

Review

The Role of Toll-like Receptors (TLRs) in Fish Immunity

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Abstract

The innate system's recognition of non-self and danger signals is mediated by a limited number of germline-encoded pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) are single, non-catalytic, membrane-spanning PRRs present in invertebrates and vertebrates. They act by specifically recognizing PAMPs of a variety of microbes and activate signalling cascades to induce innate immunity. A large number of TLRs has been identified in various teleosts and basal vertebrates. The varied evolutionary lineages of aquatic and warm-blooded higher animals result in some unique characteristics in their TLRs. In innate immunity and pathogen recognition, the majority of them share conserved signalling pathways. Comparing the immunology of teleosts, chondrichthyans, agnathans, and warm-blooded vertebrates requires a functional analysis of novel TLRs in teleosts and basal vertebrates. Furthermore, TLRs play important roles in adaptive immunity in addition to innate immunity. This review describes the discovery of TLRs and their role in fish immunity along their signalling pathway and ligand specificity.

Keywords: pathogen-associated molecular pattern; pattern recognition receptor; TLR signalling pathway; ligand specificity; innate immunity

1. Introduction

When microorganisms penetrate protective barriers such as the skin or mucosal surfaces, immune recognition by receptors expressed on dendritic cells, macrophages, B lymphocytes, endothelial cells, and other cell types initiates coordinated responses to eliminate the invading agents [1]. Macrophages and dendritic cells, traditionally classified as innate immune cells, are central to the initial sensing of pathogens and play a pivotal role in directing subsequent adaptive immune responses. Their activation is driven by germline-encoded receptors that detect conserved molecular motifs shared by diverse pathogens [2,3]. The innate immune system serves as the primary defensive barrier against invading microbial pathogens and is evolutionarily conserved across mammalian species [4,5]. Pathogen-associated molecular patterns (PAMPs) trigger innate immune responses in animals, including insects and vertebrates. In *Drosophila*, the Toll receptor was originally identified for its role in establishing dorso-ventral polarity during development. Subsequently, in 1996, Toll was shown to play a critical role in mediating innate immune responses against fungal infections. Subsequently, numerous homologues of Toll, termed



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Toll-like receptors (TLRs), were identified in mammals which recognize PAMPs. Toll-like receptors are type-1 transmembrane proteins with numerous extracellular leucine-rich repeat (LRR) motifs which together form a horseshoe-like shaped solenoid directly involved in the interaction with a ligand. The specificity of Toll-like receptors (TLRs) is determined by variations in the number and sequence of leucine-rich repeat (LRR) motifs, which enable interaction with a wide range of ligands, including lipids, carbohydrates, proteins, and nucleic acids. Ligand binding to the concave surface of the TLR extracellular domain induces conformational changes that promote receptor homo- or heterodimerization. This dimerization brings two intracellular Toll/interleukin-1 receptor (TIR) signalling domains into close proximity, thereby initiating the recruitment of adaptor proteins. Unlike the variable extracellular LRR regions, the cytoplasmic TIR domain is highly conserved across different TLRs within a species as well as among diverse animal taxa, underscoring its essential role in adaptor molecule recruitment and signal transduction [6,7].

The first identification of a Toll-like receptor (TLR) gene in fish was reported in the goldfish *Carassius auratus auratus*. Analysis of this receptor confirmed the presence of conserved vertebrate TLR structural features, including extracellular leucine-rich repeat (LRR) motifs and an intracellular Toll/interleukin-1 receptor (TIR) homology domain. In the same year, a comprehensive survey of TLR genes was conducted using the draft whole-genome sequence of fugu (*Takifugu rubripes*), leading to the identification of several unique TLR genes [8]. In addition, the zebrafish (*Danio rerio*) genome database was analyzed to identify all putative TLR genes, which were subsequently reported in 2004 [9]. In a study on the Japanese flounder (*Paralichthys olivaceus*), eleven homologous Toll-like receptor (TLR) types were identified and the findings were presented at the 7th Symposium on Diseases in Asian Aquaculture. To date, the roles of TLRs in the innate immune system of teleost fish have been progressively elucidated through TLR gene cloning and foundational functional studies. Moreover, several novel TLR genes have been identified in teleost species. Consequently, investigation of PAMP recognition and immune activation mediated by fish TLRs represents an important and compelling area of research in comparative immunology.

2. Discovery of TLRs

Toll-like receptors (TLRs) are now recognized as key molecules that alert the immune system to the presence of microbial invaders. The structural components of TLRs are conserved across plants and animals (Figure 1) and are essential for host defence against infection, indicating that TLRs represent one of the most ancient and evolutionarily conserved elements of the immune system (Figure 1). TLRs were first identified in 1985 in *Drosophila melanogaster* embryos as products of the *Toll* gene, which regulates dorso-ventral pattern formation during development. The term “Toll,” meaning “weird” or “great” in German, was later linked to immune function when the receptor was shown to regulate antimicrobial peptide production and play a crucial role in defences against fungal infections. Historically, the foundation of TLR research can be traced back to the discovery of phagocytic cells in 1883 and the subsequent identification of interleukin-1 in 1940. TLR down-stream signalling molecules such as MyD88 (Myeloid differentiation primary response 88) and IRAK (Interleukin-1 Receptor-Associated Kinase) were first discovered in IL-1 signalling. Homology between drosophila Toll and IL-1RI was noticed in 1991, which led to this discovery of toll-like receptor (TLR) in human. It was demonstrated that a Toll-like receptor, later identified as TLR4, is capable of activating specific genes that are essential for the initiation of adaptive immune responses [10]. Subsequently, the expression of TLR4 was demonstrated to be a critical determinant of cellular responsiveness to lipopolysaccharide (LPS) [11], and from then onwards a focus in this direction started [12].

