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Immune cells in teleosts

An overview of the structural and functional aspects of immune cells in teleosts

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Summary

The immune system of fish consists of two main 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. The adaptive immune system consists of the humoral and cellular components. Cytotoxic T-lymphocyte cells are the major component of the cellular immunity that frequently kills viral-, bacterial- or parasitic-infected cells. According to the anatomical location, the mucosal-associated lymphoid tissue (MALT) in teleost fish subdivides into gut-associated lymphoid tissue (GALT), gill-associated lymphoid tissue (GIALT), and skin-associated lymphoid tissue (SALT). The MALTs contain various leukocytes; including, but not limited to, lymphocytes (T and B cells), plasma cells, macrophages, and granulocytes. Macrophages are multifunctional cells that are mainly involved in the immune response, including; phagocytosis and degradation of foreign antigens, tissue remodeling, and production of cytokines, chemokines and growth factors. An interesting feature of teleost
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macrophages is their ability to form melanomacrophage centers (MMC) in the hemopoietic tissues. Dendritic cells, rodlet cells, mast cells, eosinophilic granular cells (ECGs), telocytes, osteoclasts, club cells, as well as, barrier cells have been recorded in many fish species and have many immunological roles. This paper aims to summarize the current knowledge of the immune cells present in fish tissues serving as anatomical and physiological barriers against external hazards. Increased knowledge of fish immune systems will facilitate the development of novel vaccination strategies in fish.

**Keywords:** lymphocytes, dendritic cells, macrophages, neutrophils, rodlet cells

**Introduction**

In contrast to higher vertebrates, fish are free-living organisms from the early embryonic stages of life and depend on their innate immune system for survival. Nonspecific immunity is a fundamental defense mechanism in fish. In addition, it plays a key role in the acquired immune response and homeostasis through a system of receptor proteins. Moreover, the immune system contributes to the regulation of gonadal function (Nielsen and Esteve-Gassent, 2006).

Generally, the system of phagocytic cells is widely distributed throughout the body. It is responsible for the removal of spent cells, particles, or macromolecular aggregates from its surroundings. The main cells involved in phagocytosis in fish are neutrophils, monocytes of the hemopoietic organs, and the free and fixed macrophages of the spleen and kidney. These cells remove bacteria mainly by the production of reactive oxygen species during a respiratory burst (Mokhtar, 2021).
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The head kidney in teleosts is an essential lymphoid organ for the proliferation and differentiation of B-lymphocytes (Petrie-Hanson and Ainsworth, 2001), macrophage production (Meseguer, et al., 1991), and acts as the site for capturing and processing foreign substances (Press and Evensen, 1999). On the other hand, the parenchyma of the thymus is composed of several cell types such as, T lymphocytes/thymocytes, undifferentiated cells (lymphoblasts), melano-macrophages, and to a lesser extent by nurse-like cells, immunoglobulin positive (Ig+) cells, mucous cells, rodlet cells, and neuroendocrine cells (Cao et al., 2017).

Fish possess lymphocyte populations analogous to T cells, B cells, cytotoxic cells (similar to natural killer cells), macrophages, and polymorphonuclear leukocytes. The immune system of teleosts has sub-populations of T lymphocytes that exhibit differential responses to mitogens, B cell acute allograft reactions, mixed leukocyte reactions, and cooperative interactions between T cells, B cells, and macrophages that are essential for the production of antibodies (Uribe et al., 2011).

Interest in defense mechanism of fish arises from a need to address questions concerning origins and evolution of immunity in vertebrates. Species variation in the morphology of the immune system results in a large number and diversity of species within the teleost fishes. This review aims to provide an overview of the distribution, structure and functions of immune cells in teleosts.

1. Leukocytes

Like leucocytes in higher vertebrates, fish leucocytes are classified into agranulocytes and granulocytes. The agranulocytes have no granules (lysosomes) in the cytoplasm and their nucleus is unlobed. The most frequent leucocytic cells observed in hemopoietic tissues are lymphocytes, neutrophils, and macrophages
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(Kondera, 2014). It is well known that leucocytes are migrating cells that can migrate to the connective tissue. Also, leukocytes and probably other ameboïd cells can migrate through normal mucus secretions. They are characterized by ameboïd-like cell processes, a large euchromatic nucleus and the cytoplasm contains mitochondria and vacuoles. Few studies have demonstrated a pattern of distribution of leucocytes in aquatic species. The leukocytes are widely distributed in the ovary and may represent potential in-situ modulators of ovarian function that work through local secretion of regulatory soluble factors. These factors include many cytokines that are mainly produced by the action of immune cells within the ovary. Actual follicle rupture during ovulation may depend on tissue remodeling that is characteristic of an acute inflammatory reaction and includes increased leukocyte migration, the release of various mediators and loosening of connective tissue elements around the follicles (Mokhtar, 2019).

Polymorphic granulocytes express iNOS. The expression of iNOS in granulocytes increases during stressing and inflammatory events, produces a cytotoxic environment, and supports proinflammatory reactions (Yu et al., 2005). The immunoreactivity of iNOS was also detected in leukocyte populations in the primary lymphohematopoietic organs, gills, and gut, as well as in vascular structures and MMCs of the spleen (Campos-Pérez et al., 2000).

1.1. Neutrophils

Neutrophils are the most abundant leukocytes with an irregular and occasionally segmented nucleus. The numbers of circulating neutrophils reported in fishes vary over a considerable range (1–25% of leucocytes). The teleost neutrophil
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has a grayish granular cytoplasm by Romanowsky dye. In blood smears, neutrophils are round or oval in shape and are usually larger than erythrocytes. In immature cells, the nucleus is eccentric and exhibits the human kidney-shape, but in mature cells, two- or three-lobed nuclei may be recognized. Neutrophils show peroxidase and Sudan black-positive reaction. Moreover, these cells have lysozymes and other hydrolytic enzymes in their lysosomes (Havixbeck and Barreda, 2015).

