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Review Article

Major Components of Fish Immunity: A Review

Firdaus-Nawi, M. and Zamri-Saad, M.*

Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT

Fish are fascinating creatures with a certain degree of immunity comparable to those of mammals. The fish's immune system consists of two major components, innate and adaptive immunities. Innate immunity is non-specific and acts as the primary line of protection against pathogen invasion while adaptive immunity is more specific to a certain pathogen/ following adaptation. Innate immunity consists of the non-specific cellular and the non-specific humoral components. The non-specific cellular component consists of toll-like receptors (TLRs), macrophages, neutrophils, eosinophils and non-specific cytotoxic cell while the non-specific humoral component involves lysozyme, the complement, interferons, C-reactive proteins, transferrins and lectins. They work together at the initial stage to prevent pathogen invasion. On the other hand, the adaptive immune system consists of highly specilised systemic cells and processes that are separated into two main components: the humoral and cellular components. Three types of antibodies, the IgM, IgD and IgT, are the major constituents of the humoral immunity, which act on invaded extracellular pathogens. The cytotoxic T-lymphocyte cells are the major component of the cellular immunity that frequently kills virus-infected and intracellular bacterial or parasitic-infected cells. Both innate and adaptive immunities complement each other in the host's attempt to prevent infection.

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E-mail addresses:
firdausnawi_18@yahoo.com (Firdaus-Nawi, M.),
mzamri@upm.edu.my (Zamri-Saad, M.)
* Corresponding author

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INTRODUCTION

The fish is an organism that comes in various shapes, and the general picture of a fish is of a water creature with scales that coat the whole body and which has fins to swim. However, these are not always true

since some species of fish lack scales such as clingfish, while others lack fins, such as the eel. Furthermore, some fish like the lungfish can survive for several hours outside of water. Berra (2001) defined fish as a poikilothermic chordate with gills as the major respiratory organ. Scales and/or mucus protects the whole body.

Fish are classified in the paraphyletic group of creatures that comprises all gill-bearing aquatic vertebrates lacking limbs with digits. Thus, hagfish, lamprey and cartilaginous and bony fish and the various extinct related groups are included in this class. Generally, fish are cold-blooded or ectothermic i.e. its body temperature follows the ambient temperature, and some giant fish such as tuna and white shark are able to hold a higher core temperature (Carey & Lawson, 1973; Goldman, 1997). Fish live in various water bodies of the world and they have been discovered in almost all watery environments, from the streams of high mountains, where gudgeon and char may be found to the deepest oceans where anglerfish and gulpers live. Thus, fish demonstrate the greatest species diversity compared to other classes of vertebrates on earth. So far over 32,000 species of fish have been identified (Fish Base, 2011).

The immune system is a vital physiological mechanism that prevents infection and preserves internal homoeostasis. Therefore, the immune system acts as a shield to the fish and provides protection from attacks by a broad spectrum of invading microorganisms. The system has various specialised organs designed to detect and react against any microbe that enters the host by mobilising cells and molecules in the blood stream. Failure of the system to react leads to immunodeficiency while over-reacting against foreign microbes causes autoimmunity that can cause tissue damage. Principally, the immune system is regulated by sophisticated and complicated mechanisms (Lydyard et al., 2000) and any failure leads to infection, disease and death. This review describes the major components of the fish immune system as well as its working mechanism in protecting the fish from invasive pathogens.

THE FISH IMMUNE SYSTEM

Similar to mammals, the fish immune system is built with two major parts i.e. the innate and adaptive immunities. Innate immunity reacts to invading pathogens by recognising the germ's line-encoded molecules. TLRs and phagocytosis are the key components of innate immunity that protect the host against foreign invaders by recognising and finally destroying the phagocytised cells (Silva et al., 2002). Adaptive immunity, on the other hand, recognises pathogens via molecules that are generated by somatic mechanisms (Medzhitov & Janeway, 1997) followed by humoral and cellular responses via B- and T-lymphocytes (Dixon & Stet, 2001).

The immune organs vary with the type of fish (Zapata et al., 1996). Lamprey, hagfish and other jawless fish lack a true lymphoid organ. They depend only on the lymphoid tissues that are found within other non-lymphoid organs. Thus, plasma cells, macrophages and erythrocytes are

fabricated within the pronephros or anterior kidney and certain parts of the gut, where maturation of the granulocytes occurs. A more advanced immune system is observed in cartilaginous fish such as rays and sharks. Plasma cells, lymphocytes and granulocytes are housed in Leydig's organ, the spiral valve of the intestine and the epigonal organs. These lymphoid organs are unique to the chondrichthyes and do not exist in other types of fish. Thymus and spleen, the vital immune organs where lymphocytes, plasma cells and macrophages are stored and mature are also found in cartilaginous fish. However, for paddlefish, sturgeon and bichir of the chondrostean sub-class, the major site for fabrication of granulocytes is located within the meninges. Covering the heart of the chondrostean is a tissue that comprises reticular cells, lymphocytes and some macrophages. Nevertheless, the macrophages, granulocytes, erythrocytes and lymphocytes develop inside the kidney, an important hemopoietic organ of chondrostean.

Similarly, the vital hemopoietic organ for bony fish or teleost is the kidney, specifically, the anterior kidney where various types of immune cells develop (Anderson, 1977). The teleost fish also have lymphoid organs in the form of the spleen, thymus and the scattered lymphoid cells found within the mucosal tissues such as the gonads, gut, skin and gills. Lymphocytes are the major immune cells in the thymus as well as neutrophils, erythrocytes and granulocytes (Chilmonczyk, 1992). Zebrafish are one of the teleost species reported to have a similar lymphatic system as mammals (Kutcler et al., 2006).

Innate Immunity

The innate immune system is the first layer of host defence against pathogenic organisms or invaders. It responds in a non-specific manner before the specific adaptive immune system is ready to take over the defence work (Holland & Lambris, 2002). Unlike the adaptive immune system, the innate immune system identifies and reacts to invaders in a general manner. Thus, the protection provided is shorter and weaker than that provided by adaptive immunity (Alberts et al., 2002).