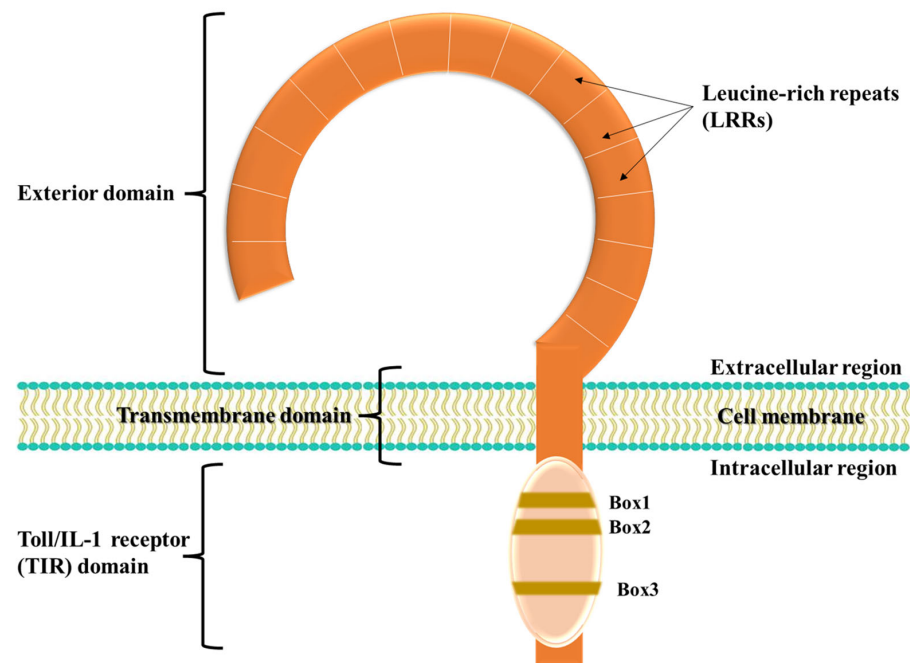


Figure 1. Schematic of a Toll-like receptor (TLR). The extracellular region contains leucine-rich repeats (LRRs), while the intracellular Toll/IL-1 receptor (TIR) domain is homologous to the cytoplasmic region of the IL-1 receptor.

The first fish member of the interleukin-1/Toll-like receptor superfamily was identified and characterized in *Oncorhynchus mykiss* [13]. Shortly thereafter, the cDNA of interleukin-1 receptor (IL-1R) was identified in Atlantic salmon [14], which comprises a C-terminal TIR domain [15]. The first piscine TLR gene was reported in gold fish (*Carassius auratus auratus*) [15]. Subsequently, TLR genes were comprehensively analyzed using the draft whole-genome sequence of fugu (*Takifugu rubripes*), leading to the identification of several unique TLR genes [16]. In 2004, a zebrafish (*Danio rerio*) genome database search revealed several predictable TLR genes [17]. To date, twenty TLR types (TLR1, 2, 3, 4, 5M, 5S, 7, 8, 9, 13, 14, 18, 19, 20, 21, 22, 23, 24, 25, and 26) have been identified across more than a dozen teleost species.

3. TLRs and Their Signalling Pathway

Fish TLRs retain key structural motifs and signalling elements (Figure 1) that are conserved in mammalian TLRs, highlighting their fundamental role in innate immunity. Nevertheless, fish TLRs exhibit greater diversity and several distinctive features, including variations in ligand specificity and extracellular domain organization. These differences are likely a consequence of their diverse evolutionary lineage and the unique aquatic environments they occupy, which have shaped both their structural adaptations and functional specializations in pathogen recognition and immune signalling [18]. In fish, six non-mammalian TLRs have been identified. TLR14 shares sequence and structural similarity with TLR1 and TLR2, while TLR19–TLR26 constitute a cluster of novel TLRs. The TLR4 gene is absent in the genomes of most fish species; in zebrafish, TLR4 does not recognize the mammalian ligand LPS and is likely paralogous rather than orthologous to mammalian TLR4. Among the twenty TLR types identified in fish, ligand specificity has been experimentally confirmed only for TLR3, TLR5M, TLR5S, and TLR22 [18]. To date, twenty-one TLRs have been identified in aquatic species, categorized into six families, namely, TLR1, TLR3, TLR4, TLR5, TLR7, and TLR11 [19,20]. The family consists of TLR1, which has been classified into subfamilies that include TLR1, TLR2, TLR6, TLR10, TLR14, TLR15, TLR16,

TLR18, TLR25, TLR27, and TLR28. These subfamilies identify the lipopeptides [21,22]. Six of these are non-mammalian TLRs, with TLR14 sharing sequence and structural similarity with TLR1 and TLR2, while TLR19–TLR26 form a cluster of novel TLRs. The TLR4 gene is absent in the genomes of most fishes; in zebrafish, TLR4 does not recognize the mammalian ligand LPS and is likely paralogous rather than orthologous to mammalian TLR4. Among fish TLRs, ligand specificity has been experimentally confirmed only for TLR3, TLR5M, TLR5S, and TLR22. In rainbow trout (*Oncorhynchus mykiss*), TLR5M signalling in response to flagellin is amplified through interaction with TLR5S, forming a positive feedback loop. Gene and genome duplications have contributed to the rich evolutionary history and genomic diversity of teleosts, with duplicate or multicopy TLR genes reported for TLR3 and TLR7 in common carp. In zebrafish, TLR4b, TLR5, TLR8, and TLR20 have been identified, whereas TLR8a and TLR22 are present in rainbow trout. These findings highlight both the structural conservation and functional diversification of TLRs in teleost fish, shaped by their evolutionary lineage and aquatic environments [23,24]. TLR25 has structural similarity to TLR1; however, it is devoid of the N-terminal cap and LRR [25]. However, precise ligands for TLR26 in fish are yet unknown but present in numerous subclades, such as catfish (*Siluriformes*) [26].

These TLRs are trafficked after glycosylation to their functional position. Specific N-glycans control the movement of TLR2 and TLR4 to the plasma membrane and the placement of nucleic acid-sensing TLRs, such as TLR3 and TLR9, in endosomal compartments [27]. The long carbohydrate chains on the LRR domains not only give the receptor structural stability and help it stay in the right place in the cell, but they also help shape the receptor's ligand-binding sites. The glycan shielding is necessary for correctly identifying pathogen-associated molecular patterns (PAMPs). The TLR4/MD-2 (Myeloid Differentiation factor 2) complex requires accessory proteins like LBP (Lipopolysaccharide-Binding Protein) and CD14 (Cluster of Differentiation 14) for the delivery and recognition of lipopolysaccharide (LPS). While the extensive N-linked glycosylation of TLR4 and MD-2 is critical for proper protein folding and stable cell-surface expression, receptor activation is strictly ligand-driven rather than controlled by glycan steric shifts. Specifically, the lipid A portion of LPS binds to the hydrophobic pocket of MD-2, creating a composite interaction surface. This ligand-bound MD-2 complex then physically bridges to a second TLR4 molecule, forcing the ligand-induced homodimerization that forms the active (TLR4/MD-2/LPS)₂ multimer [28]. The dimerization is what physically connects the extracellular recognition event to the intracellular signalling machinery. Proper glycosylation-induced conformational changes allow the intracellular Toll/IL-1 receptor (TIR) domains to come together and draw in important adaptor proteins like MyD88 and TRIF (TIR-domain-containing adapter-inducing interferon- β). Because of this, unglycosylated TLRs cannot make important signalling complexes, which makes it much harder for NF-kappaB (Nuclear factor- κ B), MAP (Mitogen-Activated Protein) kinases, and inflammatory cytokines to be activated [29]. TLR glycosylation is an important biochemical gatekeeper that controls the entire life cycle, ligand sensitivity, and signalling effectiveness of these basic innate immune sensors [29].