The nuclear chromatin material is dense and patchy in distribution. The cytoplasm is characterized by the presence of numerous granules scattered in the cytoplasm. Two types of granules can be identified based on their shape and size. Type I granules are membrane-bound, oval, large, and predominant in the cytoplasm. Type II granules are rod-like. The neutrophils-containing both types of granules are fewer in number than neutrophils, having only Type I granules. Both types of granules are observed in the differentiated cells. Moreover, their cytoplasm contained many lysosomes and phagosomes (Fig. 1A).

Myeloperoxidase-3 (MPO) is specifically expressed in neutrophil granulocytes. It is a lysosomal protein stored in azurophilic granules of the neutrophils and released into either phagosomes or the extracellular space during degranulation. Moreover, this protein in the presence of halide and hydrogen peroxide kills bacteria by halogenation of the bacterial cell wall (Klebanoff, 2005).

2.1. Eosinophils

Massive aggregations of eosinophils are recorded, particularly in association or in the vicinity of the endothelial tissues. These cells are characterized by rounded cell bodies of various sizes with an eccentric elliptical or lobed nucleus that contains coarse, clumped chromatin and strongly stained blue. Typically, these cells have been
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identified by the presence of large rounded cytoplasmic granules, which stain bright red with Romanowsky’s stain or eosin (Fig. 1C) and measuring approximately 1.15 ± 0.035 µm in diameter. These granules express s-100 protein. TEM showed the characteristic specific granules of eosinophils, lysosomes, and phagosomes. Some eosinophils contain numerous clear vacuoles or a big cytoplasmic vacuole. Mitochondria, endoplasmic reticulum and Golgi complexes are easily identified (Fig. 1B). In addition, eosinophils express CCR3 (CD193) protein, which is a receptor for C-C type chemokines. It is highly expressed in eosinophils and basophils. This receptor may contribute to the activation and accumulation of eosinophils and other inflammatory cells at sites of parasitic infection (Combadiere et al., 1995).

Eosinophils were observed in the blood of various species of salmon (*Salmo, Oncorhynchus*), but were reported rare or absent from the blood of rainbow trout. Several workers agree that eosinophils are present in the blood of Carassius. Eosinophils represent activated cells that contain degranulated or coalescent granular material in response to a parasitic infection or other inflammatory stimuli. They are the smallest among the granulocytes (Ainsworth, 1992), ranging between 13.5 ± 5.67 and 12.6 ± 1.3 µm for males and females respectively. Males have a significantly (p < 0.01) higher number of eosinophils (20.2 ± 1.2) than females (17.5 ± 1.09).

3.1. The heterophils

In fish, the existence of heterophils is still debated. In teleosts (bony fish), heterophils are absent (only a few species, such as goldfish and carp have rod-shaped and slightly eosinophilic granules), whereas in elasmobranchs (cartilaginous fish), neutrophils coexist with heterophils. These cells, also known as type I granulocytes (G1) or fine eosinophilic granulocytes, have some similarities to avian heterophils.
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Because the primary function of heterophils is phagocytosis, significant increases in the heterophil count of fish are usually associated with inflammatory disease, especially microbial and parasitic infections or tissue injury. The lack of peroxidase activity in most teleost heterophils suggests little, if any, oxidative response to stimuli (Hine, 1992).

They contain large, eosinophilic and fusiform cytoplasmic granules. The cytoplasm, which can be difficult to visualize, is light blue or clear. The nucleus is segmented and frequently displaced toward the edge of the cell and appears basophilic with moderate amounts of heterochromatin. Few mitochondria and endoplasmic reticulum are demonstrated around the electron-dense granules (Fig. 1B). No significant differences are found between males and females for size (Ainsworth, 1992); the diameter ranges from 13.9 ± 6.71 in males to 13.3 ± 1.27 in females, and the frequency is 15.4 ± 0.8% in males and 15.7 ± 1.2% in females.

4.1. Basophils

Like eosinophils, reports on the presence of basophils in the blood of fishes vary. When present (goldfish, salmonids, carp), they are easily identified by their deeply stained cytoplasm filled with dense, dark purple granules. The basophils show an irregular shaped-nucleus (7.09 ± 0.25 μm) and electron-dense cytoplasmic granules (Fig. 1D). Many factors can affect the number of basophils and the leukocytic formula, such as age, health status, ecological factors and the seasons. In addition, basophils were seen with macrophages in the epidermal layer of silver carp (Mokhtar, 2021). On the other hand, basophils have not been observed in tissues of some fish species (Murray, 1984).
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Teleost basophils endocytosed soluble antigens and expressed MHC class II and B7-H1/DC. These findings suggested that basophils can interact with T cells as antigen-presenting cells. Furthermore, some features of teleost basophils are more similar to those of mammalian mast cells than to those of mammalian basophils (Odaka et al., 2018).

5.1. Lymphocytes

Agranulocytes come in two varieties: lymphocytes and monocytes. Lymphocytes are the most numerous types of leukocytes, constituting 70 to 90% of total leukocytes. In teleosts, the average diameter of small lymphocytes varies between 5 and 8 µm (up to 12 µm in large lymphocytes) being the smallest cell of leukocytes. The lymphocytes are round cells and the nucleus occupies virtually the whole of the cell leaving only a narrow rim of basophilic cytoplasm. The scant cytoplasm contains few mitochondria, polyribosomes, endoplasmic reticulum, small electron-dense granules, and small cytoplasmic vesicles. The plasma membrane is plicate and occasionally has small pseudopodia. They possess heterochromatic nuclei with one or two well-defined nucleoli (Fig. 1E). Lymphocytes are frequently distributed in the intercellular spaces of the deeper epidermal layers of the lips and snout region, and around the blood vessels of most tissues.

