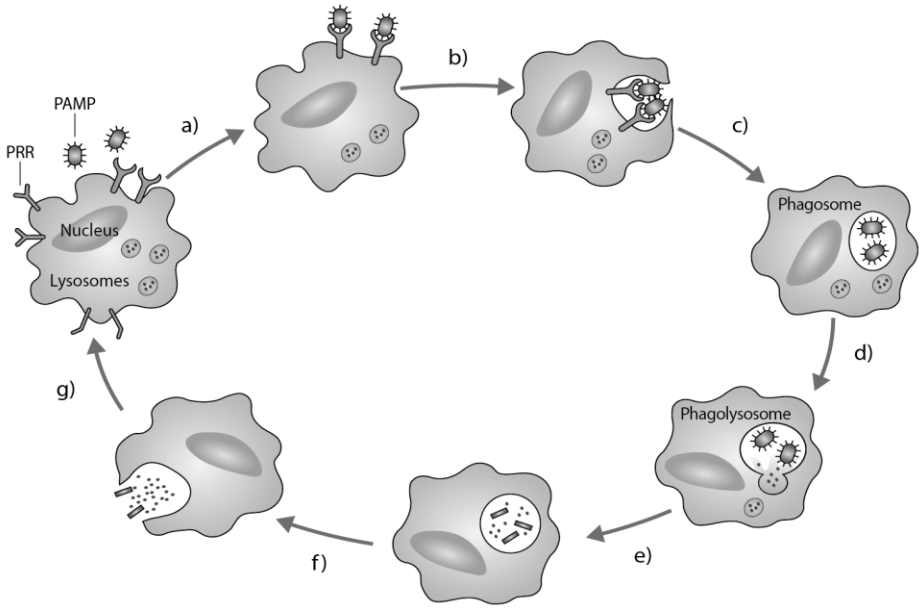

Figure 4. Antigen uptake and processing by phagocytosis in macrophages. a) Recognition: macrophage contains PRRs which can recognize and interact with PAMPs; b) Engulfment: macrophage engulfs the pathogen via endocytic PRRs; c) Phagosome formation: phagosome is formed in the cytosol; d) Phagolysosome formation: Phagosome fuses with lysosome to form phagolysosome; e) Pathogen destruction and elimination: pathogen is destroyed and killed by enzymes in phagolysosomes; f) Discharge, macrophage discharges the waste of pathogen.

such as rohu, walking catfish, and flounder (Awasthi, Rathore, Sood, Khan, & Lakra, 2015; Bodammer & Robohm, 1996; Rashid, Sardar M., & M.R., 2002).

Several studies from humans and other mammals documented the high-intensity antigen uptake at 37^{o} C; however, the antigen uptake intensity was low at background levels of endocytosis at 4^{o} C in professional APCs (Ammari, Harris, Stokes, Bailey, & Pinchuk, 2014; Boyd, Lee, Kruger, & Pinchuk, 2004). Interestingly, our research group observed active uptake of *Edwardsiella ictaluri* at 32^{o} C and 4^{o} C in catfish peritoneal macrophages, but uptake intensity of this pathogen was significantly higher at 32^{o} C (Kordon *et al*., 2018). Several factors may be necessary for antigen uptake at low temperatures. The poikilothermic nature of fish may play an important role in endocytosis, and there are phenotypic and functional interspecies differences in particular macrophages, between APCs.

In mammals, macrophages have distinct subsets based on their functions, classically activated macrophages (M1 macrophages) and alternatively activated macrophages (M2 macrophages) (Hodgkinson *et al*., 2015). M1 macrophages mediate host defense against a diverse group of pathogens, such as bacteria, viruses, and protozoa, and are activated by PAMPs and the cytokines TNF-α or IFN-γ (e.g., LPS) (Italiani & Boraschi, 2014; Murray & Wynn, 2011; Zhou *et al*., 2014). Activated M1 macrophages produce pro-inflammatory cytokines (e.g., TNFs) and IL-1) and secrete nitric oxide (Arango Duque & Descoteaux, 2014). Therefore, M1 macrophages facilitate antimicrobial and antitumor activity and also prevent cell proliferation (Mills, 2012). On the other hand, M2 macrophages have anti-inflammatory function and produce growth factors (such as transforming growth factor-beta (TGF-β)) and ornithine instead of nitric oxide, thus promoting cell proliferation and repair, wound healing, and fibrosis (Martinez & Gordon, 2014; Mills, 2012). Furthermore, M2 macrophages are divided into three subclasses: M2a, M2b, and M2c which are activated by different chemokines (Hodgkinson *et al*., 2015). Macrophages are able to switch from one functional phenotype to another type in response to environmental signals (Murray & Wynn, 2011). In teleost fish, M1 and M2 macrophages are characterized by their similarity to mammalian macrophage function and phenotype (Neumann, Stafford, & Belosevic, 2000). Recent research demonstrated the polarization of M1 macrophages into M2 macrophages in zebrafish in response to environmental cues (Nguyen-Chi *et al*., 2015).