3.1. Beyond the Leukocyte: The Mucosal Sentinel Paradigm

While classical research has historically centred on professional leukocytes as the chief arbiters of fish immunity, the expansive environmental interface of aquatic vertebrates necessitates a highly active, decentralized defence system at the mucosal borders. In fish, non-professional immune cells—specifically epithelial and mucosal sentinels—do not merely act as passive structural barriers; they function as primary immunomodulating units capable of autonomous pathogen sensing and response.

3.1.1. Mucous and Goblet Cells

The “Detect-and-Respond” Axis Goblet cells and superficial mucosal epithelial cells express a rich repertoire of pattern recognition receptors, including several conserved membrane-bound and endosomal TLRs. Rather than waiting for leukocyte infiltration, these cells operate via a localized “detect-and-respond” mechanism. Upon the ligation of specific microbial PAMPs (such as bacterial lipopeptides or flagellin) to goblet cell TLRs, intracellular signalling cascades are rapidly activated. This engagement triggers a dual-action protective output: it accelerates the regulated exocytosis of stored mucin granules to physically flush out pathogens, while simultaneously driving the immediate *de novo* transcription and secretion of localized antimicrobial peptides (AMPs), lysozymes, and lectins. This real-time tuning of mucosal fluid composition demonstrates that goblet cells are dynamic, primitive immune processors that shape the immediate chemical environment of the host interface long before systemic pathways are engaged.

3.1.2. Agnathan Specializations

Thread and Skein Cells as Evolutionary Missing Links: To map the evolutionary origins of this mucosal sentinel paradigm, we must examine the most primitive living vertebrates: the jawless Agnathans. Hagfish and lampreys lack the classical professional antigen-presenting networks found in higher teleosts, yet they maintain highly efficient mucosal barriers. In hagfish, this is exemplified by specialized epithelial “thread cells,” while lampreys possess homologous “skein cells” [30]. Recent evolutionary analyses indicate that these distinct cell types express ancestral members of the TLR2 subfamily. Thread and skein cells represent an elegant ancestral integration of mechanical defence and innate molecular sensing. When primitive TLRs on these cells encounter foreign pathogen motifs, the structural signalling induces a rapid, explosive release of dense, proteinaceous threads and muciform components that combine with water to create an immediate, high-volume protective slime layer. This unique process couples mechanical entrapment with direct localized innate sensing, providing an evolutionary bridge showing that early vertebrate immune strategies tightly interlocked physical barrier amplification with targeted receptor-mediated pathogen recognition.

3.2. Evolutionary Ontogeny: The DC-Axis

The transition from jawless vertebrates (Agnatha) to jawed ray-finned fishes (Teleostei) unveils a profound evolutionary shift in how TLR-mediated pathogen signals are translated into systemic immunity. In Agnathans, which rely on a unique adaptive architecture using variable lymphocyte receptors (VLRs), the coordination between sensing and defence is heavily decentralized, resting mostly on the aforementioned non-professional epithelial and mucosal sentinels. However, as vertebrates evolved toward the teleost lineage, an increasingly specialized “DC-axis” began to take shape. Teleosts mark the clear emergence of specialized, professional Antigen Presenting Cells (APCs) that exhibit morphological and functional homology to mammalian classical dendritic cells, governed by conserved transcription factors such as ZBTB46 and DC-SCRIPT [22].

This evolutionary milestone altered the structural hierarchy of host defence: TLR-mediated signals that were once localized within primitive epithelial cell sheets became “delegated” to highly mobile, specialized DC-like populations. In teleosts, these professional cells utilize precise endosomal and cell-surface TLR profiles (such as TLR3, TLR7, and TLR9) to sample the aquatic microenvironment. Upon TLR activation, these fish DCs undergo maturation, upregulate mandatory co-stimulatory complexes, and migrate into systemic lymphoid hubs to activate naive T-cells and direct precise immunoglobulin responses. This orderly centralization of TLR processing allowed lower jawed vertebrates

to step away from a purely localized, hyper-inflammatory tissue response and transition toward a highly targeted, energy-efficient systemic memory system [22].

3.3. The Impact of Environmental Factors on TLR Expression

As fish are strictly poikilothermic ectotherms, every facet of their biochemical, metabolic, and immunological machinery is fundamentally bound to the physical variables of their aquatic habitat. Consequently, an “eco-immunological” framework is required to accurately evaluate fish TLR dynamics, as shifts in ambient water temperature, salinity, and pH can significantly alter baseline receptor transcription, structural conformation, and ligand-binding kinetics.

3.3.1. Thermal Effects on Membrane Dynamics and Receptor Dimerization

Fluctuations in water temperature exert an immediate effect on the biophysical properties of host cellular membranes. For a fish, drops in ambient temperature led to homeo-viscous phase changes, rendering the plasma membrane increasingly rigid. This physical restriction directly stalls the lateral mobility of type-1 transmembrane proteins, severely impeding the ligand-induced homo- or heterodimerization required to bring intracellular TIR domains into close proximity (Table 1). Conversely, elevated temperatures within a species’ optimal ecological range enhance membrane fluidity, accelerating TLR mobilization and the subsequent recruitment of essential adaptor proteins like MyD88 and TRIF. This thermal dependence explains seasonal vulnerabilities frequently observed in aquaculture, where sudden cold snaps can functionally mute TLR-mediated downstream cytokine cascades, opening transient windows for pathogenic exploitation [31].

Table 1. Effect of environmental factors on fish TLR functionality.

Environmental Variable	Primary Mechanism	Impact on Fish TLR Functionality
Temperature flux	Modulates membrane fluidity and overall cellular metabolic rate.	Higher temperatures can accelerate TLR homo- and heterodimerization, whereas lower temperatures increase membrane rigidity and may hinder adaptor recruitment and downstream signalling.
Salinity gradients	Induces osmotic stress and alters ionic balance across mucosal surfaces.	Changes in salinity can shift baseline TLR expression through osmo-responsive pathways, potentially influencing seasonal susceptibility to halophilic pathogens.
pH fluctuations	Changes the ionization state of amino acid residues within receptor proteins.	Marked pH shifts may disrupt hydrogen-bonding networks within the extracellular leucine-rich repeat domain, reducing ligand-binding affinity and weakening pathogen recognition.

3.3.2. Salinity Osmoregulation and Ambient pH Disruptions

Salinity and pH changes alter the precise physical layout of the chemical interface where fish TLRs meet microbial ligands. Salinity fluctuations induce acute osmotic stress, forcing cells at the mucosal barrier to reallocate metabolic resources toward active ion transport. This homeostatic strain suppresses baseline TLR gene transcription, reducing the surface density of crucial pattern recognition molecules. Simultaneously, changes in water pH alter the charge properties of both the pathogen’s surface and the host receptor’s binding pockets. The structural stability of the extracellular horseshoe-like solenoid domain is maintained by delicate ionic and hydrogen bonding networks among its dense leucine-rich repeat (LRR) motifs. Extreme acidification or alkalinization of the water column modifies the ionization state of these critical contact residues. This can distort the concave

ligand-binding architecture or shield the hydrophobic pockets needed to secure microbial components, causing sharp declines in ligand binding affinity and compromising the host's initial defensive line.