Immunohistochemical analyses indicated the presence of two populations of lymphocytes that could be identified in the head kidney of grass carp; B lymphocytes expressed CD20 and T lymphocytes expressed CD3, the latter were randomly distributed. Moreover, a few scattered cytotoxic T lymphocytes (CTLs) expressed CD8. B lymphocytes play an essential role in the humoral immune response. The primary role of B cells is to produce antibodies against antigens. The head kidney is
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considered to be the principal lymphoid organ in teleosts for the proliferation and differentiation of B lymphocytes, for the production of macrophages, and as the site for capturing and processing foreign substances and particles. Studies suggest the presence of lymphoid cells that release immunoglobulin M (IgM) between 12 and 14 days of post-fertilization and have also shown two variants of IgM by enzyme-linked immunosorbent assay (ELISA) in embryos eight days before the end of the incubation period. This finding suggests that a source of B cells exists before the end of the incubation period in the kidney or other hematopoietic sites (Hansen and Zapata, 1998; Zapata et al., 2006). In teleosts, the immune system possesses sub-populations of T lymphocytes that show differential responses to mitogens and mixed leukocyte reactions (Fischer et al., 2006). T cells play essential roles in the adaptive immune system. Fischer et al. (2006) detected T-cell related genes such as (TCR), CD3, CD8, and CD4 as well as MHC-I and MHC-II genes in many fish species.

CD8 is the main marker for the identification of cytotoxic T lymphocytes (CTL) in fish (Fischer et al., 2013). The CLT are the main constituents of cellular immunity that often kill virus-infected and intracellular bacterial or parasitic-infected cells. A study using an anti-ginbuna CD8α -monoclonal antibody demonstrated that CD8 α+ cells were anti-viral cytotoxic cells, which further supported the theory that there were virus-specific CTLs in teleosts (Somamoto et al., 2013). Recently, CD8α homodimers in grass carp bound specifically to the peptide/MHC class I complex (Wang et al., 2018). Non-specific cytotoxic cells (NCC) of fish are reported to be the most active in the head kidney of teleosts and are considered similar to that of mammalian natural killer (NK) cells (Evans et al., 1984). Furthermore, the monoclonal antibodies DLT15 and DLIg3 appear to be promising tools for further exploring the ontogeny, physiology and molecular biology of fish T and B-
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Lymphocytes, and for providing new insights into the evolution of specific immunity of vertebrates (Romano et al., 1997)

Lymphocytes are the most abundant cell type, particularly in the outer zone of the thymus. The anti-CD3ε antibody identifies most of the lymphocytes localized in the outer zone and, to a lesser extent, those located in the inner zone of the thymus.

The undifferentiated cells that exhibit the morphological features of lymphoblasts are mainly located in the inner zone of the thymus. They are rounded cells with a centrally located circular euchromatic nucleus and are larger than lymphocytes/thymocytes, with a smaller nucleo-cytoplasm ratio (Cao et al., 2017).

**6.1. Monocytes**

The monocytes are not numerous (0.1–0.5% of the leukocytes population). They resemble mammalian monocytes histochemically, possessing a few fine and scattered granules, which stain positively with periodic acid–Schiff (PAS) and acid phosphatase (Mokhtar, 2021). The monocyte is irregular in shape and its cell membrane is thrown into pseudopodia. It contains a large amount of light blue-gray, finely granular or vacuolated cytoplasm and a large oval or kidney-shaped euchromatic nucleus with a dense chromatin pattern near the membrane and apparent large nucleolus (Fig. 1F). The cytoplasm has mitochondria, a large Golgi complex, endoplasmic reticulum and some small dense granules, in addition to vesicles of varying size and electron density. The mean diameter of monocytes is 12 µm and they do not differ significantly between males and females. Under appropriate circumstances, they develop into mature cells of the mononuclear phagocyte system (macrophages).
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The macrophages, monocytes and their precursors showed immunoreactivity for CD68. It is also important to mention that in grass carp, the monocytes are scarce. These cells have been demonstrated in very few species of teleosts such as *Sparus auratus* (Zuasti and Ferrer, 1989).

**2. Macrophages and melanomacrophage centers**

Actively phagocytosing macrophages are seen within the head kidney. They are also recognized in the spleen, thymus, epidermis, and around the ovarian atretic follicles. The plasma membrane shows long processes of pseudopodia. TEM shows that the macrophage is irregular in shape and is characterized by an indented eccentric kidney-shaped nucleus and its cytoplasm contains heterogeneous vesicles, phagosomes, electron-dense granules, mitochondria, pigments, lysosomes, and phagocytosed materials (Fig. 2A).

Enzyme histochemistry revealed that macrophages showed high acid phosphatase activity. It is well known that acid phosphatase is localized in the lysosomes, the main organelles in the cytoplasm of macrophages. They also express s-100 protein and desmin. Immunopositivity for the antigen CD45RO in sections of the head kidney, which identifies membrane receptors on T cells, is revealed in the population of T lymphocytes that constitute the lymphoid aggregates, concentrated around blood vessels and MMCs (Mokhtar, 2020).

Also, macrophages show strong immunopositivity for CD68 and the cells of the granulocytic lineage in various phases of maturation are positive for lysozyme. CD68 belongs to the lamp (lysosomal-associated membrane protein) – family of glycoproteins. It performs a role in antigen processing or in the protection of lysosomal membranes against lysosomal hydrolases (Holness and Simmons, 1993),
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and is widely used as a specific marker for both macrophages and monocytes. Barreda et al. (2004) confirmed that the gene expression of CD68 rises with the maturation of macrophages and monocytes in hematopoietic tissues of goldfish *Carassius auratus*. Moreover, the macrophages/monocytes also expressed CD163 and CD64. The hemoglobin scavenger receptor, CD163, is a macrophage-specific protein and is associated with the anti-inflammatory process (Law et al., 1993). CD64 is a type of integral membrane glycoprotein that presents in only macrophages and monocytes. CD64 plays an essential role in macrophage antibody-dependent cellular cytotoxicity and clearance of immune complexes (Hulett and Hogarth, 1998).