In addition to innate immunity, macrophages are essential in initiation of adaptive immunity as another professional APCs. Like DCs, macrophages sense signals from the microenvironment to orchestrate innate and adaptive immune responses. In mammals, intestinal macrophages recognize pathogens during inflammation, and inflammatory monocytes ($Ly6C^{+}$ monocytes) are recruited to the site of infection and differentiate into inflammatory macrophages which present antigens to T cells, thus promoting the differentiation of Th1 and Th17 cells

(Flannigan, Geem, Harusato, & Denning, 2015). Macrophages are capable of expressing MHC Class I, MHC Class II and co-stimulatory molecules; however, the expression level of these molecules is markedly lower in macrophages compared to DCs (Wu & Kaiser, 2011). In zebrafish, macrophages enable to express MHC class II molecules which showed similar role to mammalian counterparts (Lieschke, Oates, Crowhurst, Ward, & Layton, 2001; Wittamer *et al*., 2011). Furthermore, piscine macrophages also express MHC class II molecules and have a role as an APC (Brubacher, Secombes, Zou, & Bols, 2000; Vallejo *et al*., 1992). Moreover, monocytes in fugu are APCs as they expressed B7 molecules which regulate T cell responses (Sugamata, Suetake, Kikuchi, & Suzuki, 2009).

B cells

B cells in mammals are composed of different subsets with different functions and locations; B-2 cells (or follicular B cells), B-1 cells, and marginal zone (MZ) B cells (Zhang, 2013). B-2 cells respond to thymus-dependent antigens and require the cooperation of T helper cells in germinal centers; therefore, they produce high-affinity antibodies with precise antigen specificity (Tafalla, González, Castro, & Granja, 2017). However, B-1 and MZ B cells are responsible for immune responses against thymus-independent antigens and do not need help from T helper cells, thus producing low-affinity antibodies with broad reactivity which provide fast protection, in particular at the mucosal interfaces (Tafalla *et al*., 2017; Zhang, 2013). Furthermore, B-1 cells are subdivided into two classes; B-1a cells, that express CD5, a surface marker, and B-1b cells that lack the expression of CD5 (Rothstein, Griffin, Holodick, Quach, & Kaku, 2013). Although the primary function of B cells is to produce antibodies, they also have a role in innate immunity. The long-held paradigm dictates that B cells are unable to uptake large particles; however, it has been broken by the recent discovery that human primary B cells engulfed live *Salmonella typhimurium* in B cell receptor (BCR)-mediated manner (Souwer *et al*., 2009). Another study showed the phagocytic ability of murine B cells from bone marrow, spleen, blood, and the peritoneal cavity where the largest percentage of phagocytic B cells (~10-17%) were present in the peritoneal cavity, while other tissues contained B cells with phagocytic capacity less than 1.6% (Parra *et al*., 2012). Interestingly, phagocytic B cells in the peritoneal cavity were B-1 cells, which had engulfed latex beads and bacteria, and the phagocytic ability of B-1a subset (14-17%) was significantly higher than the other subset, B-1b cells (8.6-11.4%) (Parra *et al*., 2012). Moreover, both subsets of B-1 cells were capable of maturation of their phagosome into phagolysosomes and also killed internalized bacteria, and they presented ingested antigen to CD4+ T cells (Parra *et al*., 2012).

Similar to B-1 cells of mammals, teleost B cells are capable of ingesting particles and intracellular killing of ingested microbes (Li J *et al*., 2006; Sunyer, 2013). For example, B cells in zebrafish have strong phagocytic ability for both soluble and particulate antigens (Zhu *et al*., 2014; Zhu *et al*., 2013). Moreover, phagocytic ability of B cells was described in the rainbow trout, and the phagolysosome formation was observed; therefore, B-cells in rainbow trout contribute the bacterial killing (Li J *et al*., 2006; Sunyer, 2012). Furthermore, in Atlantic salmon and Atlantic cod, B cells were also able to phagocyte fluorescent beads and also showed higher phagocytic capacity than neutrophils in cod (Øverland, Pettersen, Rønneseth, & Wergeland, 2010). Our research showed that B cells in AK of channel catfish were able to uptake and destroy *E. ictaluri* (Kordon *et al*., unpublished observation).