4. TLRs and Their Ligand Specificity

Toll-like receptors (TLRs) are critical regulators of both innate and adaptive immune responses, and their functional complexity continues to attract significant research interest. Mutations that impair TLR function are associated with the development of autoimmune, inflammatory, and infectious diseases. Such conditions are believed to arise from defective negative regulation of TLR signalling, resulting in uncontrolled activation and excessive production of pro-inflammatory cytokines [32–34]. Polymorphisms in genes encoding Toll-like receptors have been identified and are associated with disease susceptibility and progression [35].

In teleost fish, Toll-like receptors (TLRs) participate in the recognition of a wide range of pathogens through diverse mechanisms (Figure 2). In zebrafish, TLR activation has been reported following infection with *Aeromonas salmonicida* and *Staphylococcus aureus* [36]. In *Sparus aurata*, the expression of TLR5 and TLR9 in purified phagocyte populations is upregulated in response to various pathogen-associated molecular patterns (PAMPs) [37]. In piscine parasites, several molecules capable of functioning as pathogen-associated molecular patterns (PAMPs) have been identified, particularly in fish flagellates. In *Trypanosoma carassii*, glycosylphosphatidylinositol (GPI)-anchored, mucin-like surface glycoproteins have been characterized as PAMPs. Table 2 summarizes Toll-like receptors (TLRs) identified in aquatic animals, along with their corresponding ligands and downstream signalling components (Figure 2). In fish, TLR1, TLR2, TLR4, TLR5, and TLR9 are generally considered sensors of bacterial ligands, whereas TLR3, TLR22, TLR7, and TLR8 are primarily associated with the recognition of viral ligand (Table 2) [38].

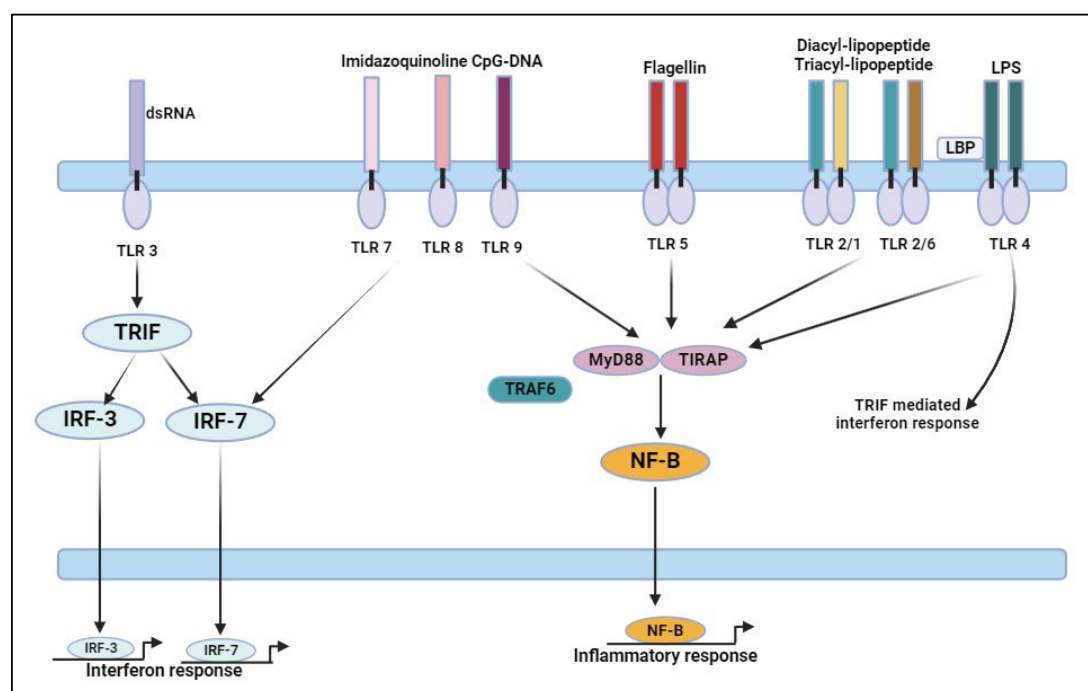


Figure 2. Schematic representation of Toll-like receptor (TLR) signalling. This simplified diagram illustrates how key conserved signalling molecules integrate inputs from TLR agonists to drive the coordinated induction of specific gene expression programs. The pathway topology is intentionally simplified for clarity; for example, TLR3, TLR7, TLR8, and TLR9 typically recognize their ligands within endosomal compartments.

4.1. Sentinel Mucous Cells

These cells function as decentralized sensors. TLR activation here triggers a localized “firewall” response, preventing pathogen colonization before systemic involvement. The traditional view of fish mucous cells as mere lubricant producers is outdated. In an evolutionary context, these cells represent the primary “front-line” of the innate immune system. TLR expression in these cells enables a sentinel function, where the detection of waterborne PAMPs triggers an immediate physiological response. This includes the rapid modulation of mucus viscosity and the secretion of localized antimicrobial peptides (AMPs), establishing a decentralized immune surveillance system that operates independently of systemic leukocyte recruitment. Goblet cells are specialized epithelial cells that play a pivotal role in mucosal homeostasis. Beyond their secretory capacity, fish goblet cells act as active participants in the immune cascade. Upon TLR activation (specifically TLR2 and TLR5 clusters), these cells undergo regulated exocytosis, releasing mucins and immune-modulating factors. This direct recognition mechanism suggests that goblet cells are integral to the mucosal fire-wall, providing a rapid-response sensing mechanism crucial for survival in pathogen-rich aquatic environments [39].

4.2. Specialized Integumentary Cells (Thread/Skein)

These represent an immuno-structural hybrid. In Myxine, TLR-sensing in thread cells suggests that slime release is a regulated immunological event, not just a physical reflex.

4.2.1. Hagfish Thread Cells: Primitive Integration of Defence

In basal vertebrates like the hagfish (Myxine), thread cells are unique evolutionary features responsible for the production of defensive slime [39]. The identification of TLRs within these specialized cells indicates a sophisticated integration of mechanical and biochemical defence. It suggests that the release of the slime-thread matrix is not merely a mechanical reflex but a regulated process that can be fine-tuned by the detection of specific microbial signatures, marking a primitive yet highly effective form of cell-specific innate sensing [40].