In fish, the innate immune response is a crucial initial component in preventing infection due to slow lymphocyte proliferation and a limited antibody repertoire that leads to a delay in the adaptive immune response (Magnadottir, 1998). Therefore, the innate immune response acts as an alarm that allows the adaptive immune system time to mount a response (Fearon & Locksley, 1996).

The innate immunity is subdivided into cellular and humoral immune responses. The cellular immune response provides a physical barrier in the form of mucus and epithelial cells that line the skin, gills and stomach, responsible for preventing invasion of microorganisms into the body. If the pathogen passes these barriers, specialised cells like granulocytes, monocytes or macrophages and the non-specific cytotoxic cells are ready to kill and digest the pathogens. Non-specific immune cells are

recruited into the site of infection primarily by inflammatory cytokines. The humoral component of the innate immune system employs a wide variety of proteins and glycoproteins that are capable of destroying or inhibiting the growth of microorganisms (Aoki et al., 2008).

Non-specific cellular immunity. Many types of leukocyte are involved in the innate, non-specific cellular immunity of fish. They include toll-like receptors (TLRs), granulocytes, macrophages and non-specific cytotoxic cells (NCCs). TLRs are the small protein molecules that have ability to recognise the conserved molecules of microbes. Granulocytes and macrophages are mobile phagocytic cells found circulating in the blood and within the secondary lymphoid tissues. Both cells play vital roles in inflammatory reaction, which actually is the cellular immune response to any invaders or tissue injuries. Eosinophilic granular cells (EGCs) are less mobile granulocytes that target parasites. EGCs are the host's innate cellular immune response against helminth infestation at the mucosal sites such as the gut and gills. Similarly, protozoa and virus-infected host cells are the targets for NCCs, making them appear in mucosal sites, blood circulation and lymphoid tissues. They are able to spontaneously kill the affected cells through apoptotic and necrotic mechanisms (Secombes, 1996).

Innate immunity lacks specificity to the pathogen, thus making innate immunity cells to mobilise quickly in large numbers. Unlike the specific immune system, there is no memory component in non-specific innate immunity. Therefore, exposure to a similar pathogen does not lead to better and quicker secondary immune response. However, the cells that are involved in the non-specific cellular immunity may interact with the cells of the adaptive immunity system and can be recruited by them or their products (Secombes, 1996).

Toll-like receptors (TLRs). Toll-like receptors (TLRs) are one of the vital components of innate immunity. They are able to recognise the pathogen's unique molecules. The word 'toll' originated from vernacular German and means fantastic or super (Chtarbanova & Imbler, 2011). Nu¨sslein-Volhard first discovered TLRs in the early 1980s following his mutagenesis studies of the fruit fly *Drosophila melanogaster* (Anderson et al., 1985). However, some years later in 1996 Lemaitre et al. (1996) discovered that this receptor also played major roles in adult-fly immunity as well as a key role in the mammal's innate immune system (Chtarbanova & Imbler, 2011).

The first report of TLRs in fish was by Stafford et al. (2003) in goldfish, *Carassius auratus auratus*, followed by Oshiumi et al. (2003) in pufferfish, Jault et al. (2004) in zebrafish while Takano et al. (2011) identified 11 types of TLR homologues in Japanese flounder (*Paralichthys olivaceus*). As in mammals, TLRs of fish work by recognising the unique conserved molecules of the microbes, known as pathogen-associated molecular patterns (PAMPs). This recognition stimulates an inflammatory response that initiates the innate immunity (Akira et al., 2006).

Macrophages. Macrophages are mononuclear, non-specific esterase positive and peroxidase-negative leukocytes. They are avidly phagocytic and emit nitrogen-free radicals and oxygen that kill various pathogens (Secombes, 1990). Macrophages have both complement and antibody (Fc) receptors (Secombes & Fletcher, 1992) and express the class II MHC molecules (Secombes, 1994). Since they belong to cellular innate immunity, macrophage-specific antibodies are not being made in fish, although antibodies to a related cell in the brain (the glial cells) exist (Dowding et al., 1991). Their actions usually rely on another immune component, the antibody (Thuvander et al., 1992).

Production of macrophages in fish occurs during primary hematopoiesis. The resident populations are self-maintained with contribution from monocytes that are circulating in blood to mature as tissue macrophage (Hodgkinson et al., 2015). Macrophages are commonly found in both layers of fish thymus, the cortex and medulla. Three kinds of macrophages have been observed in the thymus of the teleost, the melanomacrophages (Gorgollon, 1983; Pulsford et al., 1991), the monocytes (Castillo et al., 1990) and the multinucleated giant cells (Pulsford et al., 1991). Macrophages are strongly positive for non-specific esterase, acid phosphatase and 5'-nucleotidase (Castillo et al., 1990). Other than in the thymus, macrophages are also found scattered throughout the area in between the inner and outer zones of the pharyngeal epithelium and in the lymphoid organs, blood and peritoneal cavity (Secombes, 1990).

Granulocytes. Fish granulocytes have a distinctive structure and are sometimes referred to as the polymorphonuclear (PMN) leukocytes. Their cytoplasm contains numerous fine granules that give rise to the three types of granulocytes, the neutrophils, basophils and eosinophils. The neutrophil is stainable by neutral dyes but has no affinity for acidic or basic dyes. It is the most abundant granulocyte that migrates from blood into the affected tissues to engulf bacteria. On the other hand, acid dyes like eosin are suitable for staining the eosinophil that plays a primary role in allergic inflammatory reactions as well as destruction of internal parasites. Finally, basophil can be stained with basic dyes and is found only in low numbers (Secombes, 1996).

The granulocytes are distributed differently in the different parts of fish. Fletcher (1986) concluded that many factors influence the distribution of granulocytes in blood, tissues and other body fluids. These factors include season of the year, disease, environmental pollutants and the various stressors. Lowe-Jinde (1986) and Steinhagen et al. (1990) supported this and revealed an increased numbers of leukocytes, especially the granulocytes following infection. Lamas and Ellis (1994) reported that the numbers of granulocytes in the blood were greatly increased within 24 hours of stressing the fish. As macrophages, the granulocytes can also be isolated from the lymphoid tissues, blood and the peritoneal cavity (Lamas & Ellis, 1994).

