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Toll-like receptor recognition of bacteria in fish: Ligand specificity and signal pathways

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Pattern recognition receptors (PRRs) recognize the conserved molecular structure of pathogens and trigger the signaling pathways that activate immune cells in response to pathogen infection. Toll-like receptors (TLRs) are the first and best characterized innate immune receptors. To date, at least 20 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 found in more than a dozen of fish species. However, of the TLRs identified in fish, direct evidence of ligand specificity has only been shown for TLR2, TLR3, TLR5M, TLR5S, TLR9, TLR21, and TLR22. Some studies have suggested that TLR2, TLR5M, TLR5S, TLR9, and TLR21 could specifically recognize PAMPs from bacteria. In addition, other TLRs including TLR1, TLR4, TLR14, TLR18, and TLR25 may also be sensors of bacteria. TLR signaling pathways in fish exhibit some particular features different from that in mammals. In this review, the ligand specificity and signal pathways of TLRs that recognize bacteria in fish are summarized. References for further studies on the specificity for recognizing bacteria using TLRs and the following reactions triggered are discussed. In-depth studies should be continuously performed to identify the ligand specificity of all TLRs in fish, particularly non-mammalian TLRs, and their signaling pathways. The discovery of TLRs and their functions will contribute to the understanding of disease resistance mechanisms in fish and provide new insights for drug intervention to manipulate immune responses.

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1. Introduction

The innate system, the most ancient and universal form of host defense, is an efficient first line of defense against invading microbes in invertebrates and vertebrates [1]. Fish in aquatic environments protect themselves from various microbial pathogens mostly with the help of innate or non-specific immunity [1]. Pattern recognition receptors (PRRs), part of the ancient innate arm of the immune system, are conserved in invertebrate and vertebrate lineages. They recognize the conserved molecular structure of pathogens, known as pathogen-associated molecular patterns (PAMPs), and trigger the signaling pathways that activate immune cells in response to pathogen infection [2].

Among various PRRs, Toll-like receptors (TLRs) are the first and best characterized innate immune receptors. All TLRs are type I transmembrane proteins that contain three parts: an extracellular N-terminus with leucine-rich repeat (LRR) domain, a transmembrane domain and an intracellular C-terminus with a Toll/IL-1 receptor (TIR) domain [3,4]. TLR specificity is determined by sequence variation and the number of LRR domains, which is involved in pathogen recognition. Contrary to the extracellular LRR domain, the cytoplasmic TIR domain which activates downstream signaling pathways is highly conserved not only between the different TLRs of one species but also between different animal species [5,6].

Toll receptors have originally been identified in fruit fly (*Drosophila melanogaster*) embryos as the product of the Toll gene, which controls the establishment of drosoventral polarity [7,8]. These receptors have been related to the synthesis of anti-microbial peptides and play a critical role in immunity against fungal infection in flies [9]. An ortholog of Toll from *D. melanogaster* has been identified in humans and could activate certain genes necessary for innate or adaptive immune responses. Given its structural and functional similarities to the Toll receptors, this ortholog was named “Toll-like receptor” (now known as TLR4) [8,10,11]. The

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Abbreviations

PRR	pattern recognition receptor
TLR	toll-like receptor
PAMP	pathogen-associated molecular pattern
LRR	leucine-rich repeat
LRRNT	N-terminal LRR capping region
TIR	Toll/IL-1 receptor
IL-1	interleukin-1
LTA	lipoteichoic acid
LPS	lipopolysaccharide
PGN	peptidoglycan
Pam2CSK4	synthetic diacylated lipopeptides
Pam3CSK4	synthetic triacylated lipopeptides
MALP-2	macrophage-activating lipopeptide-2
Poly(I:C)	polyinosinic:polycytidylic acid
LBP	LPS-binding protein
CD14	cluster of differentiation 14
MD2	myeloid differentiation protein 2
TRIL	TLR4 interactor with leucine-rich repeats
CpG DNA	DNA-containing unmethylated CpG motifs
CpG ODN	CpG-containing oligodeoxynucleotides
ER	endoplasmic reticulum
ECD	ectodomain
IFN	interferon
MyD88	myeloid differentiation primary-response protein 88
MAPKs	mitogenactivated protein kinases
IRAK	IL-1 receptor-associated protein kinase
TRAF	TNFR-associated factor
TNFR	TNF receptor
TNF	tumor necrosis factor
TAK	TGF-β-activated kinase
TGF	transforming growth factor
TAB	TAK 1 binding protein
IKK	IκB kinase
IκB	inhibitor of NF-κB
NEMO	NF-κB essential modulator
NF-κB	nuclear factor κB
AP-1	activator protein
IRF	interferon regulatory factor
TIRAP	TIR domain-containing adaptor protein
TRIF	TIR domain-containing adaptor inducing IFN-β
TRAM	TRIF-related adaptor molecule
TICAM	TIR domain-containing adaptor molecule
TANK	TRAF-family-member-associated NF-κB activator
TBK	TANK binding kinase
RIP	receptor-interacting protein