Macrophages are multifunctional cells that are mainly involved in the immune response, including phagocytosis and degradation of foreign antigens, tissue remodeling, and production of cytokines, chemokines and growth factors. Their specific localization and variations in the distribution in the ovary during the spawning season suggest that macrophages play diverse roles in intra-ovarian events, including folliculogenesis and tissue restructuring after atresia (Mokhtar and Hussein, 2020).

An interesting feature of teleost macrophages is their ability to form aggregates once they are replete. Usually, these aggregates are in the areas of the melanomacrophage center (MMC) of the hemopoietic tissues (they are prevalent in the head kidney, thymus, and spleen). However, such aggregates are frequently pigmented, around or within chronic inflammatory lesions (Vogelbein et al., 1987). These pigments, melanin and related pigments, are considered to play a defensive role in many organisms, in their capacity for generating hydrogen peroxide (Agius and Roberts, 2003).
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the liver of grass carp and they are concentrated in the adventitial layer of venous vessels, between hepatocytes and around the bile duct.

The MMCs are heterogeneous in composition, containing iron, melanin, lipofuscin, lipid, and glycogen. They are characterized by golden-brown cytoplasm with HE methods. In addition, these cells give a positive reaction with PAS. Their lipofuscin contents appear pink with Long Ziehl-Neelsen. Also, the iron contents of these aggregates appear blue by Perls Prussian blue (Mokhtar, 2018). Hemosiderin is a waste product of iron metabolism and occurs as yellow to brown intercellular granules, which often phagocytize senescent red blood cells. These centers play an important role in the storage of iron resulting from the breakdown of erythrocytes, breaking down the destroyed tissue, catching free radicals in an immune response and blood purification from suspended harmful substances. The MMs are characterized by the presence of eccentric nucleoli and cytoplasm with heterogeneous populations, lysosomes, phagosomes, myelin figures, senile RBCs or plenty of melanin granules of variable size and electron density (Fig. 2 B). The MMCs show lipid droplets and dark black deposits mostly for melanin pigments (Fig. 3A, B).

Clinical studies have shown the association of MMCs with a range of highly resistant intracellular bacteria and parasites. Furthermore, these aggregates may be a site of primary melanogenesis rather than melanin storage. In addition, melanomacrophage centers are included in antigen trapping and presentation to lymphocytes (Press and Evensen, 1999). The MMCs are considered a scavenger structure but their role in the immune system is ambiguous. Chronically stressed fishes, including those that are unhealthy, tend to have more and larger MMCs. The size and number of MMCs also increase with fish age. Some authors reveal that MMCs are suggestive of an analogy with the germinal centers of higher vertebrates.
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(Lamers and de Haas, 1985), while Mokhtar (2020) found that these centers contained macrophages and not lymphocytes.

3. Dendritic cells (DCs)

Dendritic cells (DCs) share many functional and morphological features reported in mammals. DCs are one of the antigen-presenting cells with dendritic morphology, motility, phagocytic ability and strong T cell stimulatory properties (Bassity and Clark, 2012). ATPase-positive dendritic cells and IgM-positive lymphocytes are seen in the epidermis. They were also observed in the liver as wandering lymphocytes located in the sinusoidal lumen and perisinusoidal connective tissue adjacent to endothelial cells and associated with macrophages and lymphocytes (Mokhtar, 2018). DCs are characterized by irregular shape, obviously high nuclear to cytoplasmic ratio, multiple dendrite-like cytoplasmic processes, heterochromatic nucleus, few cytoplasmic Birbeck-like granules, and rER (Fig. 2C, D). The Birbeck-like granules are electron-dense and exhibit different sizes and shapes (round and tennis-racket). The cytoplasm contains lysosomes and sometimes showing phagocytized materials. They also display macropinocytotic vesicles peripherally located, vacuoles of various sizes, mitochondria, and phagosomes.

Furthermore, functional dendritic cells have been identified in some teleosts, such as rainbow trout, salmonids, medaka, zebrafish, grass carp, and Channel catfish in different tissues; kidney, spleen and gills. Dendritic cells (DCs) are also found around the ovarian theca cells and blood vessels of tilapia in association with macrophages and constitute 8.14% of the cellular constituents of the ovarian stroma. They express S-100 protein and c-kit in the ovary of tilapia as they are involved in phagocytosis and immune surveillance in the ovary. Furthermore, these cells are
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involved in providing the interactions necessary for the function of both lymphoid and
endothelial cells of blood vessels (Meseguer, et al., 1995). This latter system is the
major constituent for blood filtering due to its ability to perform endocytosis. In
addition, dendritic cells are arranged in a layer of epithelial reticular cells of the
macrophage type in the HK, forming a network of mechanical support for the
hematopoietic cells. Moreover, these cells show ultrastructural characteristics that
permit them to exert a function associated with the removal of particles and senescent
cells, as described previously by Meseguer et al. (1995).