Non-specific cytotoxic cells. Non-specific cytotoxic cells (NCC) of fish are considered to be similar to that of mammalian natural killer (NK) cells. They share several similarities, particularly the competent lytic cycle, the target cells for lysis, recognition of target cell and the effecters to lyse the infectious microorganisms (Jaso-Friedmann et al., 1993). However, there are also differences, which include the kinetics of killing and the morphology and specificity of the target cells (Evans & Jaso-Friedmann, 1992). Studies on NCCs of teleosts found that they tend to target various cells including tumor cells, virus-transformed cells and some protozoa (Whyte, 2007). NCCs are reported to be most active in the head of kidney of teleosts but spleen and peripheral blood leukocytes (PBL) also demonstrate the cytolytic abilities (Evans et al., 1984). In sharks, however, macrophages are the cells that are responsible for spontaneous cytotoxicity (McKinkey et al., 1986).

Non-specific humoral immunity. Teleost fish have been shown to have substances of non-specific humoral defence. These substances include the lysozyme, alkaline phosphatase, complement, interferon, C-reactive protein, transferrin, lectin and several other substances. They are extremely important for fish (Ingram, 1980) and play significant roles in maintaining homoeostasis (Saurabh & Sahoo, 2008).

Lysozyme. Lysozyme involves in mediating defence against invasion by pathogens. It is one of the major substances in the saliva, mucus and blood of vertebrate. Lysozymes are also broadly distributed in invertebrates, bacteriophages, microbes and plants (Jollès & Jollès, 1984). Lysozyme is a leukocyte-released enzyme and has a broader activity in fish compared to in mammals (Demers & Bayne, 1997). It has an antibiotic effect and is normally used as an indicator of non-specific immune functions.

Neutrophils are the major producers of lysozyme in fish (Ellis, 2001) but monocytes can also produce lysozyme (Fletcher & White, 1973). Therefore, fish lysozyme is mainly distributed in the leukocyte-rich organs, especially the head kidney and at sites of antigenic invasion such as the gills, skin, gastro-intestinal tract and eggs (Murray & Fletcher 1976; Lie et al., 1989). Lysozyme is also detected in the body mucus, peripheral blood and various tissues of both freshwater and marine fishes (Fletcher & Grant, 1968; Ebran et al., 2000; Fagan et al., 2003).

Lysozyme works by further disrupting the bacterial cell wall after an earlier disruption of the outer wall by the complement and other enzymes (Yano, 1996; Saurabh & Sahoo, 2008). Therefore, fish lysozyme attacks the lipopolysaccharide layer leading to damage of the outer cell membrane, allowing additional lysozymes to reach and injure deeper structures (Day et al., 1978; Iacono et al., 1980), increasing permeability that results in the loss of cell viability without lysis. Therefore, fish lysozyme has substantial antibacterial activity over the mammalian lysozymes against both Gram-positive and Gram-

negative bacteria (Itami et al., 1992). Furthermore, lysozyme plays an important role in preventing vertical transmission of some bacterial pathogens of fish (Yousif et al., 1994).

Alkaline phosphatase. Alongside with lysozyme, alkaline phosphatase (AP) is also an important enzyme in fish, especially in their innate immune system. It is a lysosomal enzyme and can be found in various body secretions such as body mucus, intestinal mucus and blood serum (Nigam et al., 2012). Concentration of AP increases when the host in stress making it as a potential stress indicator (Ross et al., 2000). Fast et al. (2002) in their study of Atlantic salmon found that the activity of mucus AP increases following parasitic infections and suggested that AP is one of the important enzymes in the innate immune system. Another study on catfish indicates high activity of AP during skin regeneration due to wound healing, demonstrating the role of AP as a protective enzyme (Rai & Mittal, 1983).

The complement. The complement is one of the major mechanisms of the humoral component of the immune system. It is involved in both initiation of the innate immune response and mounting of an adaptive immune response (Alvarez-Pellitero, 2008; Nakao et al., 2011) using its more than 35 soluble proteins (Sunyer & Lambris, 1999; Gasque, 2004). The complement works via a combination of three pathways: the alternative, the lectin and the classic pathways. The alternative pathway is active in the serum of fish than in that of mammals (Yano, 1996), and is important in the defence mechanism of fish (Ellis, 2001; Holland & Lambris, 2002). The classic pathway is more common in mammals, involving the formation of a complex blend of antigen and antibody (Gasque, 2004). This pathway is activated by the binding of the Fc portion of the IgG to the C1q component of the C1 complex (Muller-Eberhard, 1986; Kishore & Reid, 2000; Pangburn & Rawal, 2002). The lectin pathway requires interaction between lectins of the complement with sugar moieties found on the surface of microbes (Turner, 2003; Fujita et al., 2004), activating lectin-associated enzymes, the MBL-associated serine proteases (MASPs) that enhance the complement activation (Chen & Wallis, 2004). Microbes that are fixed with the complement are readily phagocytosed and lysed by the macrophages or the cytotoxic cells.

Interferons. Interferons (IFNs) are potent cytokines that act as key effectors of antiviral activity in the vertebrates (Castro et al., 2008). They are secreted proteins or glycoproteins that induce antiviral capability in cells and defends against virus infection by inhibiting viral replication (Yano, 1996; Samuel, 2001). IFN-like activity was first detected in fish in 1965 and has since been detected in cells and organs of many fish species infected with virus (Robertsen, 2006). The first IFN gene of fish was cloned in 2003 (Robertsen et al., 2003). Nevertheless, IFNs production has been confirmed in bony but not cartilaginous fish (Yano, 1996).

It is now established that fish cells secrete IFN-α and IFN-β molecules in response to virus infection (Kelly & Loh, 1973; Rio et al., 1973; Okamoto et al., 1983; Snegaroff, 1993). Type I IFNs are involved in the first line of defence against virus infection (Robertsen, 2006). They have five exon and four intron genes that are not found in the classic type I IFNs of birds and mammals (Lutfalla et al., 2003; Robertsen et al., 2003). Now, fish type I IFNs has been shown to have the same exon/intron structure as the IL-10 and IFN-λ gene families (Lutfalla et al., 2003).

C-reactive protein. C-reactive protein (CRP) is the first protein to exist in the blood plasma of humans and most animals as a response to tissue damage, infection and inflammation. It was first found reacted with the C-polysaccharide (CPS) of *Pneumococcus* bacterium in the serum of patients with acute inflammation, and was thus named C-reactive protein.