human genome contains 10TLRs, contrary to the 13TLRs in the mouse genome [12]. In mammalians, TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are expressed in the plasma membrane, whereas TLR3, TLR7, TLR8, and TLR9 are localized within intracellular vesicles [13]. In bony fish, Sangrador-Vegas et al. [14] isolated a cDNA sequence of an interleukin-1 receptor from rainbow trout (*Oncorhynchus mykiss*), and it is the first piscine member of the interleukin-1/TLR superfamily. The first teleost TLR gene was characterized in goldfish (*Carassius auratus*) [15]. To date, at least 20 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 found in more than a dozen of fish species (Table 1). However, orthologs of mammalian TLR6 and TLR10 have not been identified in fish. Among all these TLRs, TLR1, TLR2, TLR4, TLR5, and TLR9 are presumed as sensors of bacterial ligands in fish [16], although some reports have suggested that the TLR7 and TLR8 in humans can also sense bacterial RNA [17,18]. In this review, the ligand specificity and signal pathways of TLRs that recognize bacteria in fish are summarized.

2. Specificity of TLRs in bacterial recognition

2.1. TLR1 and TLR2

In mammals, TLR2 recognizes various ligands from bacteria by forming homodimer or heterodimer with TLR1 or TLR6 [19]. Triacylated lipopeptides are considered as ligands for TLR2-TLR1, whereas diacylated lipopeptides are recognized by TLR2-TLR6 heterodimers [12,20]. In addition, TLR2 recognizes lipoteichoic acid (LTA) and peptidoglycan (PGN), which are characteristic cell wall components of Gram-positive bacteria [21,22]. In fish, similar to other vertebrates, TLR1 molecules do not have LRRNT modules in the N-terminal, which is believed to be important for dimerization with TLR2. Thus, fish TLR1 also possibly forms a dimmer with TLR2 similar to that in mammals [23]. Comparative sequence analysis showed high conservation of the position of the critical PGN recognition leucine residues in carp TLR2 LRR domain [24]. Ribeiro et al. [24] investigated the role of the TLR2 in the recognition of

Table 1
Known ligands of TLRs in fish.

TLRs	Fish species	Ligands	References
TLR1	Rainbow trout, pufferfish large yellow croaker, orange-spotted grouper	Unknown	[29–32]
TLR2	Commom carp, rohu, channel catfish, orange-spotted grouper	PGN, LTA, Pam_3CSK_4, Lipopeptides	[23,24,27,28,31]
TLR3	Fugu, zebrafish, rohu, orange-spotted grouper	dsRNA, poly(I:C)	[38,104–107]
TLR4	Channel catfish, grass carp, mrigal, zebrafish, rare minnow	Unknown	[23,39–44]
TLR5M	Japanese flounder,channel catfish, gilthead seabream, fugu, rainbow trout	Flagellin	[23,54,57–60]
TLR5S	Japanese flounder,channel catfish, gilthead seabream, fugu, rainbow trout	Flagellin	[23,54,57–60]
TLR7	Fugu, rainbow trou, Zebrafish, channel catfish	Unknown	[38,58,78,108,109]
TLR8	Fugu, channel catfish, Atlantic salmon, rainbow trout	Unknown	[38,58,78,108–110]
TLR9	Japanese flounder, cobia, zebrafish, rainbow trout, Atlantic salmon	CpG DNA	[68–75]
TLR13	Atlantic salmon, channel catfish	Unknown	[111,112]
TLR14	Lamprey, fugu, Japanese flounder	Unknown	[38,76,77]
TLR18	Zebrafish, channel catfish	Unknown	[23,78]
TLR19	Zebrafish, channel catfish	Unknown	[23,78]
TLR20	Zebrafish, common carp, channel catfish	Unknown	[23,80,81]
TLR21	Zebrafish, channel catfish, orange-spotted grouper	CpG DNA	[23,38,83,87,113–115]
TLR22	Fugu, zebrafish, grass carp channel catfish	dsRNA, poly(I:C)	[23,38,84–88]
TLR23	Fugu	Unknown	[38]
TLR24	Lamprey	Unknown	[89]
TLR25	Channel catfish, fathead minnow, Nile tilapia	Unknown	[23]
TLR26	Channel catfish	Unknown	[23]