4.2.2. Lamprey Skein Cells: Evolutionary Precursors of Sentinel Function

Skein cells in lampreys represent another critical cell type for understanding the evolution of vertebrate immunity. As components of the epidermis, their TLR-mediated sensing capabilities suggest they serve as immuno-structural sentinels. The presence of TLRs in skein cells provides evidence that pattern recognition was already integrated into the specialized integumentary anatomy of Agnathans, pre-dating the complex adaptive systems seen in higher vertebrates [41].

4.3. The TLR-DC Bridge

Understanding the migration of TLR-activated signals from epithelial barriers to specialized DCs is crucial for a modern understanding of the fish immune-axis. The ontogeny and functional evolution of dendritic cells are vital for understanding the link between TLR detection and the activation of the adaptive arm. From Agnathans to teleosts, the differentiation of DC-like populations marks the transition from simple PAMP sensing to complex antigen presentation. Integrating this perspective allows for a more holistic view of the TLR-DC axis, illustrating how ancestral sentinel signals (from mucous or thread cells) were eventually funnelled into specialized professional APCs [39].

Table 2. Known ligands of TLRs in different fish species.

TLRs	Fish Species	Ligands	Reference
TLR1	Rainbow trout, orange-spotted grouper	Unknown	Zhang et al. [42]
TLR2	Common carp, rohu, channel catfish, orange-spotted grouper	PGN, LTA, Lipopeptides	
TLR4	Mrigal, channel catfish, rare minnow	Unknown	
TLR5M	Japanese flounder, channel catfish, gilthead seabream, fagu, rainbow trout	Flagellin	
TLR5S	Japanese flounder, channel catfish, gilthead seabream, fagu, rainbow trout	Flagellin	
TLR7	Fagu, rainbow trout, channel catfish, zebrafish	Unknown	
TLR8	Fagu, rainbow trout, channel catfish, salmon	Unknown	
TLR9	Japanese flounder, rainbow trout, zebrafish	CpG DNA	
TLR13	Atlantic salmon, channel catfish	Unknown	
TLR14	Fagu, Japanese flounder	Unknown	
TLR21	Orange-spotted grouper, channel catfish, zebrafish	CpG DNA	
TLR25	Nile tilapia, channel catfish, fathead minnow	Unknown	
TLR26	Channel catfish	Unknown	
TLR3	Zebrafish, rainbow trout, grass carp, channel catfish	dsRNA (Poly I:C)	Secombes and Zou [43]

5. TLRs and Innate Immune Training

Until recently, immunological memory was considered an exclusive feature of the adaptive immune system. However, accumulating evidence indicates that innate immune cells, including myeloid cells (monocytes, macrophages, and dendritic cells) and lymphoid cells such as natural killer and innate lymphoid cells can undergo functional reprogramming through metabolic and epigenetic modifications. This reprogramming enables innate cells to display enhanced or diminished responses upon subsequent stimulation (Figure 3). This phenomenon, known as innate immune memory or trained immunity, provides a mechanistic explanation for observations that vaccination with *Bacillus Calmette–Guérin* (BCG) reduces childhood mortality by conferring long-lasting protection against sepsis and respiratory infections [44]. It has also been established that immunization with BCG or other mycobacterial preparations induces heterologous protection against a broad range of microbial pathogens and provides protection against multiple tumour types, with effects lasting for several months. Mechanistic studies investigating this phenomenon demonstrated that prior exposure to BCG leads to a sustained increase in pro-inflammatory cytokine production following lipopolysaccharide (LPS) stimulation, compared with responses observed in unvaccinated individuals. Notably, this enhanced inflammatory response persists for at least three months after vaccination [45]. Subsequent studies demonstrated that monocytes stimulated *in vitro* with BCG and subsequently rested for several days exhibited enhanced production of TNF- α (Tumour Necrosis Factor-alpha) and IL-6 upon secondary stimulation with TLR2 and TLR4 ligands [46]. This enhanced response in monocytes was subsequently shown to result from alterations in chromatin architecture and shifts in cellular metabolism. Trained cells displayed increased trimethylation of histone H3 at lysine 4 (H3K4me3) at the promoters of pro-inflammatory and metabolic genes, including mTOR (mammalian target of rapamycin) [47]. Trained monocytes undergo metabolic reprogramming charac-

terized by a shift from oxidative phosphorylation to aerobic glycolysis, and the resulting hyperinflammatory response to TLR ligands can be abrogated by the addition of the mTOR inhibitor rapamycin during the initial BCG stimulation [48]. Collectively, these findings demonstrate that innate immune training is a cell-intrinsic process in which monocytes are reprogrammed into a hyperinflammatory state through coordinated alterations in cellular metabolism and chromatin architecture. While the term trained immunity is a recent addition to the immunological lexicon, the alteration of TLR signalling by prior environmental exposures likely relates to the long-standing appreciation of the process of LPS tolerance. Prior exposures of subjects (or cells) to LPS result in a decreased subsequent responsiveness to TLR4 ligands. Much work has been done on this topic, and several mechanisms have been proposed to explain lipopolysaccharide (LPS) tolerance. Notably, both tolerance and trained immunity appear to be regulated at the level of cytokine gene expression, with significant alterations in histone modifications identified as key factors influencing these processes (Figure 3) [49]. Despite these advances, the manner in which receptor-proximal signalling events are modulated during innate immune training or tolerance remains unclear, and the underlying drivers of the metabolic reprogramming observed in trained cells require further investigation.