The liver, in response to factors released by fat cells, synthesises CRP. It is a member of the pentraxin family of proteins (Pepys & Hirschfield, 2003) and was the first pattern recognition receptor (PRR) to be identified (Mantovani et al., 2008). Since the first discovery in 1930, CRP has been found in many animal species, horseshoe crab and mollusk, *Achatina fulica* (Yano, 1996). Baldo and Fletcher (1973) reported CRP that binds to pneumococcal CPS in plaice serum. CRP has also been isolated from the smooth dogfish, *Mustelus canis* (Robey & Liu, 1983), Japanese eel (Nunomora, 1991), channel catfish (Szalai et al., 1994), rainbow trout (Winkelhake & Chang, 1982; Murai et al., 1990), lumpsucker, *Cyclopterus lumpus* (Fletcher & Baldo, 1976; White et al., 1978), tilapia, *Tilapia mossambica* (Ramos & Smith, 1978) and murrel fish (Mitra & Bhattacharya, 1992). However, there are fish species that lack CRP such as flounder, *Platichthys flesus* while bacterial endotoxin (LPS) was found to be able to stimulate the production of CRP following exposure to fish (White et al., 1981; White & Fletcher, 1985).

Transferrin. Transferrin (Tf) is a multi-functional protein or bi-lobed monomeric iron-binding glycoprotein actively involved in iron metabolism that is associated with innate immune response (Garcia-Fernandez et al., 2011). The primary role of Tf is transporting iron in a safe state from absorption, utilisation or storage sites around the body (Gomme & McCann, 2005). Although iron is a vital element for growth and survival, excess free iron is toxic to the cells (Kohgo et al., 2008). Therefore, tight regulation of iron metabolism maintains a balance between beneficial and toxic effects and this is accomplished by the interactions of several genes, such as the iron transporter transferrin, that are also involved in the response to infection (Neves et al., 2009).

Transferrin is synthesised in the liver and secreted into the blood but also found in the brain and central nervous system, testes, ovary, spleen, mammary gland and the kidney (Lambert et al., 2005). Transferrin contributes to the immune system through binding to iron, creating a low iron environment where few microorganisms

can survive and the infectivity of pathogenic microorganisms becomes limited (Suzumoto et al., 1977; Chen et al., 2009; Jurecka et al., 2009a, b).

Tf has been detected in almost all fish species (Yano, 1996), including the Pacific hagfish (Aisen et al., 1972) and the lamprey (Boffa et al., 1967; Macey et al., 1982). For cartilaginous fishes, Tf has been detected in the cat shark, *Scyllium stellare* (Got et al., 1967) and the lemon shark (Clem & Small, 1967). In bony fish, Tf has been detected in more than 100 species of fish (Turner & Jamieson, 1987; Jamieson, 1990).

Lectins. Lectins are primordial molecules that have multiple functions. They have existed in fish and other animals for decades and were initially identified as hemagglutinins (Russell & Lumsden, 2005) as they bind carbohydrate and agglutinate cells (Ewart et al., 2001). Lectins comprise at least two sugar-binding sites but the monosaccharide or glycosaccharide that inhibits lectin-induced agglutination or precipitation provides lectins' specificity (Goldstein et al., 1980). Lectins have been divided into several types, which include the C- and S-type lectins (Yano, 1996). The C-type is calcium-dependent.

A number of lectins have been reported in fish, but most have been characterised only in terms of agglutination activity and carbohydrate specificity (Ewart et al., 2001). In fish, C-type lectins, galectins and pentraxins have been identified from the earliest jawed vertebrate (sharks) to the more advanced teleost species such as salmon and carp (Vasta et al., 2004).

Ingram (1980), Ellis (1981) and Fletcher (1982) found many antipathogenic materials in the fish mucus, including lectins. Lectins have also been isolated from the skin mucus of scaleless hagfish, freshwater eel, moray eel, loach, sea catfish, ayu, cusk eel, dragonet and flounders (Yano, 1996), suggesting that lectin is produced by club cells (Al-Hassan et al., 1986). Furthermore, lectin was also isolated from the eggs of many species of fish such as lamprey, herring, carp, loach, Japanese catfish, smelts, ayu, salmonid fishes, sea bass, perch, porgy and flounder (Yano, 1996).

There are many studies on the function of fish lectins. Kamiya and Shimizu (1980) reported the ability of lectins from windowpane flounder skin mucus to agglutinate marine yeast, *Metschnikowia reukafii.* Kamiya et al. (1990) revealed the same ability of conger eel skin mucus lectins to agglutinate *Vibrio anguillarum*. Blue gourami lectins were reported to agglutinate fish pathogen *Aeromonas hydrophila* and at low concentrations (<1 ng/ml) promoted phagocytosis of the same bacterium (Fock et al., 2001). A mannan-binding lectin in the plasma of the Atlantic salmon was showed to bind to fish pathogens *Vibrio anguillarum* and *Aeromonas salmonicida* in a calcium-dependent manner (Ewart et al., 1999) and to increase phagocytosis and killing following incubation with *A. salmonicida* (Ottinger et al., 1999). Voss et al. (1978) reported Chinook salmon egg lectins inhibited the growth of pathogenic bacteria such as *Vibrio anguillarum, Yersina ruckeri, Aeromonas hydrophila* and *Edwardsiella tarda*. Fish

egg lectins were suggested to provide some protection to the developing egg and to prevent the transmission of pathogenic organisms from mothers to their offspring.

Adaptive Immunity

Adaptive immunity or specific immune system is the third line of the immune system that invaders face after surviving the physical barrier and the innate immunity. The adaptive immune system is composed of highly specialised, systemic cells and processes that eliminate or prevent pathogenic growth. The term adaptive refers to the differentiation of specific from non-specific and the tailoring of response to a particular foreign invader. Adaptive immunity is activated by the non-specific or innate immunity (Rubio-Godog, 2010).