4. Rodlet cells

Rodlet cells (RCs) are unique cells found exclusively in teleosts (Fig. 3C) that
are considered a part of a generalized host response not only to various stressors,
especially parasitic infection, but also to toxins, neoplasia, and general tissue damage
(Mokhtar, 2019). RCs are encountered in the epidermis of the head region and dermis
of Red-tail shark and silver carp. RCs react positively to bromophenol blue.
Ultrastructurally (Fig. 3D), each RC consists of a plasmalemma in close contact with
a fibrous capsule measuring 0.6–0.8 µm in thickness. The capsules consist of a dense,
compact network of microfibrils that form a continuous structure in close contact with
the internal portion of the cell. The inner cytoplasm of RCs is occupied by several (up
to 15) longitudinal rodlets (club-shaped sacs) surrounded by several light vesicles and
vacuoles. Each rodlet is comprised of a peripheral cortex and a central dense core
(rod). The cortex forms a club-shaped sac with a fine, granular inner zone and a
coarse outer zone. These cores have been reported to be protein-rich and have
peroxidase activity (Iger and Abraham, 1997). These results suggested to some
authors that the rodlet-core material shows proteolytic activity that may have a toxic
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effect on parasites (Dezfuli, et al., 1998). Other cytoplasmic organelles such as the Golgi apparatus, rough endoplasmic reticulum, and free ribosomes are observed. The irregularly shaped nucleus occupies a lateral or basal position in RCs in contact with the capsule. The heterochromatin is distributed irregularly in the nucleoplasm.

The number of rodlets increases with the growth of the cell, and the rodlets gradually change from round to elongate. In parallel, their sacs inflate, elongating to match the rodlet form, and become less opaque. Concomitantly, the RCs’ nuclei change from oval to irregular. Rodlets can be forcefully ejected into extracellular spaces or the external environment. The rodlets themselves appear to be passive, stable elements, but their ejection has been suggested to play a role in reducing the parasitic infection. The capsule reacted positively to PAS, while the rodlets reacted negatively to PAS. The rodlet cells of HK of grass carp expressed α-SMA (Mokhtar, 2020). Rodlet cells contain an actin cytoskeleton that may have a role in the contraction to eject their contents. The rodlet cell undergoes a one-way contraction that eventually destroys the cell during the expulsion process; the rodlets along with the entire contents of the cell are expelled (Rideout, et al., 2015).

These cells are also commonly observed in organs of fish, including the thymus, spleen, kidney, gills, heart, gall bladder, skin, pancreas, liver, and blood vessel endothelium (Mokhtar, 2021). These cells were initially described as intracellular parasites and named Rhabdospora thelohani. They may be involved in water or electrolyte transport or have functions like those of mucous cells, for example, pH control, lubrication, and antibiotic effects. They may be modified goblet cells or may have a regulatory role of these cells in ion transportation and osmoregulation. They may also be considered as non-specific immune cells, involved in immunity, as their number is increased in parasitic infection. Recent studies have
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supposed a secretory function to these cells with a holocrine mode of secretion (Mokhtar, 2015). The location of RCs is probably linked to their potential role as host response cells and the need to secrete into extracellular spaces, usually the external environment or passages leading to the external environment (Manera and Dezfuli, 2004).

5. **Mast cells**

Numerous mast cells are recorded in the ovarian stroma of tilapia (which represent 10.11% of the cellular constituents), particularly in association with or in the vicinity of the blood vessels. These cells are characterized by rounded cell bodies of various sizes with an eccentric nucleus and eosinophilic granules with HE. The mast cells are positive for bromophenol blue (Fig. 4A) due to the high protein content in their granules, as well as showing a metachromatic reaction with toluidine blue. The granules of mast cells express the staining affinity for s100 protein, desmin, and c-kit. Furthermore, these granules show strong staining affinity for Safranin O. By electron microscopy, mast cells are characterized by a large oval euchromatic nucleus surrounded by numerous cytoplasmic electron-dense granules. Mast cells in teleosts are like those in mammals and can generate many mediators, chemokines, and cytokines (Mokhtar, 2019).

6. **Eosinophilic granule cells (EGCs)**

Eosinophilic granular cells (EGCs) are spherical cells that occur in the epidermis of the upper lip, snout and nostrils of silver carp. EGCs are common immune cells encountered in many species, including salmonids and cyprinids. EGCs
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are commonly found in the intestinal mucosa, gills, the connective tissue that surrounds the spinal cord, and in the epidermis of the skin. They are also found in the connective tissue stroma around the bile duct (Reite, 2005). Their nuclei are eccentrically placed and their cytoplasm contain rounded granules stained bright red by hematoxylin and eosin (HE) due to the presence of basic proteins. In addition, EGC granules stain deep magenta with PAS and alcian blue, indicating their content of sulfated and neutral glycosaminoglycans. They react positively with bromophenol blue that indicates their contents of protein as well as showing a metachromatic reaction with toluidine blue (Fig. 4B).

The marked histological and biochemical similarity between fish eosinophils and mammalian mast cells has been noted by many authors and suggests that they release toxic proteins and oxygen radicals onto the body surface of multicellular parasites in areas of inflammation. Acute tissue damage causes EGC degranulation and release of mediators of inflammation, whereas an increase in the number of these cells is often found in chronically inflamed tissues (Jordanova et al., 2007).

7. Kupffer cells

Kupffer cells are pleomorphic cells, situated in the hepatic sinusoids. They project slightly to the sinusoidal lumen and establish close contact with endothelial cells (Fig. 4C). They possess irregular cell surfaces and contain lysosomes, phagosomes in the form of vacuoles varying widely in diameter, density, and shape as well as a few fat droplets (Figs. 4D). Small groups of parallel tubular structures can be observed in their cytoplasm, termed tubulosomes, that are arranged in longitudinal profiles in the vicinity of lysosomes. The nuclei of Kupffer cells are indented and
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frequently eccentrically located. Clumps of heterochromatin are distributed
throughout the nuclei and form a distinct rim along with the nuclear envelope.