ligands from Gram-positive bacteria in European common carp (*Cyprinus carpio carpio* L.). The overexpression of carp *tlr2* in carp macrophages, using the expression of downstream cytokines to assess activation, suggested that carp *tlr2* could sense both PGN and LTA from Gram-positive *Staphylococcus aureus*, but not diacylated lipopeptide macrophage-activating lipopeptide-2 (MALP-2), and was less sensitive to stimulation by synthetic triacylated lipopeptide (Pam_3CSK_4) [24]. The absence of TLR6 in fish genome could explain the unresponsiveness to MALP-2, which is recognized by TLR2-TLR6 heterodimers in mammalian vertebrates [25]. However, contrary to the carp *tlr2*-transfected HEK (human embryonic kidney) 293 cells, fish cells exhibit low responsiveness to Pam_3CSK_4, which is recognized using a TLR2-TLR1 heterodimer in mammals [24,26]. Ribeiro et al. [24] claimed that the overexpression of carp *tlr2* homodimers in HEK293 cells overcame the requirement for TLR2-TLR1 heterodimerization and was sufficient for the recognition of Pam_3CSK_4. In Indian major carp mrigal (*Cirrhinus mrigala*) and rohu (*Labeo rohita*), inductive expression of *tlr2* was observed following PGN and LTA exposure and Gram-positive (*Streptococcus uberis*) or Gram-negative (*Edwardsiella tarda* and *Aeromonas hydrophila*) bacterial infections [27,28]. In the anterior kidney leukocytes of rainbow trout (*O. mykiss*), *tlr1* expression was unaffected by the human TLR2/6 and TLR2/1 agonists diacylated lipopeptides (Pam_2CSK_4) and Pam_3CSK_4 or by stimulation with other mammalian TLR agonists (flagellin, poly(I:C), loxoribine and R848) [29]. However, Palti et al. [29] still hypothesized an important role for *tlr1* in anti-microbial immunity because of the up-regulation of *tlr1* mRNA in response to lipopolysaccharide (LPS) and bacterial infection in other fish species. For example, the expression level of *tlr1* in the spleen of pufferfish (*Tetraodon nigroviridis*) injected with LPS was markedly up-regulated [30]. *Tlr1* and *tlr2* mRNA expression in the spleen of orange-spotted grouper (*Epinephelus coioide*) were up-regulated by LPS and poly(I:C) treatment; these genes were also up-regulated in the spleen and head kidney with the bacterial pathogen *Vibrio alginolyticus* infection [31]. Large yellow croakers (*Pseudosciaena crocea*) exhibited an obvious increase in *tlr1* mRNA expression in their anterior kidney with LPS stimulation, whereas no significant changes in the *tlr1* mRNA expression were detected after PGN or poly(I:C) stimulation [32]. These different assays suggested that the ligand-binding of the TLR1 family in fish may be different from and more complex than those found in mammals. TLR1 and TLR2 in fish probably participate in the recognition of LPS, poly(I:C), and lipopeptides.

2.2. TLR4

Mammalian TLR4 recognizes LPS, the major component of the outer membrane of Gram-negative bacteria and an important endotoxin, together with accessory molecules [3]. The complex of CD14 (a GPI-anchored surface protein) and LBP (a soluble plasma protein) is responsible for the transfer of LPS to TLR4 [33,34]. Myeloid differentiation protein 2 (MD2) is also necessary for LPS recognition by forming a complex with TLR4 [35,36]. Carpenter et al. [37] first described TLR4 interactor with leucine-rich repeats (TRIL), which is highly expressed in mammalian brains. They also found that TRIL could be induced through LPS in the human astrocytoma cell line U373 in murine brains following intraperitoneal injection and in human peripheral blood mononuclearcells. TRIL is knocked down using small interfering RNA (siRNA) attenuated LPS signaling and cytokine production, and it could interact with components of the TLR4 receptor complex and LPS [37]. These results indicate that TRIL is a key component of the TLR4 complex and is important for LPS responses. In addition to LPS, the coat proteins of the respiratory syncytial virus and the mouse mammary tumor virus are recognized by TLR4 [13].