Adaptive immunity consists of two major components: the antibodies and lymphocytes, or often called the humoral and the cell-mediated immune response, respectively (Uribe et al., 2011). Cells of the adaptive immunity are the lymphocytes, both B and T cells. The B cells, derived from the bone marrow, become the cells that produce antibodies. The T cells, which mature in the thymus, differentiate into cells that either participate in lymphocyte maturation or kill virus-infected cells. A key feature of adaptive immunity is 'memory', which differentiates it from innate immunity.

Adaptive immunity is highly adaptable due to the mechanisms of somatic hypermutation and V(D)J recombination. These mechanisms allow a small number of genes to generate a huge number of different antigen receptors that are uniquely expressed on each individual lymphocyte. This gene re-arrangement leads to an irreversible change in the DNA of each cell and all progenies of that cell inherit the genes that encode the same receptor specificity, including Memory B and Memory T cells, which are the key to long-lived specific immunity.

Humoral Immunity

Humoral immunity refers to antibody secretion and the accessory processes that accompany it. These include the Th2 activation and cytokine production, germinal centre formation and isotype switching, affinity maturation and memory cell generation. The humoral immunity involves substances found in the humours or body fluids, which include pathogen and toxin neutralisation, complement activation, opsonin promotion of phagocytosis and pathogen elimination (Janeway, 2001). Thus, humoral immune response is one of the branches of adaptive immunity that are mediated by secreted antibodies produced by B lymphocyte lineage or the B cells. The B cells transform into plasma cells, which produce and secrete antibodies. The CD4+ T-helper cells provide co-stimulation that aids this entire process, allowing the secreted antibodies to bind to the antigens located on the surface of the invading microorganisms and send them for destruction (Pier et al., 2004).

Humoral immunity in fish is variable and quite different from other animals. It depends on the external conditions and the species of fish (Lukjanenko, 1971).

However, the fish humoral immune response does share several basic characteristics with that of mammals. These include the basic immunoglobulin (Ig) structure, the cellular requirement for stimulation of antibodies and the functions of antibodies in neutralisation, complement fixation and opsonisation of antigen.

Antibodies. Antibodies, also known as immunoglobulins (Ig), are the primary humoral component of the adaptive immune system (Magnadottir et al., 2005). The Ig molecule has a dual functions i.e. as antigen receptor on the surface of B-cells and as an antibody secreted into blood and other body fluids. Thus, there are two forms of H-chains in the immunoglobulins, one with a hydrophobic C-terminal peptide that can bind to a cell membrane and the other with a hydrophilic N-terminal region that is secreted. The same gene encodes the two forms and processing of the pre-mRNA determines which form should be synthesised. The N-terminal on both H and L chains is called the variable (V) domain and is the structure of the antibody that binds to the antigen (Pilstrom & Bengten, 1996).

The most prevalent immunoglobulin in the serum of teleosts is the IgM tetramer with eight antigen-combining sites. It has been detected in many species of fish including chondrichthyean and osteichthyean. It consists of 70 kDa heavy chain and 22-25 kDa light chains (Tort et al., 2003). In general, fish Igs are of lower affinity and diversity than those of mammals and birds (Du Pasquier, 1982). Therefore, better understanding of the structure and function of fish IgM becomes extremely important for effective prevention and control of various fish diseases (Magnadottir, 1998).

IgM is tetrametric in teleost (Acton et al., 1971) but pentametric in higher vertebrates and cartilaginous fish (Kobayashi et al., 1984). Since isotypic repertoire of Ig is limited in fish (Kaattari et al., 1998), the degree of similarity between mucus and serum derived Ig is unknown. However, there are reports that monoclonal antibodies developed against serum of carp, *Cyprinus carpio* L do not react with the mucus Ig (Rombout et al., 1993a) due likely to the existence of varied redox forms of Ig in some teleost species (Kaattari et al., 1998) or perhaps the existence of different glycosylation patterns (Kenneth et al., 2000). Kenneth et al. (2000) revealed the different protein band patterns between the mucus and the serum Ig where the mucus Ig possesses four primary bands, the 72, 68, 43 and 28 kDa, while the serum Ig possesses two primary bands; the 72 and 28 kDa.

Recently, scientists have discovered the existence of IgD and IgT isotypes in teleost but not as abundant as the IgM (Tian et al. 2009). Wilson et al. (1997) first discovered IgD that was homologous with the mammalian IgD. Then, Hanzen et al. (2005) discovered IgT, sometimes referred to as IgZ, in rainbow trout while Danilova et al. (2005) reported the occurrence in zebrafish. However, unlike IgM, the roles of these new Igs are still obscure. Nevertheless, IgD might be involved in innate immunity as Edholm et al. (2010) found that the IgD secreted by channel catfish lacked the

antigen-specific V domain and could bind to basophils to stimulate the pro-inflammatory cytokines. According to Zhang et al. (2010), IgT might involve in the interactions between the host intestinal mucosa and the microflora.

Immunoglobulins of fish are found in the skin mucus, gut, gill mucus, bile and systemically in the blood plasma (Morrison & Nowak, 2002). The presence of Ig on the skin and gill surface is important since these organs are consistently exposed to a wider natural environment. The systemic and mucosal immune responses are autonomous because specific antibodies against certain antigens can be elicited from the skin, gills and gut. However, intravenous injection of antigen stimulates little activity in the mucus (Lobb & Clem, 1981), indicating that the mucosal Ig is exclusive from the systemic plasma cells. Grabowski et al. (2004), on the other hand, showed the stimulation of the mucus antibody response following intraperitonium vaccination with sonicated formalin killed *Flavobacterium columnare*. Similarly, Firdaus-Nawi et al. (2011) demonstrated increasing mucus antibody following oral vaccination with killed *Streptococcus agalactiae*. This pattern was also observed with *Flavobacterium psychrophilum* (LaFrentz et al., 2002), indicating that the systemic antibodies may disseminate to mucosal sites from blood circulation (Di Conza & Halliday, 1971; St. Louis-Cormier et al., 1984; Cain et al., 2000).

B cells. B cells are a type of lymphocyte that plays an important role in the humoral immune response. The primary functions of B cells are to produce antibodies against antigens, to perform the role of antigen-presenting cells (APCs) and finally, to develop into memory B cells after activation by antigenic interaction. The head of kidney (HK) or pronephros is the source of B cells in teleost fish, making HK the primary lymphoid tissue (Zapata et al., 2006). The spleen is considered secondary lymphoid tissue in which plenty of B cells are found in teleost fish. Bromage et al. (2004) revealed that the spleen is a site for B cell activation, plasmablast formation and differentiation into plasma cells. Plasma cells then migrate to the HK, which explains the presence of few Ig-secreting cells in the spleen compared to HK.