Kupffer cells are situated in the hepatic sinusoids in grass carp, spotted
pimelodus and juvenile crocodile. However, no classical Kupffer cells are present in
the liver of Kareius bicoloratus and Salmo trutta fario. Kupffer cells play an
important role in removing degenerated blood cells, degrading hemoglobin and
eliminating toxic and foreign substances by the presence of many lysosomes in their
cytoplasm. In addition, tubulosomes are evident in the cytoplasm of Kupffer cells in
association with phagosomes. These structures may be specialized lysosomes
involved in the breakdown of phagosomal contents (Wilpe and Groenewald, 2014)

8. Barrier cells

Barrier cells are among the cells found in the head kidney. They are few in
number with electron-dense, elongated, and branched appearance (Fig. 5A).
Numerous ribosomes, well-developed secretory organelles, and electron-lucent
vesicles characterize the cells. Large granules and microfilaments are also present.
Dense reticular-like cells recognized as barrier cells in the head kidney of grass carp
appear as pericytes or line the blood sinusoids, resembling those described in the
spleen and bone marrow of mammals (Secombes and Wang, 2012). The processes of
the barrier cells extending among the extracellular matrix may increase the filtration
capacity and clearance of blood. Furthermore, barrier cells are observed to be closely
associated with clusters of haemopoietic and lymphopoietic cells. They thus form part
of the haemopoietic microenvironment, with the function of isolating putative stem
cells, concentrating haemopoietic factors and regulating the migration of blood cells
in circulation. As such, the presence of barrier cells in fish head kidney reinforces the
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proposed homologies between this organ and the mammalian bone marrow (Alvarez, et al., 1996).

9. Osteoclasts

Osteoclasts are specialized large multinucleated bone cells (mononucleated in juveniles) with phagocytic properties. They dissolve the matrix by releasing lysosomal enzymes. During bone remodeling, many osteocytes are released from their lacunae as osteoclasts, which actively reabsorb the bone. The relationships between bone, connective tissue, and cartilage are more complex than those in higher vertebrates (Sire et al., 1990). They are derived from undifferentiated cells of the monocyte-macrophage lineage. Osteoclasts are characterized by a cytoplasm with a homogeneous, "foamy" appearance. This appearance is due to a high concentration of vesicles and vacuoles. These vacuoles include lysosomes filled with acid phosphatase. This permits the characterization of osteoclasts by their staining for high expression of tartrate-resistant acid phosphatase (TRAP), MMP9, and cathepsin K (Väänänen et al., 2000).

10. The thrombocytes

The thrombocytes (Fig. 5B) are elongated, fusiform cells with an indentation in the plasma membrane. They are predominantly smaller than the lymphocytes, with a central compact nucleus. The nucleus shows deep heterochromatic sulci and the cytoplasm is characterized by the presence of numerous coated vesicles of different shapes and sizes. The thrombocytes of fish should not be considered as the platelets of higher vertebrates because they are true cells. Thrombocytes have been described in goldfish as cells with dense chromatin and to be morphologically similar to
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lymphocytes (Stosik et al., 2019). The total number of thrombocytes ranges from 60000 to 70000/mm³.

The thrombocytes are clearly differentiated from lymphocytes by their spindle shape, clear vacuoles, marginal microtubules and electron-dense granules in the cytoplasm. The nucleus contains peripherally located moderate heterochromatin. The cell edges present some finger-like projections. The cytoplasm is hyaline when stained with a Romanowsky-type dye. Thrombocytes clearly differ from all other leucocyte populations in possessing clear canalicular structures and small variably dense granules in their cytoplasm. Glycogen granules are also found. By Giemsa stained preparations, the chromatin-rich nucleus exhibits a dark blue or dark violet color. The cytoplasm is extremely scant and usually has a light blue area adjacent to one of the poles of the nucleus. In addition to taking part in blood clotting, it has been reported that the piscine thrombocytes are blood macrophages that form one of the protective barriers against foreign agents and might be considered true digestive cells, because they may remove circulating cell fragments directly by phagocytosis (Köllner et al., 2004).

11. Club Cells

Club cells were frequently observed in the skin of fish and esophagus of some fish (Abdelhafez, 2013). The cytoplasm of club cells is rather poor in organelles and rich in non-vesicle secretion. The few observed organelles (endoplasmic reticulum, Golgi complexes, polyribosomes, and mitochondria) are located in the perinuclear region, while the rest of the cytoplasm is filled with a filamentous substance. Therefore, cytoplasmic content can be separated into two regions: one light and electron-lucent around the nucleus and the other abundant and electron-dense, which
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occupies nearly the entire cytoplasmic volume. Large vacuoles are occasionally displayed in the peripheral cytoplasm. The plasma membrane shows invaginations throughout its length, making the cell surface irregular and slightly interdigitated with the epidermal cells.

The club cell cytoplasm shows the absence of glycoprotein content, as determined by the negative reaction to PAS and toluidine blue stains (Fig. 5C). The club cells of many Cyprinidae such as Red-tail shark secret proteinous substances as they stain positive with bromophenol blue. Club cells are related to the production, storage, and release of the alarm substance, leading to an alarm reaction in phylogenetically close species. The alarm reaction is triggered when fish are threatened and this reaction is induced by substances released from injured skin. This event causes disruption of the plasma membrane of the club cells, resulting in exposition and releasing of cytoplasmic content into the water. Anti-pathogenic and phagocytic functions for these cells are also suggested. Chondroitin and keratin were also found in the cytoplasm of club cells of some fish, suggesting a healing function, thus helping the repair of damaged tissue. Serotonin immunoreactivity was found in the club cells of *Heteropneustes fossilis* and *Carapus acus*. The serotonin contained in the club cells can affect the pheromonal or other possible functions of these cells (Zaccone et al., 1990).

12. Mucous cells

Skin mucus acts as the first barrier to infection. The mucus of fish contains lectins, pentraxins, lysozymes, complement proteins, antibacterial peptides and immunoglobulin M (IgM), which have an important role in inhibiting the entry of pathogens. In addition, the epidermis is able to react to different attacks (thickening...
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and cellular hyperplasia), and its integrity is essential for osmotic balance and to prevent the entry of foreign agents (Shephard, 1994).