Like mammals, teleost B cells serve as a professional APC. Zebrafish B cells are able to present both soluble and particulate antigens to prime naïve $CD4^+$ T cells (Zhu *et al*., 2013). This study showed that the expression of MHC class II molecules and co-stimulatory molecules (CD86 and CD83) was upregulated in B cells (Zhu *et al*., 2013). Other *in vivo* and *in vitro* studies have demonstrated that B cells in teleost fish are important APCs which activate T cells and initiate adaptive immunity (Lewis, Del Cid, & Traver, 2014; Zhu *et al*., 2014).

Granulocytes

Professional phagocytes in vertebrates, including fish, are monocytes/macrophages, dendritic cells, and neutrophils, which belong to granulocytes (Esteban *et al*., 2015; Neumann, Stafford, Barreda, Ainsworth, & Belosevic, 2001; Rabinovitch, 1995). We described the features and functions of DCs and monocytes/macrophages in the APC section. In this part, we will briefly characterize the other professional phagocytes/granulocytes of teleost fish.

Fish granulocytes, also known as polymorphonuclear (PMN) leukocytes due to the distinct structure, contain a large number of granules in their cytoplasm; therefore, they are classified into three types of granulocytes: neutrophils (heterophil), eosinophils, and basophils (Firdaus-Nawi & Zamri-Saad, 2016). Fish granulocytes are present in the different compartments of fish. They can be isolated from peripheral blood, lymphoid tissues, and peritoneal cavity (Ainsworth, 1992). Many factors affect the distribution and numbers of granulocytes in fish. These factors include season of the year, environmental pollutants, diseases, and stressors, such as transport, handling, trauma, and exposure to some chemicals, which lead to increase or decrease the granulocyte numbers (Ainsworth, 1992; Hine, 1992; Schultz & Grieder, 1987).

Neutrophils

Neutrophils, being the most abundant granulocytes, hsave a low affinity binding to acidic and basic dyes and mediate the acute inflammatory response in fish similar to mammals (Hine, 1992). Teleost neutrophils are professional phagocytes and the first leukocytes which are recruited to the site of infection by chemokines (Havixbeck & Barreda, 2015; Katzenback & Belosevic, 2009). Activated neutrophils ingest pathogens and produce reactive oxygen species, release toxic substances from intracellular granules, thus becoming very potent killers (Flerova & Balabanova, 2013; Meseguer, López-Ruiz, & Esteban, 1994; Rieger *et al*., 2012; Wilhelm, 2007). Neutrophils also secrete neutrophil extracellular traps (NETs) which consist of antimicrobial granular proteins that prevent the dissemination of invading pathogens (Brinkmann *et al*., 2004; Palić, Andreasen, Ostojić, Tell, & Roth, 2007; Pijanowski *et al*., 2013).

Eosinophils

Eosinophils are stained by acidic dyes, such as eosin, and have a role in allergic inflammatory reactions and immune responses to parasites in mammals (Acharya & Ackerman, 2014). Similar to mammals, the eosinophils of teleosts are effective in controlling parasitic infections. A recent study showed that the morphology of zebrafish eosinophils resembled mammalian eosinophils, and they responded to the helminth *Pseudocapillaria tomentosa* infection by degranulation and increasing the number of eosinophils in the intestines (Balla *et al*., 2010).

Basophils

Basophils can be stained with basic dyes (e.g., toluidine blue pH 9.0) and are very rarely found in teleost blood (Tavares-Dias, 2006). Secreted IgD, a class of immunoglobulins, bind to the surface of basophils and trigger production of the antimicrobial factors, such as cathelicidin, pentraxin-3, and defensin (Edholm, Bengten, & Wilson, 2011).

Non-specific Cytotoxic Cells

Non-specific cytotoxic cells (NCCs) are unique to fish and considered to be functionally similar to mammalian natural killer (NK) cells (Firdaus-Nawi & Zamri-Saad, 2016). NCCs target various cells which include tumor cells, virus-infected cells, and some protozoa, and are able to kill the affected cells through lysis (Jaso-Friedmann, Leary, & Evans, 1993; Whyte, 2007). Like NK cells, cell to cell contact is required to deliver the lethal cytotoxic impact of NCCs (Donald & Jaso-Friedmann, 1992). The binding of NCCs to the antigens of parasites occurs in the presence of Mg^{+2}, and the lysis of target cells requires Ca^{+2} (Carlson, Evans, & Graves, 1985).

Compared to mammalian NK cells, NCCs are more effective in killing the infected target cells in less time (Graves, Evans, Cobb, & Dawe, 1984). Furthermore, NCCs are able to lyse target cells at wide temperature ranges (16 °C, 26 °C, and 37 °C), but the optimum temperature is 37 °C for lysis by NK cells (Graves *et al*., 1984). Although the NCCs of channel catfish are functionally similar to NK cells, the NCCs of catfish are the smallest nucleated cells and lack granules in their cytoplasm (Shen *et al*., 2002). NCCs are commonly found in the head kidney and spleen of teleost; however, they are rare in peripheral blood (Evans, Carlson, Graves, & Hogan, 1984).