Other than the lymphoid tissues, B cells are also found in various organs and tissues including the intestine, skin and gills. In the intestine, the distribution of B cells is low and variable among different species of fish. Studies in sea bass, carp and rainbow trout demonstrated between 2% and 12% of the leukocytes in the intestine were IgM-positive, mainly in the lamina propria of both anterior and posterior intestines. However, a small number of these cells were also detected in the epithelium (Salinas et al., 2011). B cells are also detectable in the skin of cartilaginous and teleost fish (Wolfle et al., 2009) and in the epithelium of carp skin (Rombout et al., 1993b). Another study in rainbow trout revealed the large numbers of B cells in the basement membrane area followed by the epithelial layer and the cells in the dermis or sub-epidermal layer (St.

Louis-Cormier et al., 1984). Furthermore, Zhao et al. (2008) reported that the skin of channel catfish contains B cells and antibody secreting cells (ASC), which most likely serve as the major source of mucosal antibody. On the other hand, Grove et al. (2006) reported a large number of IgM-positive cells in the stratified epithelium of the gill arch and filaments of Atlantic halibut fish, while Grøntvedt and Espelid (2003) reported an abundance of B cells in primary gill lamellae and filaments along the blood vessels of spotted wolfish.

Mucosal Immunity

Mucosal immunity is vital because it is the first line of adaptive humoral defense that effectively blocks or neutralises the pathogen. However, fish lack secretory IgA, Peyer's patches and tonsils that play important role in mammalian mucosal immunity (Kaattari & Piganelli, 1996). Instead, massive intraepithelial lymphocytic aggregations are observed in the central region of the spiral intestine of elasmobranch (Tomonaga et al., 1986). They are believed to play a similar role as the Peyer's patch of mammals. Furthermore, minor subepithelial lymphoid accumulations were reported in the intestine of roach and perch (Zapata & Solas, 1979; Rombout & van den Berg, 1989). A recent study by Firdaus-Nawi et al. (2011) demonstrated aggregations of lymphoid cells in the lamina propria of red tilapia following oral immunisation against *Streptoccoccus agalactiae*. Antibody-secreting cells (ASCs) were observed in the lamina propria of perch following immunisation with sheep red blood cells (Pontius & Ambrosius, 1972). Fletcher and White (1973) reported increased antibody titers within the intestinal mucus of plaice upon oral immunisation with heat-killed *Vibrio anguillarum*. Similarly, Firdaus-Nawi et al. (2011) demonstrated increased antibody titers in the intestinal mucus of red tilapia following oral immunisation against *Streptoccoccus agalactiae*.

Mucosal immunity also gives protection against parasitic infestation. A study by Sitja-Bobadilla et al. (2006) using co-habitation challenge of turbot with *Enteromyxmum scophthalmi* resulted in leukocyte infiltration in the intestine. The infiltration consisted of lymphocytes but no specific IgM was detected in the serum. On the other hand, Zhang et al. (2010) demonstrated the unchanged numbers of IgM-positive cells in the gut of surviving trout that were naturally infected with parasite *Ceratomyxa shasta*. However, parasite-specific IgM were detected in the serum. This suggests that different fish species respond differently to different parasites.

Cellular Immunity

Cellular immunity, also referred to as cell-mediated immunity, is a specific immune response that involves macrophages, natural killer cells (NK), mast cells, basophils, eosinophils and neutrophils (Broere et al., 2011). Various cytokines are released in response to the antigen. Cellular immunity protects the body by activating the antigen-specific cytotoxic T-lymphocytes that induce apoptosis of cells that display epitopes of

foreign antigen on their surface. These include virus-infected cells, intracellular bacteria-infected cells and cancerous cells. Cell-mediated immunity is directed primarily at the pathogen that survived phagocytosis and the pathogens that infect non-phagocytic cells. It is most effective in removing virus-infected cells, but also participates in protection against fungi, protozoans, cancer cell and intracellular bacteria (Kerry & Hansen, 2011).

Cellular immunity also plays a major role in transplant rejection. Graft-versus-host reaction (GVHR) is a representative phenomenon of cell-mediated immunity involving CD4 and CD8 T-lymphocytes. Nakanishi and Ototake (1999) employed a model system of clonal triploid ginbuna and tetraploid ginbuna-goldfish, *Carassius auratus* hybrids to demonstrate the presence of GVHR in a teleost fish. The sensitised triploid cells were injected into tetraploid recipients and a typical GVHR was induced that led to the death of the recipients within one month. Post-mortem conducted during the course of the clinically apparent graft-versus-host disease (GVHD) showed several pathological changes including enlargement of the spleen, infiltration of mononuclear cells and focal necrosis particularly in the skin, liver and lymphoid tissues. Most features of acute GVHR are similar to those found in mammals and birds, providing evidence for the presence of allo-reactive cytotoxic T cells in teleosts (Manning & Nakanishi, 1996).

T cells. T cells are lymphocytes that play a vital role in cell-mediated immunity as well as the adaptive immune system (Nakanishi et al., 2015). The presence of an antigen-specific receptor or T-cell receptor (TCR) on the cell surface distinguishes them from other lymphocytes (Manning & Nakanishi, 1996). They are called T cells because they mature in the thymus. They are also sometimes called thymocytes.

There are several types of T cell, which include T helper cells (T_H cell), cytotoxic T cells (CTL), memory T cells, regulatory T cells (Treg cell) and gamma delta T cells. Naive $CD4^+$ T cells can differentiate into the five types of effector T cells (Th1, Th2, Th17, Th9 and Th22), three subsets of regulatory T cells (Treg, Th3, Tr-1) and memory T cells (Annunziato & Romagnani, 2009; Wan & Flavell, 2009). Thus, these $CD4^+$ T cells play vital roles in regulation of the immune system, immune pathogenesis and host defense mechanism. According to Zhu and Paul (2010), $CD4^+$ T cells are characterised by their plasticity in addition to heterogeneity. Fischer et al. (2006) detected T-cell-related genes such as TCR, CD3, CD4 and CD8 as well as MHC class I and class II genes in several fish species. Additionally, mRNA expression of T cell surface marker genes in alloantigen or virus-specific effector cells has been reported in several fish such as TCRβ and CD8α in ginbuna (Somamoto et al., 2006) and rainbow trout (Fischer et al., 2013) and TCR in channel catfish (Stuge et al., 2000). This suggests the presence of $CD4^+$ helper T cells and $CD8^+$ CTL in fish similar to their presence in higher vertebrates (Fischer et al., 2013).