Unlike in mammals, TLR4 is present in some but not all fish species [2,38]. To date, TLR4 genes have been cloned and characterized in ictalurids [e.g., channel catfish (*Ictalurus punctatus*)], and cyprinids [e.g., zebrafish (*Danio rerio*), rare minnow (*Gobiocypris rarus*), common carp (*C. carpio*), and grass carp (*Ctenopharyngodon idella*)] [16,23,39–43]. However, lower vertebrates, especially fish and amphibians, are resistant to the toxic effect of LPS [44,45]. Several *in vitro* studies on leukocytes from different fish species have shown that much higher concentrations of LPS (microgram per milliliter) have been used to activate immune cells [15,46–49] compared with mammals (nanogram per milliliter) [50]. Additionly, the accessory molecules, which include LBP, CD14, and MD2, have not been isolated from fish [16], although TRIL has been identified in teleost fish such as zebrafish, common carp, and fugu (*Takifugu rubripes*) [51]. Quiniou et al. [23] discovered that the channel catfish *tlr4* gene encoded a protein that lacks the key features required for LPS-binding in mammals. Based on these results, the authors hypothesize that the mechanism of LPS recognition in fish should be different from that in mammals. TLR4 has been well studied in the zebrafish model [43,44,51,52]. Zebrafish have two *tlr4* orthologs (*tlr4a* and *tlr4b*). Sepulcre et al. [44] examined the role of fish TLR4 in LPS signaling by knocking down the expression of *tlr4a*, *tlr4b* and *myd88* through a morpholino-mediated translation blocker and a morpholino-mediated splice blocker. It did not disrupt the immune response of the zebrafish to LPS exposure. Additionally, leukocytes from seabream and pufferfish stimulated by different ultrapure LPS preparations responded to this PAMP with much lower sensitivity than mammals [44]. However, the presence of TLR4 in seabream is unknown and TLR4 is absent in pufferfish [44]. These results indicate that fish TLR4 is not involved in the recognition of LPS and that LPS induces NF-κB activation and the expression of proinflammatory cytokines via a MyD88-independent pathway [44]. This finding had been confirmed by Sullivan et al. [43], who demonstrated that, despite the presence of intracellular intact signaling cascades in zebrafish liver cells or HEK293 cells, zebrafish *tlr4a* alone, *tlr4b* alone, and *tlr4a* combined with *tlr4b* exhibited no induction (1.0-fold) of the NF-κB-luciferase reporter when cells were stimulated with LPS. Interestingly, while the overexpression of the TIR domain of TLRs (human TLR4, zebrafish TLR3 and TLR9) induced NF-κB activation, overexpression of the TIR domain of zebrafish TLR4b slightly decreased basal NF-κB activation [44]. Combined with other analysis, Sepulcre et al. [44] determined that engaging zebrafish TLR4 by its unknown ligand would negatively regulate the MyD88-dependent NF-κB signaling in the zebrafish. However, this result was directly contradicted by the findings of Sullivan et al. [43], who found that transmembrane and intracellular domains of zebrafish TLR4a and TLR4b positively regulated the NF-κB pathway and the overexpression of the TLR4b TIR domain alone may not provide an accurate representation of the TLR4b function. Furthermore, they demonstrated that the lack of responsiveness to LPS was most probably caused by the inability of the extracellular LRR domains to recognize the molecule rather than by changes in the TIR domains that use chimeric molecules, in which portions of the zebrafish TLR4 proteins were fused to portions of the mouse TLR4 protein [43]. Previous studies have also determined the intracellular downstream adaptor proteins in zebrafish. Studies on MyD88 and TIRAP have indicated that molecules downstream of the MyD88-dependent signal pathway are available for activation [52]. Therefore, the lack of sensitivity to LPS in zebrafish may be determined using the molecules upstream of MyD88 [52]. For example, LBP, MD2, CD14, and TRAM are absent in teleosts [16,51,53], and the conformation of zebrafish TIRAP may weaken its function in recruiting MyD88 to transfer the signal from TLRs to downstream molecules, resulting in low sensitivity to LPS [52]. Hence, the three presumptions on the role of TLR4 in fish are

as follows: (1) They respond to unidentified PAMPs of bacterial, fungal, protistan, helminthic, and/or viral origins; (2) TLR4a and TLR4b may function as coreceptors for each other or for other zebrafish TLR proteins in a manner similar to the role mammalian TLR1 and TLR6 play in supporting the alternative ligand specificities of TLR2; and (3) the zebrafish TLR4s may behave as "decoy" receptors, modulating the activation of other TLR pathways [43].However, in these studies, the *tlr4* expression in fish cells stimulated by LPS was not detected. Su et al. [42] found that Gram-negative *A. hydrophila* can be used to induce *tlr4* in rare minnow (*G. rarus*). In catfish, *tlr4* genes were apparently up-regulated in the spleen and down-regulated in the head kidney in response to Gram-negative *Edwardsiella ictaluri* infection [54]. Recently, *tlr4* in the Indian major carp mrigal (*C. mrigala*) has been first identified, and its expression was significantly up-regulated in all the tested tissues after 4 h of LPS treatment [41]. These three studies suggest that the fish TLR4 retains the functionality of LPS responses. Additionally, several reports have suggested that fish TLR4 might play an important role in viral infection. For example, *tlr4* expression in common carp was up-regulated during the spring vireamia of carp virus infection [16]. In grass carp, four *tlr4* genes were isolated and significantly increased in grass carp reovirus (GCRV)-infected liver and muscle, suggesting the significance of *tlr4* genes in GCRV infection [39]. Further analysis should be performed to ascertain the ligand of TLR4 and the recognition of LPS in fish. However, studies on the historical origins of zebrafish *tlr4* have raised questions regarding nomenclature. Sullivan et al. [43] used comprehensive syntenic and phylogenetic analysis to support that zebrafish *tlr4a* and *tlr4b* genes are paralogous rather than orthologous to human *tlr4*. Therefore, they proposed to use the nomenclature of *tlr4ba* and *tlr4bb* for the zebrafish *tlr4* genes, and *TLR4A* for human gene [43].