Furthermore, the mucous cells of most teleosts are PAS-AB positive as the sulfate groups provide acidification of glycoproteins, which is effective to prevent bacterial and viral invasion. They also show metachromatic reaction (Fig. 5C). Skin mucus has evolved to have robust mechanisms that can trap and immobilize pathogens before they can contact epithelial surfaces. This occurs because in this mucus layer particles, bacteria, or viruses are entrapped and removed from the mucosa by the water current. Furthermore, mucus in most fishes is continuously secreted and replaced, which prevents the stable colonization of potentially infectious microorganisms as well as the invasion of metazoan parasites (Moron, et al., 2018). The different functions that have been suggested for fish mucus and its role as a clue component of fish immunity have been considered. The skin mucus provides a medium in which the antibacterial mechanisms may act. Fish skin mucus, thus, serves as a repository of a variety of biologically active substances as well as numerous defensive molecules of both the innate and acquired immune system. Mucus performs various functions (besides inhibition of the invasion and proliferation of pathogenic microorganisms) including ion regulation, osmoregulation, lubrication, and parenteral care behavior (Mokhtar, 2017).

Many substances with biostatic and biocidal activity (e.g., complement, C-reactive proteins, proteases, lectins, lysozyme, hemolysins, agglutinin, proteolytic enzymes, antimicrobial peptides, antibodies, and immunoglobulins) are present and have been identified in the fish epidermis and/or skin mucus. Although the protective role of the epidermal mucus of fishes has been known for many years, of great interest
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at the present is to see the skin mucus as a source for isolation of new and potent antimicrobial components (Ourth, 1980).

The most studied enzyme present in fish mucus is lysozyme. Lysozyme (N-acetylmuramide glucanohydrolase or muramidase) is a ubiquitous bactericidal enzyme identified in a wide range of organisms including fishes. Lysozyme is present in mucus, lymphoid tissue, and serum of most fish species, but not in others (such as cod and wolfish). The bacteriolytic activity of lysozyme in fish skin mucus and other tissues contributes to its host defense mechanism against bacterial infection (Shailesh Saurabha and Sahoo, 2008).

13. Telocytes

Telocytes (TCs) are interstitial cells that establish relations to various types of cells. Telocytes have unique morphological characteristics. They are characterized by spindle-shaped cell bodies and multiple cell prolongations called telopodes. Telopodes (Tps) may give rise to dichotomous branches and establish cellular connections to form a complex labyrinthine system. Telopodes are composed of thin segments called podomers and thick ones termed podoms. The podoms are rich in calcium release units that consist of mitochondria, endoplasmic reticulum, and caveolae. TCs are reported in gonads, gills, skin, around the bile ducts and blood vessels of grass carp, silver carp, and tilapia. In the dermis of silver carp, numerous TCs are observed among the collagen fibers in neighboring fibroblasts (Mokhtar, 2018). Their cell body contains an oval euchromatic nucleus. The telopodes possess many secretory vesicles (Fig. 6). These TCs establish a network in the dermis by their Tps. The Tps are connected to fibroblasts and extend to the epidermal cells.
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Telocytes are multifunctional cells (Cretoiu and Popescu, 2014); they contribute to the generation and transmission of nerve impulses to involuntary muscles. They serve in mechanoreception and exhibit receptors for excitatory and inhibitory neurotransmitters. They establish contact with immunoreactive cells such as eosinophils, mast cells, and macrophages. Telocytes play a role in the regeneration of some organs. Telocytes exert their effect on cells either by establishing cellular contact or through paracrine mode. Two types of cellular contact are documented for telocytes; homocellular and heterocellular contact. Homocellular contact is formed between two telopodes or between the cell body of two adjacent telocytes. Heterocellular contact occurs between telocytes and stromal cells.

14. Plasma cells

Secretory immunoglobulins (Ig) are produced mainly by plasmablasts and plasma cells and play key roles in the maintenance of mucosal homeostasis. The plasma cells were identified in the anterior kidney, as well as the spleen and appeared to be primarily or entirely responsible for the long-term antibody responses in teleost (Fast, 2002). Three types of antibodies, IgM, IgD and IgT, are the major constituents of humoral immunity, which act on invading extracellular pathogens. In teleosts, plasma cells are crucial to provide lifetime protection against pathogenic infections, which is important for vaccine development for aquaculture (Firdaus and Zamri, 2016).

15. Antigen-sampling cells

As in mammals, a common mucosal immune system has been proposed in teleost fish, as antigen uptake in the gut triggers elevated levels of specific antibodies
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at other mucosal surfaces, such as gills and skin (Vervarcke et al., 2005). In mammals, surveillance of the intestinal luminal content is carried out by specialized M-cells in the intestinal epithelium, which sample antigens and transport them to underlying macrophages or dendritic cells. In the steady state of mammals, induction of intestinal immune responses is mainly dominated by local production of immunoglobulin A (IgA) secreted into the intestinal lumen to prevent pathogens from entering tissue compartments (Menard et al., 2010).

Teleost fish lack lymphoid follicles in the intestine as found in mammals, and proper mammalian M cells have until now not been detected. However, in the Atlantic salmon, certain enterocytes in the second segment of the mid-intestine located at the fold base were described to absorb BSA-conjugated gold particles (10 nm) (Fuglem et al., 2010). Scanning electron microscopy further proved these cells to exhibit a depressed surface with irregular and short microvilli, while ultrastuctural studies showed more vacuolated but more electron-dense cytoplasm compared with other enterocytes. Moreover, lectin-binding assays revealed the presence of cells in similar positions as the gold-BSA absorbing cells to express the lectin UEA, which is characteristic of mammalian M cells. Additionally, IgT has been presumed to be an immunoglobulin with prevalent function in mucosal tissues in teleost fish (Zhang et al., 2010).