Overall Working Mechanisms of Fish Immune System

In an attempt to prevent establishment of infection, both innate and adaptive immunities work in complement. Upon exposure to pathogenic organism, the innate immunity is activated to prevent the invasion through TLRs that recognise the pathogen-associated molecular patterns (PAMPs). The first hurdle faced by the microorganism is the efficient physical barrier in the form of mucus and epithelial cells of the mucosal organs such as the intestines, gills and skin (Figure 1). Should the pathogen successfully pass these barriers, invasion starts.

Successful invasion by pathogens stimulates two major mechanisms i.e. the innate cellular and humoral immunities, and the specific adaptive immunity. Innate immunity involves granulocytes, phagocytes and the non-specific cytotoxic cells that are employed by inflammatory cytokines to kill and digest the invading pathogens through a process known as phagocytosis (Secombes & Fletcher, 1992). At the same time, the humoral component of the innate immune system employs a wide variety of proteins and glycoproteins described earlier that are capable, either alone or in collaboration with the cellular innate immunity, of destroying or inhibiting the growth of the microorganisms (Aoki et al., 2008). The phagocytosis process especially by residence macrophages results in full elimination of an invading pathogen (Figure 2).

After the degradation process, selected small protein fragments from the pathogen are displayed by macrophages on the Class II MHC (MHC II). This turns macrophages into antigen presenting cells (APC) and activates the adaptive immunity where helper T-cells (T_h) attract and bind to the MHC II of APC by T-cell Receptors (TCR). Formation of APC-T_h complex releases signalling molecules known as cytokines by the activated T_h that triggers the proliferation and maturation of B cells as well as other immune mechanisms. Maturation of B cell leads to formation of two types of cell, the plasma cells that produce specific antibody and the memory B cells that remember the specific antigen for certain periods of time (Kum & Sekkin, 2011) (Figure 3). The released antibodies act to disable the pathogen through the mechanism of opsonisation or neutralisation before the disabled pathogen is destroyed by the complement system and cleared by macrophages via phagocytosis.

Intracellular antigens (Ag) such as virus undergo another effective mechanism of elimination by the body. The pathogen is phagocytised and processed before being displayed on Class I MHC (MHC I) of APC, which attracts the $CD8^+$ cells to bind to the MHC I of APC via $CD8^+$ receptors. Then the activated $CD8^+$ cells start to clone themselves into two types of cell, the memory T cell and the Cytotoxic T-Lymphocyte (CTL) cell that destroy the virus-infected cells using various enzymes and cell apoptosis (Figure 4).

Subsequent exposure to the same antigen predisposes the pathogen to the same physical barrier of the mucosal organs.

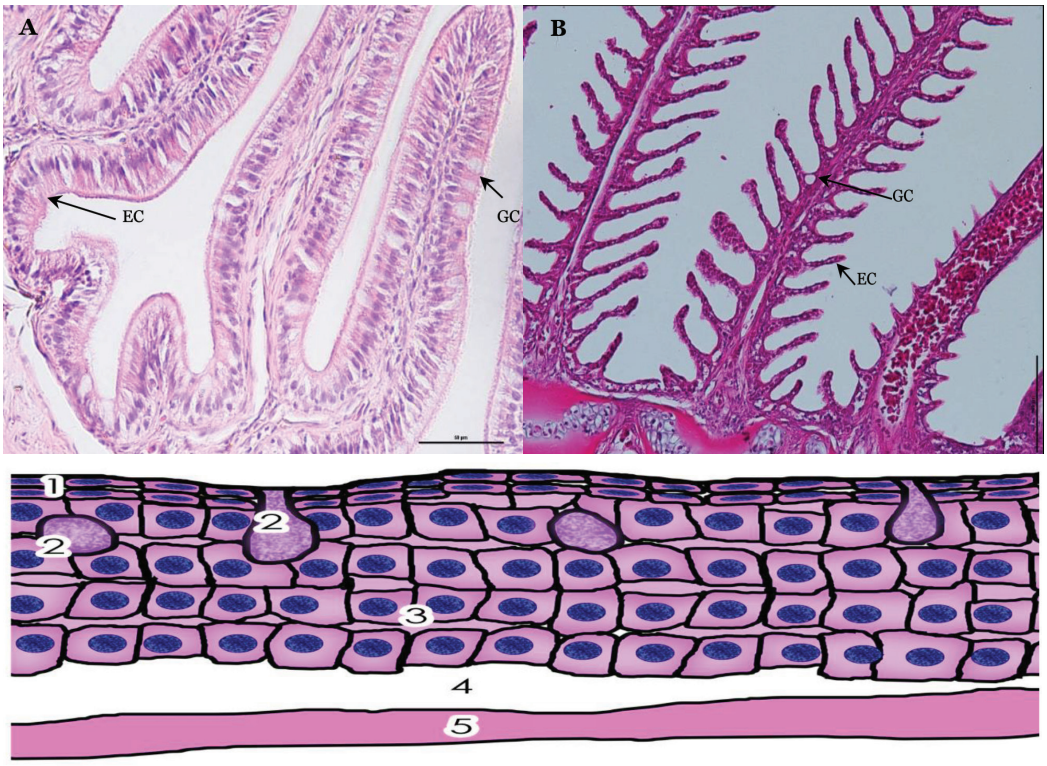

Figure 1. Intestine and gills of the fish are coated with mucus layer produced by goblet cells (GC) as primary innate protection, followed by the layer of epithelial cells (EC) (A & B). Similarly, the mucus also covers the skin of the fish and the epidermal layer is made up of epithelial cells (C) that provide both a physical and chemical barrier against invading pathogens.