2.3. TLR5

In mammals, TLR5 is responsible for the detection of flagellin, a component of the bacterial flagellum, and specifically recognizes the constant domain, which is relatively conserved among different bacteria species through close physical interaction between TLR5 and flagellin [21,55,56]. In fish, two forms of TLR5 that sense bacterial flagellin have been reported [16]. The first form, the "membrane form" (TLR5M), is orthologous to mammalian TLR5 and contains the typical structure of TLRs (LRR domain, transmembrane region, and TIR domain). The second form, the "soluble form" (TLR5S), is unique in fish and lacks the transmembrane region and TIR domain. Both forms have been identified in some species such as in channel catfish (*I. punctatus*) [54], rainbow trout (*O. mykiss*) [57], fugu (*T. rubripes*) [58], and Japanese flounder (*Paralichthys olivaceus*) [59]. However, the expression profile of *tlr5s* is different from that of *tlr5m*. For example, in Japanese flounder, *tlr5m* is mainly expressed in the gill, head kidney, heart, and liver, whereas *tlr5s* is mainly expressed in head kidney, heart, and brain of the healthy fish. Through the challenge of flagellin, *tlr5m* was down-regulated for 3 h in peripheral blood leukocytes (PBLs) and liver cells, while strong gene expression of *tlr5s* in PBLs and liver cells was observed [59]. The expression of *tlr5m* in rainbow trout was ubiquitously found, whereas the *tlr5s* was only expressed in the liver [57]. Tsujita et al. [57] have suggested that stimulation of *tlr5m* with *Vibrio anguillarum* or its flagellin activated the expression of *tlr5s*. Flagellin-mediated NF-κB activation was more significant in the presence or simultaneous expression of *tlr5s* [57]. Thus, a two-step flagellin response has been proposed to occur for host defense against bacterial infection in fish: (a) Flagellin first induces the basal activation of NF-κB via TLR5M, which facilitates the production of TLR5S and minimal acute phase proteins, and (b) the inducible TLR5S amplifies TLR5M-mediated cellular responses in a positive feedback profile [57]. Similarly, Muñoz et al. [60] demonstrated that recombinant TLR5S could physically bind to *V. anguillarum* flagellin in gilthead seabream (*Sparus aurata*) using pull-down assays and found that subsequent inflammatory response was initiated after initial flagellin recognition via TLR5M, and TLR5S would be induced to modulate this response by binding to flagellin. However, the exact role of TLR5S remains controversial. On the one hand, TLR5S could bind flagellin and enhance innate immune response as reported in rainbow trout [57,60]. On the other hand, the interaction of TLR5M and flagellin is hampered, and an exaggerated inflammatory response is avoided because of the binding of TLR5S to flagellin [60]. Thus, further investigation should be conducted to elucidate the molecular mechanisms of TLR5S in the modulation of the flagellin-mediated immune response in fish.

2.4. TLR9

Contrary to the TLRs mentioned in section 2.1–2.3, TLR3, TLR7, TLR8, and TLR9 are induced by the nucleic acid from bacteria or viruses but not the microbial components located on the surface or outer/inner membranes [12]. The DNA that contains unmethylated CpG motifs (CpG DNA) is the ligand recognized by mammalian TLR9 within the endosomal compartment, and it is usually found in prokaryotes but rarely in eukaryotes [12,61]. Hemmi et al. [62] found that *tlr9*-deficient ($tlr9^{-/-}$) mice did not respond to CpG DNA and suggested that cellular response to CpG DNA from bacteria is mediated by TLR9. In humans, TLR9 is retained in the endoplasmic reticulum (ER) in resting immune cells [63]. Once these immune cells are exposed to CpG DNA, TLR9 is redistributed from the ER to endosomal compartments, CpG DNA-containing structures, where it could interact with its ligand [64]. Zhou et al. [65] constructed the three-dimensional structure of human TLR9 ectodomain (ECD)-CpG ODN complex and calculated the TLR9 ECD-CpG ODN interaction patterns using bioinformatics tools, and the geometrically complementary interface between the human TLR9 and the CpG-containing oligodeoxynucleotides (CpG ODN) molecule has been suggested. In addition, among 25 LRRs, which constitute the extracellular domain of human TLR9, LRR11 was considered as the main region that binds to CpG ODN with five positively charged residues that were involved in the binding of the TLR9 ECD to the CpG ODN [65]. These results were consistent with previously reported experimental data [66]. Yet, the exact process of TLR9 activation in mammalians is controversial, and further analysis on this process should be conducted [67].