Concluding Remarks and Future Research

The functions of immune parameters at the individual, species, and population levels are ambiguous, and the relationships between the various immune parameters remain poorly understood. Increased knowledge of the mucosal innate immune factors could be advantageous in fish farming and possibly human health, beyond the area of
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immune evolution. A detailed analysis of the innate immune-related molecules in fish, including their function and network will certainly generate new technologies that can be applied to improve aquaculture.

The sites of antigen capture and presentation and the sites of antibody production are still unknown with regard to mucosal immunity. Although many genes have started to be studied, the cellular source(s) has not yet been determined. New technologies based on gene studies will reveal novel patterns of teleost mucosal gene expression and will highlight unexpected roles for candidate genes and pathways. The utilization of these findings will improve strategies for selection of disease-resistant brood stock and evaluation of prevention and treatment options.

Another important research field at present is to study the effects of the diet on fish mucosal immunity. Probiotics are gaining scientific and commercial interest and are now quite commonplace in health-promoting functional foods to therapeutic, prophylactic, and growth supplements. Future research will analyze the effects of oral administration of probiotics at mucosal levels (skin, gills, and gut), considering that the mechanisms by which probiotics exert their beneficial effects on the host are largely unknown and new molecular works are needed. This underscores the interconnection of mucosal tissues in the body, potentially permitting the application of functional feed additives to improve fish skin health.

Furthermore, each fish species secretes AMPs with structural differences, which can be used by the pharmaceutical industry in its search for novel drugs to treat drug-resistant pathogens. Furthermore, AMPs possess other desirable characteristics which may be exploited in the near future as antimicrobial agents, vaccine adjuvants, inactivated vaccines, and antitumor agents, even for human beings. For that, a deeper knowledge of mucosal immunity and of the immunological progression from mucosal
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innate to acquired immune systems is needed to prevent and control infectious diseases in fishes.

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Figure legends:

Fig. 1: leukocytes distribution in fish tissues

(A): Neutrophils in the liver of grass carp. (B) Eosinophils (violet) in the trunk kidney of grass carp showed rounded electron-dense granules. Note heterophils (red) characterized by many vacuoles and rod-like granules. (C) Eosinophils (arrowheads) by HE in the ovarian stroma of tilapia. (D) Macrophages (blue) extended their processes (arrowhead) around mature basophils (pink). (E) Higher magnification of lymphocyte in the head kidney of grass carp showing a large heterochromatic nucleus and few cytoplasmic vacuoles (arrowhead). (F) Monocyte (orange) in the head kidney of grass carp showing mitochondria (arrowhead). Note the presence of many lymphocytes (blue).

Fig. 2. Digital colored TEM micrographs of the macrophages and dendritic-like cells in the liver of grass carp.
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(A): Macrophage (pink) with many lysosomes (L) and phagosomes (arrowheads).

(B): Melanomacrophage (pink) contains many melanin pigments (arrowheads).

(C): Dendritic-like cell (blue, arrowhead) in perisinusoidal c.t. extends its processes to neighboring endothelium (E) and melanomacrophage cell (MC).

(D): Dendritic cell (blue, DC) is in the sinusoidal lumen in the vicinity of lymphocyte (LC). Note the connection of their dendritic processes with degenerated endothelium and the presence of vesicles (arrowhead) in the processes and few granules (arrow) in the cytoplasm.

Fig. 3: (A) Semithin section of melanomacrophage center in the kidney of grass carp showing melanin pigments (white arrowhead) and digested particles (black arrowhead). 

(B) TEM of melanomacrophage center showing melanin-containing vesicles (white arrowhead), lysosomes (black arrowhead), and phagosomes (arrow).

(C) Semithin section in the kidney of grass carp showing many rodlet cells (arrowheads). 

(D) TEM of rodlet cell showing thick fibrillary capsule (arrow), rodlet granules (white arrowhead) and polyribosomes (black arrowhead).

Fig. 4. (A) Mast cells (arrowheads) in the ovarian stroma of tilapia stained by bromophenol blue. 

(B) Semithin section stained by toluidine blue showing EGCs (arrowheads) in the epidermis of silver carp.

(C) Digital colored TEM micrographs of hepatic sinusoids in the liver of grass carp that are lined with endothelial cells (EC, blue) and Kupffer cells (KC, violet) and surrounded by space of Disse (SD) and melanomacrophages (MC). Note fenestration (arrowheads) in the endothelial cells.

(D): Kupffer cells (KC, violet) contain many lysosomes (L) and phagosomes (arrowhead).
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Fig. 5: (A) Digital colored TEM image of barrier cells (blue, white arrowhead) in the head kidney of grass carp extended their cytoplasmic processes around the blood vessel (BV). Note the mature erythrocyte (red, arrow) and lymphocyte (black arrowhead). (B) The blood smear with Giemsa's stain of Nile tilapia showing (1) erythrocytes are oval in shape and always contain a central nucleus, (2) neutrophils. Note the thrombocytes (arrowhead). (C) Semithin section of the anterior portion of the oesophagus of grass carp showing mucous goblet cells (gc), club cells (cc), and basal cells (bc). Note the apical microridges (arrow).

Fig. 6. Digital colored TEM micrograph of telocytes in the liver of grass carp. (A): Bile duct (BD) is surrounded by telocytes (TC, blue) and their telopodes (TP). TC contain mitochondria (M in inserted figure) and caveolae (C). Note the presence of fibrocyte-like cells (F) and melanomacrophage (MM). Lymphocyte (LC) is situated in a sinusoidal lumen, adjacent to Kupffer cell (KC). Ito cell (IC) and sinusoidal endothelium (pink, E) establish direct contact with TC. (B): Two telocytes (blue, TC) surround the blood capillary (pink, BC) and connected with their telopodes (Tp). (C): TCs with their telopodes (Tps) encircle a bile duct (violet, BD). Note the presence of many secretory vesicles (arrowheads) in the podoms of telopodes in Figs. A–C.