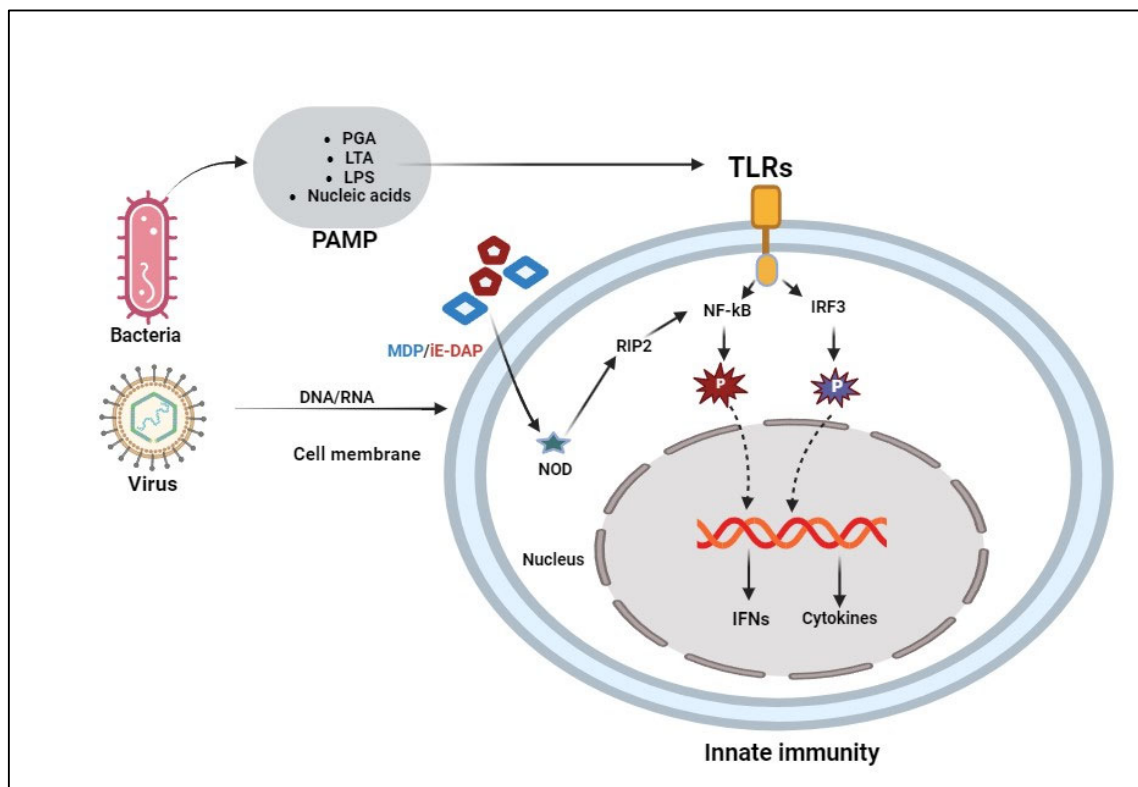


Figure 3. Simplified schematic of fish innate immune TLR signalling. TLRs detect bacterial, viral, and fungal PAMPs/MAMPs (Microbe-Associated Molecular Patterns) and transmit signals through their cytoplasmic domains to activate downstream pathways, inducing cytokines and interferons (IFNs). Intracellular PAMPs such as MDP and iE-DAP (γ -D-glutamyl-meso-diaminopimelic acid) are recognized by NOD (Nucleotide-binding Oligomerization Domain) receptors, leading to effector activation and processing of pro-cytokines into active cytokines.

6. TLR-Mediated Stimulation of Adaptive Immunity

Toll-like receptors (TLRs) play a pivotal role in regulating adaptive immunity by influencing both T- and B-cell responses. Dendritic cell maturation, driven by TLR signalling, is essential for the initiation of antigen-specific immune responses [50]. Upregulation of

co-stimulatory molecules such as CD80 and CD86 on DCs following TLR ligation endows DCs with the capacity to prime naïve T cells [51]. Toll-like receptors (TLRs) play a pivotal role in regulating adaptive immunity by influencing both T- and B-cell responses. Dendritic cell maturation, driven by TLR signalling, is essential for the initiation of antigen-specific immune responses [51]. TLRs also facilitate the fusion of MHC (Major Histocompatibility Complex) class I-containing endosomes with phagosomes, thereby promoting cross-presentation of exogenous antigens and enhancing CD8⁺ T-cell activation [51]. Beyond enhancing MHC peptide loading, TLR signalling—particularly in response to lipopolysaccharide (LPS)—induces the redistribution of MHC class I and class II molecules to the dendritic cell surface [52]. Dendritic cells express a diverse array of pattern recognition receptors (PRRs), some of which mediate antigen uptake and direct it to intracellular compartments for processing and presentation [53]. Engagement of pattern recognition receptors by ligands on phagocytic cargo activates intracellular signalling pathways, highlighting the importance of the context in which antigens are encountered. Antigens captured and presented by dendritic cells under steady-state conditions typically promote immune tolerance. In contrast, antigen uptake during infection or inflammation, accompanied by the upregulation of co-stimulatory molecules, leads to sustained T-cell activation [54]. Activation of pattern recognition receptors also induces dendritic cell migration from infected tissues to draining lymph nodes, where they initiate the activation of naïve T cells.

Detection of microbial products by dendritic cells (DCs) is translated into distinct classes of effector immune responses. Toll-like receptor (TLR) activation informs the immune system about the nature of the invading pathogen and guides lymphocytes to mount the most appropriate response for clearance. The specific effector response is determined by the receptors engaged and the cytokines secreted by DCs. Cytokines from activated DCs also direct the differentiation of naïve CD4⁺ T cells. For instance, IL-12 and IL-18 produced downstream of TLR and inflammasome signalling promote Th1 polarization, resulting in IFN- γ -producing (interferon-gamma-producing) Th1 cells. MyD88 is essential for these responses, as Th1 differentiation and IFN- γ production are impaired in MyD88-deficient mice [55]. The precise mechanism by which IL-18 is released from dendritic cells in this context remains unclear. The involvement of the inflammasome and the potential role of gasdermin D as a conduit for IL-18 secretion have not been established. Meanwhile, the production of IL-1, IL-6, and IL-23- activated downstream of TLRs and C-type lectin receptors works in concert with TGF- β to drive the differentiation of Th17 cells, which produce IL-17. However, the regulatory mechanisms controlling IL-1 release in these pathways are still not fully understood and warrant further investigation.

Recent studies suggest that interactions between dendritic cells (DCs) and T cells can enhance IL-1 β release from TLR-stimulated DCs through a mechanism dependent on TNF superfamily proteins produced by the responding T cells. DCs are functionally heterogeneous, with distinct subsets defined by the expression of subset-specific pattern recognition receptors (PRRs) [53]. As a result, these cells respond to different stimuli, engage distinct signalling pathways, and produce specific cytokines that in turn define the Th cell subset that will be generated [56]. For example, CD8⁺ dendritic cells, which are critical for activating CD8⁺ T cells, express TLR3 as well as RIG-I-like receptors (RLRs). These cells produce type I interferons and IL-12 in response to viral infections or when endosomal TLR3 detects virus-infected cells that have been internalized [57]. Plasmacytoid dendritic cells (pDCs) represent another specialized DC subset, primarily dedicated to the induction of type I interferons and the control of antiviral immunity. pDCs express endosomal TLRs, including TLR7 and TLR9, which recognize single-stranded RNA and CpG DNA, respectively, but lack TLRs that detect bacterial cell wall components. Consequently, pDCs are specialized for sensing viral infections. Additionally, B-cell responses and antibody

production are regulated in part by TLR signalling [58]. B-cell responses can be classified into T-cell-independent and T-cell-dependent types. Innate-like B cells, including B1a and B1b subsets, express Toll-like receptors (TLRs) and respond directly to stimuli derived from the microbiota [59]. Toll-like receptors (TLRs) cooperate with B cell receptors (BCRs) to activate both canonical and non-canonical NF- κ B signalling pathways, driving the production of natural IgM antibodies that provide protection against bacteria and viruses, including influenza. TLRs play a crucial role in shaping the immunoglobulin repertoire of B-1a cells and regulating their antibody output. These findings have significant implications for the maintenance of steady-state B-1a responses to both self-antigens and microbiota-derived antigens [59].