In fish, the ligand-binding properties of TLR9 have, so far, not been well evaluated. To determine the effect of CpG ODN stimulation on cobia (*Rachycentron canadum*) *tlr9*, Byadgi et al. [68] used qRT-PCR to quantify gene expression [68]. The results indicated that CpG ODN 1668-and 2006-injected group showed high expression of *tlr9* and proinflammatory cytokines in the spleen and liver compared with the other groups. The broad expression of *tlr9* was found during the larval, juvenile, and adult stages irrespective of the tissues, which is consistent with the results reported using other fish species such as zebrafish, seabream, Atlantic salmon, rainbow trout, and half-smooth tongue sole [68–73]. In addition, the protective role of TLR9 in the early stages of development under pathogenically hostile environments was suggested because of the *tlr9* gene expression in all larval stages of fish [68]. Takano et al. [74] cloned Japanese flounder *tlr9* cDNA and characterized its functional domains in the amino acid sequence. They found that TLR9 had two CXXC motifs separated by six amino acid residues; similar motifs were also involved in cobia TLR9 [68], and this region was considered to bind directly to CpG ODN [5]. Additionally, tumor necrosis factor (TNF) promoter activation was observed in *tlr9*-

transformed hirame natural embryo (HINAE) cells treated with CpG ODN, but not with GpC ODN, and Japanese flounder TLR9 was suggested to be a receptor for CpG ODN [74]. These data demonstrate that the DNA-binding properties of TLR9 are conserved between teleosts and mammals. However, direct evidence of the interaction between CpG ODN and TLR9 in non-mammalian vertebrates remains ambiguous. A pull-down approach was used to determine whether Atlantic salmon TLR9 could bind CpG ODNs. Western blot results demonstrated that TLR9 could bind both CpG and inverted CpG ODNs with similar avidity. Moreover, the efficiency of coprecipitation of both CpG ODNs with TLR9 increased gradually as pH was reduced from 7.5 to 5.5 [75]. Therefore, the authors believed that Atlantic salmon TLR9 interacted with synthetic oligonucleotides via a CpG-independent but a pH-dependent mechanism [75]. However, this result is contrary to that in cobia and Japanese flounder [68,74]. Thus, in-depth study in this area should be conducted to clarify the ligand-binding properties of TLR9. Additionally, Iliev et al. [75] also suggested that transgenic TLR9 can spontaneously activate the promoter of genes that contains IFN-stimulated response elements involved in the IFN