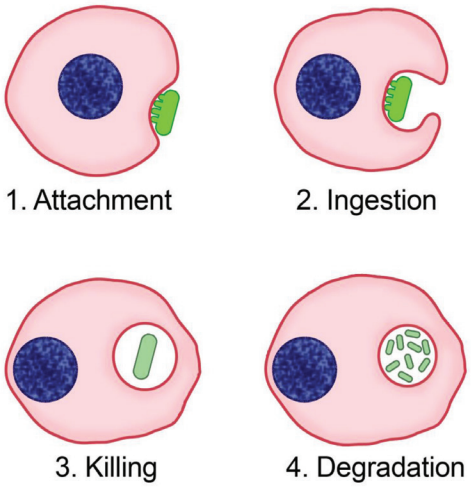

Figure 2. Process of phagocytosis by macrophages starting with attachment of pathogen such as bacterium followed by ingestion before it is killed and lysed into small fragments by the enzyme lysosome. Then the degradation process takes over.

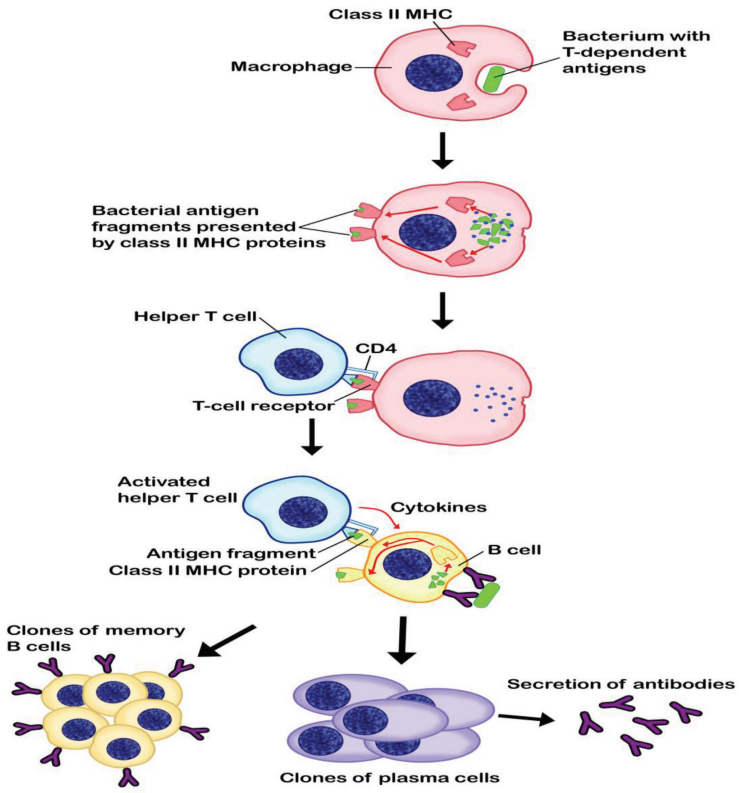

Figure 3. Mechanism of B cell activation and maturation by helper T-cell (Th) resulted in formation of specific antibody secreting plasma cells and memory B cells that have the ability to remember the infection.

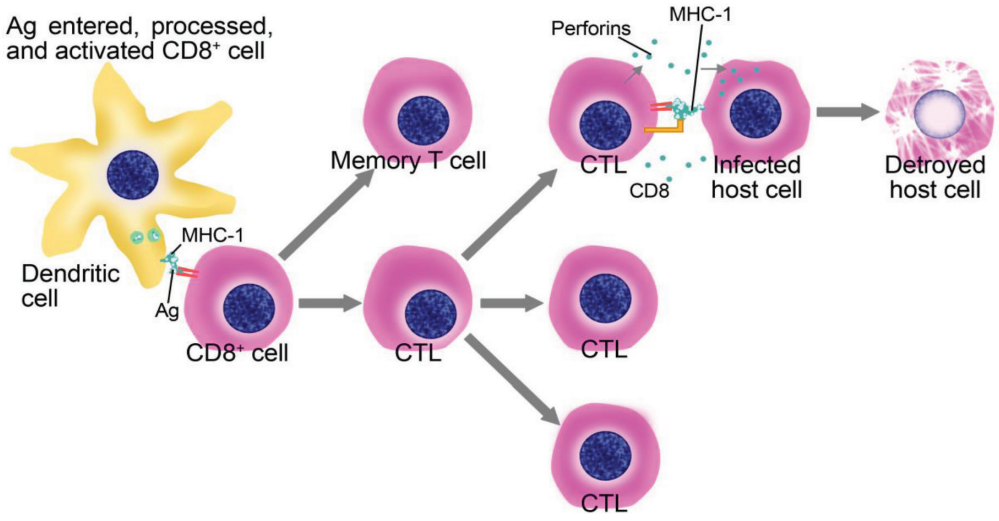

Figure 4. Elimination of virus-infected cell by the Cytotoxic T-Lymphocyte (CTL). This mechanism does not involve antibody production and is also known as Cell-Mediated Immunity (CMI).

At the same time, the Memory B and T cells located in the mucosal layer of the exposed fish stimulate production and release of specific antibodies and/or cytotoxic cells against the pathogen onto the mucosal surface to prevent adhesion and invasion of the pathogen. This forms the basis for mucosal immunity.

Inability of the mucosal layer to efficiently prevent invasion leads to a second invasion of the pathogen into the host. As described earlier, this invasion stimulates the non-specific cellular and humoral innate immunities to kill and remove the invading pathogen. Simultaneously, this second invasion activates processing of the pathogen by phagocytes to be presented to the adaptive immune system for the Memory B cells to enhance production and release of antibodies specific to the pathogen (humoral immunity) or the Memory T cells to enhance the cytotoxic T-lymphocytes (cell-mediated immunity), depending on the type of invading pathogen. These form the basis for vaccination against diseases.

CONCLUSION

The immune system of fish is not as complex as that of mammals because of the absence of several components. However, the immune system of fish is adequate in providing protection against antagonistic pathogens from the surroundings. Recent findings in fish immunology and its components help to provide a better understanding of fish immunology as well as to pave the way for further research. This will help in efforts to improve the health and disease protection of fish.

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