T-cell-dependent antibody responses require antigen engagement by the B-cell receptor (BCR). Macrophages and dendritic cells deliver antigens to naïve B cells within the follicle. While small antigens can reach B cells via the conduit system, larger particulate antigens are transported by subcapsular sinus macrophages. These macrophages are less efficient at phagocytosis than other subsets but function primarily as carriers, presenting antigens on their surface for B-cell recognition. In addition to antigen recognition, B cells require help from follicular helper T cells (T_{fh}) through CD40L, as well as cytokines that drive class switching to specific heavy chain isotypes. TLRs expressed on B cells further regulate antibody responses, particularly IgG2 in mice and IgG1 or IgG3 in humans [60]. In this context, TLRs provide B cells with critical information about the nature of the antigen and whether it is associated with a pathogen-associated molecular pattern (PAMP). TLRs on B cells play a key role in coordinating antibody responses during infection. They are also implicated in autoimmune diseases, where co-engagement of the BCR and TLR7 or TLR9 occurs in response to self-antigens complexed with nucleic acids, such as RNA- or DNA-containing immune complexes [61].

In this section, the role of TLRs in adaptive immunity is discussed. In fish, however, the function of TLRs has been primarily demonstrated in the context of innate immunity. Although the adaptive immune response in fish is generally slower, it is essential for long-lasting protection and plays a critical role in the success of vaccination. For disease resistance, innate immune parameters remain the first line of defence and are crucial for overall immune protection. As lower vertebrates, fish possess relatively less robust immunity. Key components of their adaptive immune system include T-cell receptors (TCRs), major histocompatibility complex (MHC) molecules, and immunoglobulins (Igs).

7. TLRs in Fish and Their Role in Diseases

7.1. *Agnatha*

Agnatha is a superclass of jawless fish within the phylum Chordata and subphylum Vertebrata. The surviving jawless fishes, including lampreys and hagfish, represent the most primitive class of vertebrates. These species possess a unique adaptive immune system based on variable lymphocyte receptors (VLRs), which are composed of leucine-rich repeat (LRR) subunits [62]. Two Toll-like receptors, laTLR14a and laTLR14b, were first identified in *Lampetra japonica* using a PCR-based approach. These receptors share 56% sequence homology and are closely related to the human TLR2 subfamily. The lamprey TLR repertoire includes both fish- and mammalian-type TLRs, reflecting a combination of evolutionary features from lower and higher vertebrates [63]. Currently, three types of TLRs belonging to the TLR2 subfamily have been identified in lampreys: TLR24 (pmTLR2a–d), TLR14 (pmTLR14a–c), and an ortholog of the jawed vertebrate TLR14 (TLR14d). Phylogenetic analysis shows that these receptors form clearly distinct clusters, highlighting their evolutionary divergence within the TLR2 subfamily [63]. TLR7, TLR8, and three TLR21 genes have been identified in the lamprey genome, which are capable of recogniz-

ing foreign RNA molecules and unmethylated CpG DNA, respectively. Members of the TLR2 subfamily, along with TLR3, TLR5, TLR7/8, and TLR21/22, are conserved between lampreys and teleosts, indicating that lampreys and jawed vertebrates share a common set of TLR families present in both fish and mammals.

7.2. Chondrichthyes

The class Chondrichthyes comprises cartilaginous fishes—jawed vertebrates with paired fins—within the phylum Chordata and subphylum Vertebrata. Information on TLRs in cartilaginous fishes remains limited. A BLAST analysis of *Chiloscyllium griseum* (family Hemiscylliidae) TLR2 reveals high sequence homology with mammalian TLR2a. SMART analysis of the deduced amino acid sequence indicates the presence of a partial TIR domain. In the genome of *Callorhinchus milii*, two gene models encoding TICAM and TIRAP have been predicted as potential TLR signalling components. In *Chiloscyllium* species, the TLR-TIR domain exhibits 87% and 76% nucleotide identity with TLR2 sequences from warm-blooded vertebrates and bony fishes, respectively [64]. In sharks, exposure to peptidoglycan (PGN) induces upregulation of TLR2-TIR expression across multiple tissues. These findings confirm both the presence of TLRs in sharks and their ability to be specifically induced by ligand recognition.

7.3. Teleostei

The infraclass Teleostei, within the class Actinopterygii and subphylum Vertebrata, comprises the ray-finned bony fishes. In teleosts, twenty different TLRs have been identified. Notably, within the TLR1 family, orthologs of mammalian TLR6 and TLR10 are absent [65], but another member of the same family, TLR14, is present which shares same features with TLR1, TLR6 and TLR10. TLR families 3 and 7 (including TLR7, TLR8 and TLR9) share teleost and mammalian orthologs. In the majority of the teleost species, TLR4 gene is lost and whenever it is present, as in the case of *D. rerio* and *Gobiocypris rarus*, it does not recognize the mammalian agonist, LPS [66]. The TLR21 family includes members specific to the teleost lineage, such as TLR13, TLR19, TLR20, TLR21, TLR22, and TLR23. Additionally, paralogous or duplicated TLR genes—likely arising from the third or fourth rounds of whole-genome duplication—have been identified in *Danio rerio* [9,17], and *Cyprinus carpio* [67]. Recently, two novel TLRs, TLR25 and TLR26, have been identified in catfish. Their TIR domains exhibit multiple conserved regions shared with previously characterized TLRs [68]. Beyond catfish, TLR25 has also been reported in GenBank for the fathead minnow (*Pimephales promelas*), Nile tilapia (*Oreochromis niloticus*), medaka (*Oryzias latipes*), and ayu (*Plecoglossus altivelis*). To date, TLR26 homologs have not been identified in any other fish or tetrapod species. Phylogenetic analysis of TIR domains places TLR26 within the TLR20 cluster, although its ligand specificity remains unknown [68]. A distinctive feature of teleost TLRs is the presence of a soluble form of TLR5, known as TLR5S. This variant lacks both the transmembrane and TIR domains and has been reported in *Oncorhynchus mykiss* [69] and *T. rubripes* [16], and *Ictalurus punctatus* [70]. The tissue-specific expression pattern of soluble TLR5 (TLR5S) differs from that of the membrane-bound form, TLR5M. In catfish, TLR5S expression is markedly upregulated following infection with *Edwardsiella ictaluri* [71]. In the Indian major carp, Mrigal (*Cirrhinus mrigala*), constitutive expression of TLR5M has been observed across all embryonic developmental stages and in multiple organs and tissues. The role of TLR5 signalling in inducing IL-8 and TNF highlights its importance in enhancing innate immune responses in fish [72]. In the genome of *D. rerio*, MyD88, MAL, TICAM, TRIF and SARM have been identified [73]. The functionality of teleost MyD88 has been demonstrated through infection studies in *Danio rerio* with *Salmonella enterica* [74] and in *Paralichthys olivaceus* with *Edwardsiella tarda* [75]. NF-κB has been characterized in *P.*

olivaceus [76], *D. rerio* [77] and *Catla catla* [78], indicating that teleost NF- κ B plays a critical role in gene transcription downstream of TLR signalling cascades.