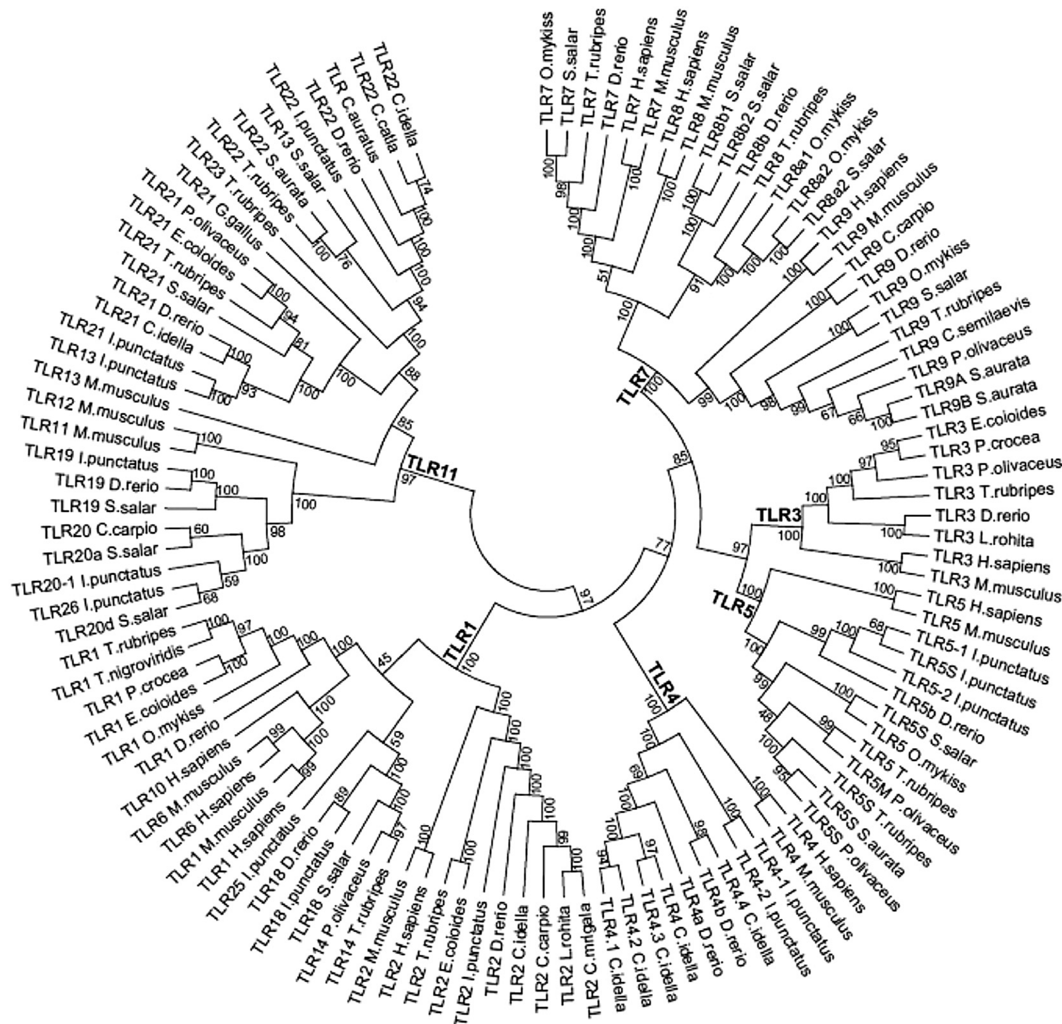

Fig. 1. Circular phylogenetic tree of fish TLR based on full-length amino acid sequences. MEGA6.0 is used to construct the tree according to the neighbor-joining method using the Poisson correction model with 1000 bootstrap replicates [2]. All positions containing gaps and missing data were eliminated from the dataset (pairwise deletion). The numbers at the branches indicate bootstrap values. Accession numbers of sequences used to build the tree are as follows: *Carassius auratus* (TLR: AAO19474.1); *Catla catla* (TLR22: AGW43269.2); *Cirrhinus mrigala* (TLR2: AHI59129.1); *Ctenopharyngodon idella* (TLR2: ACT68333.1, TLR4: ACT68334.1, TLR4.1: AEQ64877.1, TLR4.2: AEQ64878.1, TLR4.3: AEQ64879.1, TLR4.4: AEQ64880.1, TLR21: AGM21642.1, TLR22: ADX97523.2); *Cynoglossus semilaevis* (TLR9: ACL68661.1); *Cyprinus carpio* (TLR2: ACP20793.1, TLR9: ADE20130.1, TLR20: AHH85805.1); *Danio rerio* (TLR1: AAI63271.1, TLR2: AAQ90474.1, TLR3: AAI07956.1, TLR4a: ACE74929.1, TLR4b: AAQ90475.1, TLR5b: NP_001124067.1, TLR7: XP_003199309.1, TLR8b: XP_003199440.1, TLR9: NP_001124066.1, TLR18: AAI63840.1, TLR19: XP_002664892.3, TLR22: AAI63527.1); *Epinephelus coioides* (TLR1: AEB32452.1, TLR2: AEB32453.1, TLR3: ADZ76423.1, TLR21: AEK49148.1, TLR21: NP_001186264.1); *Ictalurus punctatus* (TLR2: AEI59663.1, TLR4-1: AEI59665.1, TLR4-2: AEI59666.1, TLR5-1: AEI59668.1, TLR5-2: AEI59669.1, TLR5S: AEI59667.1, TLR13: AHH38552.1, TLR18: AEI59674.1, TLR19: AEI59675.1, TLR20-1: AEI59676.1, TLR21: AEI59678.1, TLR22: AEI59679.1, TLR25: AEI59680.1, TLR26: AEI59681.1); *Labeo rohita* (TLR2: ADQ74644.1, TLR3: AFD97495.1); *Oncorhynchus mykiss* (TLR1: ACV92063.1, TLR5: BAC65467.1, TLR7: ACV41797.1, TLR8a1: ACV41799.1, TLR8a2: ACV41798.1, TLR9: ACM18118.1); *Paralichthys olivaceus* (TLR3: BAM11216.1, TLR5M: BAJ16366.1, TLR5S: BAJ16368.1, TLR9: BAE80690.1, TLR14: BAJ78226.1, TLR21: AFW04263.1); *Pseudosciaena crocea* (TLR1: AHB51065.1, TLR3: ADZ52858.1); *Salmo salar* (TLR5S: AAV35178.1, TLR7: CCX35457.1, TLR8a2: CCX35458.1, TLR8b1: CCX35459.1, TLR8b2: CCX35460.1, TLR9: ABV59002.1, TLR13: NP_001133860.1, TLR18: CDK60413.1, TLR19: CDH93609.2, TLR20a: CDH93610.2, TLR20d: CDH93613.1, TLR21: CDH93614.1); *Sparus aurata* (TLR5S: CCP37739.1, TLR9A: AAW81698.1, TLR9B: AAW81699.1, TLR22: CDK37745.1); *Takifugu rubripes* (TLR1: AAW69368.1, TLR2: AAW69370.1, TLR3: AAW69373.1, TLR5: AAW69374.1, TLR5S: AAW69378.1, TLR7: AAW69375.1, TLR8: AAW69376.1, TLR9: AAW69377.1, TLR14: AAW69369.1, TLR21: AAW69371.1, TLR22: AAW69372.1, TLR23: AAW70378.1); *Tetraodon nigroviridis* (TLR1: ABO15772.1); *Homo sapiens* (TLR1: CAG38593.1, TLR2: AAH33756.1, TLR3: ABC86910.1, TLR4: AAF05316.1, TLR5: AAI09119.1, TLR6: BAA78631.1, TLR7: AAZ99026.1, TLR8: AAZ95441.1, TLR9: AAZ95520.1, TLR10: AAY78491.1); *Mus musculus* (TLR1: AAI41322.1, TLR2: AAH14693.1, TLR3: AAH99937.1, TLR4: EDL31078.1, TLR5: NP_058624.2, TLR6: BAA78632.1, TLR7: AAI32386.1, TLR8: AAI32055.1, TLR9: AAK29625.1, TLR11: NP_991388.2, TLR12: EDL30230.1, TLR13: EDL14060.1); *Gallus gallus* (TLR21: NP_001025729.1).