Mast Cells

Mast cells (MCs) are present in all vertebrates including fish, amphibians, reptiles, birds, and mammals (Mulero, Sepulcre, Meseguer, García-Ayala, & Mulero, 2007; Reite, 2005). In mammals, MCs are indispensable for innate immunity; they initiate inflammation during bacterial infection by releasing lipid mediators, proteases, biogenic amines, cytokines, and chemokines, which are capable of facilitating the active recruitment of neutrophils (Heib, Becker, Taube, & Stassen, 2008; Stassen, Hültner, Müller, & Schmitt, 2002). In addition to innate immunity, MCs are also important players in adaptive immunity. Previous reports demonstrated that MCs contain classical PRRs, such as TLRs, and RIG-1 family receptors; therefore, they are able to phagocytose adherent bacteria and kill them (Feng, He, Zheng, Wu, & Yang, 2007; Malaviya *et al*., 1994; St John & Abraham, 2013). Moreover, MCs can process the engulfed bacteria and present antigens to specific T-cells, thus mediating T- and B-cell responses (e.g., lymphocyte growth, recruitment, and production of antibodies) (Henz, Maurer, Lippert, Worm, & Babina, 2001; Malaviya, Twesten, Ross, Abraham, & Pfeifer, 1996; Maurer *et al*., 2003). Furthermore, MCs possess FcεRI, the high-affinity receptor for the Fc region of IgE, that are produced during parasite infections (da Silva, Jamur, & Oliver, 2014; Nadler, Matthews, Turner, & Kinet, 2000).

Mast cells have an irregular shape, and their cytoplasm is filled with a wide range of electron-dense and membrane-bound granules (Dezfuli, Giari, Lui, Lorenzoni, & Noga, 2011). The granules of MCs in teleost fish contain biologically highly active histamine, serotonin, lytic enzymes, and antimicrobial peptides (piscidins) (Galindo-Villegas, Garcia-Garcia, & Mulero, 2016; Vadstein *et al*., 2013). Mast cells in teleosts are involved in defense mechanisms against multiple infections responding to infection by migration and degranulation (Mulero *et al*., 2007). Critical locations of MCs in teleosts include connective tissues, skin, gill filaments, intestinal

submucosa, and brain suggest that MCs are involved in pathogen recognition, and activated MCs can produce substances involved in the inflammatory response (Dezfuli, Pironi, Giari, & Noga, 2010; Reite & Evensen, 2006). Importantly, heterogeneity observed in MCs of fish by distribution, staining properties, and abundance, and many studies demonstrated that eosinophilic granular cells (ECGs) are mucosal mast cells (Dezfuli, Manera, Giari, DePasquale, & Bosi, 2015; Reite, 1998; Reite & Evensen, 2006; Vallejo & Ellis, 1989). ECGs are less mobile granulocyte cells and respond to parasites, such as helminth invasions at the mucosal sites, gut, and gills (Firdaus-Nawi & Zamri-Saad, 2016).

Conclusions

Similar to mammals, the fish immune system constitutes of innate and adaptive immune responses. The innate immune response is of vital importance to combat pathogens and to provide resistance to the disease due to delayed adaptive immune response. The innate immune response contains three major parameters including physical, humoral, and cellular parameters. Pathogens are engulfed and killed by professional phagocytes (monocytes/macrophages, DCs, B cells and neutrophils), or killed by NCCs and mast cells. If pathogens overcome innate immunity, adaptive immune responses will be activated by APCs (DCs monocytes/macrophages, and B cells). Molecular studies have shown in teleosts the conservation of almost all the sets of immune-related genes present in vertebrates, but despite the impressive advancement of molecular immunology, investigations into the functional immunology of fish are scarce and still need much work.

Following a lengthy gap, in the last decades, considerable progress has been made in the mechanistic understanding of fish professional APC-dependent immune responses. Namely, the morphological and functional characterization of the DC subsets and their role in priming of naïve T cells have been reported in several teleost fish. Also, fish B cells and macrophages have been described as professional APCs capable of inducing adaptive immune responses. The number of fish APC, T and B cell-specific markers in fish is increasing thus allowing characterization of lymphocyte subsets. More detailed knowledge about the phenotype and the function of teleost APCs will not only help gain insight into the evolution of the vertebrate adaptive immune system but will provide valuable information for development and optimization of immunotherapies and vaccination protocols for aquaculture use.

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