8. Structural Conservation of TLRs Between Teleost Fish and Mammals

Toll-like receptors (TLRs) are single-pass transmembrane proteins consisting of an N-terminal extracellular leucine-rich repeat (LRR) domain and a C-terminal intracellular Toll/IL-1 receptor (TIR) domain. This fundamental architecture is highly conserved across animal TLRs. The LRRs mediate recognition of specific pathogen-derived ligands, and the extracellular region typically contains 20–30 LRR motifs, flanked by LRR N-terminal (LR-RNT) and C-terminal (LRRCT) domain modules to shield the hydrophobic core of the first LRR at the N-terminal or last LRR at the C-terminal [79]. The 'LXXLXLXXN' elements are conserved in the LRR [80]. Bell et al. [81] suggested that the variable sequence and length of insertion is contained at the following position of 10 and 15 amino acid residue in the LRR consensus sequence to generate the variety function of TLRs. Each LRR typically adopts an arc-shaped conformation, and the alignment of multiple LRR loops forms a solenoid-like structure. Variation in the conformation of these solenoid structures within the extracellular domain is thought to underlie the ability of TLRs to specifically recognize different pathogen-associated molecular patterns (PAMPs) [81]. Interestingly, a degree of sequence conservation in the LRR motifs has been observed between teleost and mammalian TLRs, notably within TLR5 and TLR9, suggesting that these receptors may recognize common PAMPs across species. The intracellular TIR domain of TLRs mediates signal transduction by interacting with the TIR domains of adaptor proteins. This domain is approximately 200 amino acids in length and contains three functionally critical regions—Box 1, Box 2, and Box 3—which are conserved in mammalian TLRs [82]. The role of these three TIR domain regions in signal transduction was confirmed through mutagenesis studies in *Homo sapiens*. Similar conservation of these functional motifs has also been observed in teleost fish TLRs [83]. The assessment of fugu, zebra fish and mammalian TIR domain evolution suggested coincidental evolution among the same class of TLRs even between fish and mammals. "Coincidental evolution" is a term describing phylogenies with branches that do not evolve independently [84]. A plausible explanation for this phenomenon is the conservation of TLR signalling cascades in fish. Indeed, homologues of four key TIR domain adaptor proteins—MyD88, MAL, TRIF, and SARM—have been identified in zebrafish and *Fugu*, highlighting the evolutionary preservation of these signalling components [9]. Collectively, these findings demonstrate that the TLR system plays a crucial role in the immune defence of fish.

9. Discussion

Innate immunity plays a critical role in defending against pathogenic challenges, with Toll-like receptors (TLRs) mediating both immediate tissue-specific and systemic responses. TLR signalling is essential for controlling and clearing infections, as well as promoting tissue repair, primarily through the production of cytokines and chemokines. Among these, chemokines are particularly important for recruiting phagocytes to infection sites. Beyond innate immunity, TLRs also contribute to adaptive immune responses by activating antigen-presenting cells. Understanding TLR-mediated pathways offers valuable insights into host defence mechanisms and presents new opportunities for therapeutic interventions to treat infectious diseases by modulating immune responses [85]. TLRs are mostly associated with initiation of the innate response and inflammation [86] and in some cases, inhibition of TLR signalling may help to combat the overactive innate response characteristic of numerous inflammatory disorders. The role of TLRs in the adaptive immune response is not well understood, and is an important area of future study [87]. Vaccine efficacy

against many aquatic diseases could be enhanced by incorporating TLR agonists, which stimulate dendritic cells and promote stronger adaptive immune responses. Consequently, comparing immune modulation mediated by the diverse TLR repertoire across different species is crucial for developing effective therapeutic strategies in aquaculture. Functional characterization of novel fish TLRs represents a promising avenue for studying comparative immunology between fish and warm-blooded vertebrates.

It is evident that many molecules involved in the immune defence system are conserved between mammals and teleost fish. The high level of conservation of TLRs across these groups highlights the crucial role of PAMP recognition by TLRs in mediating innate immunity in fish. In addition, teleosts possess several novel TLRs whose functions remain uncharacterized. Considerable interspecific diversity of TLRs has also been observed within fish genomes, with zebrafish, in particular, exhibiting relatively recent TLR gene duplication events [9]. Therefore, comparing immune modulation across the diverse TLR repertoires of different species is important. Moreover, some of the novel TLRs in teleost fish are hypothesized to compensate for the functions of mammalian TLRs, such as TLR4, TLR6, and TLR10, which are absent in teleosts [88]. Functional characterization of novel fish TLRs represents an important area for comparative immunology between fish and mammals. Epithelial tissues, including mucosal surfaces, are key sites for surveying infectious agents and are populated by resident innate leukocytes such as dendritic cells (DCs), macrophages, and mast cells. In mammals, antigen-presenting cells (APCs), particularly DCs, play a central role in orchestrating immune responses. TLR signalling is especially important for modulating DC activity, which in turn drives the generation of multiple effector cell types, including Th1 cells, cytotoxic T lymphocytes (CTLs), and B-cell responses [89]. Consequently, research on mammalian TLR-expressing cells has been extensively pursued for therapeutic applications, including the development of vaccines and vaccine adjuvants.

On the other hand, fish leukocytes expressing TLRs have not been classified. Transcription of fish TLR genes were highly occurring in the leukocytes. Further, we demonstrated the proliferation and infiltration of Japanese flounder TLR9-expressing cells at the site of bacterial infection [75]. Similarly, fish leukocytes are likely to play important roles in modulating immune responses. Therefore, identifying teleost TLR-expressing cells and analyzing their roles in immunomodulation through TLR signalling is essential for developing effective therapeutic strategies in aquaculture.

10. Conclusions

Key features of fish TLRs and the components of their signalling cascades exhibit strong structural similarity to the mammalian TLR system. At the same time, fish TLRs display unique characteristics and remarkable diversity, likely reflecting their complex evolutionary history and adaptation to diverse aquatic environments. Advances in genomic technologies have uncovered the extensive repertoire of TLRs in fish, yet the precise functional roles of many individual TLRs within specific species or closely related groups remain largely unexplored. Among the at least 16 TLR types identified in fish, ligand specificity has been directly confirmed only for TLR2, TLR3, TLR5M, TLR5S, and TLR22. A key focus for current and future research is the development of robust assays to identify the ligands for all fish TLRs. Achieving this will not only enhance our understanding of comparative immunology and the mechanisms underlying disease resistance in fish but also provide a foundation for designing more effective vaccines, adjuvants, and therapeutic strategies in aquaculture.

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