response. Nevertheless, unlike in primary leukocytes, it failed to colocalize with CpG ODNs when overexpressed in salmonid cell lines. These results indicated that only specific immune cell types can relocate TLR9 to the endosomal compartment, where it may become activated by its ligand [75].

2.5. Non-mammalian TLRs in fish

TLRs have been massively expanded in fish (Fig. 1). In mammals, the TLR1 family comprised TLR1, TLR2, TLR6, and TLR10. Fish have no orthologs of TLR6 and TLR10, but some TLR14 and TLR18 are non-mammalian. TLR14 have been discovered in lamprey (*Lampetra japonica*), fugu (*T. rubripes*), and Japanese flounder (*P. olivaceus*) and shares some features with TLR1, TLR6, and TLR10 [38,76,77]. Thus, TLR14 might be a functional substitute for mammalian TLR6 and TLR10 in terms of generating immune response against various pathogens in water [77]. The Japanese flounder *tlr14* gene was upregulated in response to infection with Gram-positive *Streptococcus iniae* and Gram-negative *E. tarda* [77]. TLR18 in zebrafish and channel catfish are the homologs of human TLR1 and may correspond to TLR14 of other fish [78,79]. Pietretti et al. [80] identified full-length cDNA sequences for the *tlr20* of zebrafish and common carp, and phylogenetic analyses indicated that this is close to TLR11 and TLR12 in mice, which sense ligands from protozoan parasites. This result is consistent with previous results found in channel catfish [81]. TLR21 is common to birds, amphibians, and fish. In chickens, the homolog of mammalian TLR9 is absent, and TLR21 recognizes CpG DNA similar to mammalian TLR9 [82]. However, both TLR9 and TLR21 have been identified and characterized in fish, and the biological functions of these two receptors in fish remain unclear. Yeh et al. [83] comparatively investigated zebrafish TLR9 and TLR21. They found that the two TLRs had similar expression profiles in zebrafish and cooperatively mediated the anti-microbial activities of CpG ODN. The results suggest that these two zebrafish TLRs are functional and recognize CpG ODN. Interestingly, different CpG motifs have been involved in the TLR9-mediated recognition of CpG ODN, and TLR9 respond preferentially to CpG ODN with GACGTT or AACGTT motifs. By contrast, CpG ODN with GTCGTT motifs exhibits better activity in TLR21 [83]. TLR22, a fish-specific TLR that recognizes dsRNA, was identified from several fish species such as in gilthead seabream [84], orange-spotted grouper [85], grass carp [86], and zebrafish [87]. Unlike TLR3, which is expressed in the ER as a sensor for short-sized dsRNA, TLR22 is a sensor of long-sized dsRNA on the cell surface [88]. TLR24 was found only in lampreys, a jawless fish [89]. To date, TLR25 has been identified and characterized in channel catfish (*I. punctatus*), fathead minnow (*Pimephales promelas*), Nile tilapia (*Oreochromis niloticus*, improperly annotated as TLR1-like), medaka (*Oryzias latipes*), and ayu (*Plecoglossus altivelis*); phylogenetic analyses have indicated that TLR25 represents a new member of the TLR1 family [23]. In addition, TLR25 was found to lack a LRRNT cap but possesses the same number of LRRs as TLR1. Hence, TLR25 may potentially be a partner heterodimerized with TLR2 as well as TLR1, thus extending the range of possible PAMP ligands [23]. TLR26, a new member of the TLR11 family, was found only in channel catfish to date and is highly related to its TLR20 genes. Phylogenetic analyses have suggested that TLR26 could be an ancient duplicated gene of TLR20 because of the distinct subclades (i.e., catfish TLR20-1, TLR20-2, and TLR26 subclades) within the clade of TLR20 [23,54]. However, the precise ligands of TLR26 in fish are unclear.

3. TLR signaling pathways in bacterial recognition

After the recognition of the corresponding ligands via interactions with LRRs, TLRs are activated and the related adaptor proteins are recruited within the cytoplasm, and different signaling cascades are triggered (Fig. 2). The intracellular components downstream of TLRs are generally highly conserved between mammalian and teleosts [90]. TLR signaling pathways are roughly classified into two distinct pathways: the MyD88-dependent and MyD88-independent (TRIF-dependent) pathway [1]. MyD88, the first identified TIR domain-containing adaptor protein, is used by

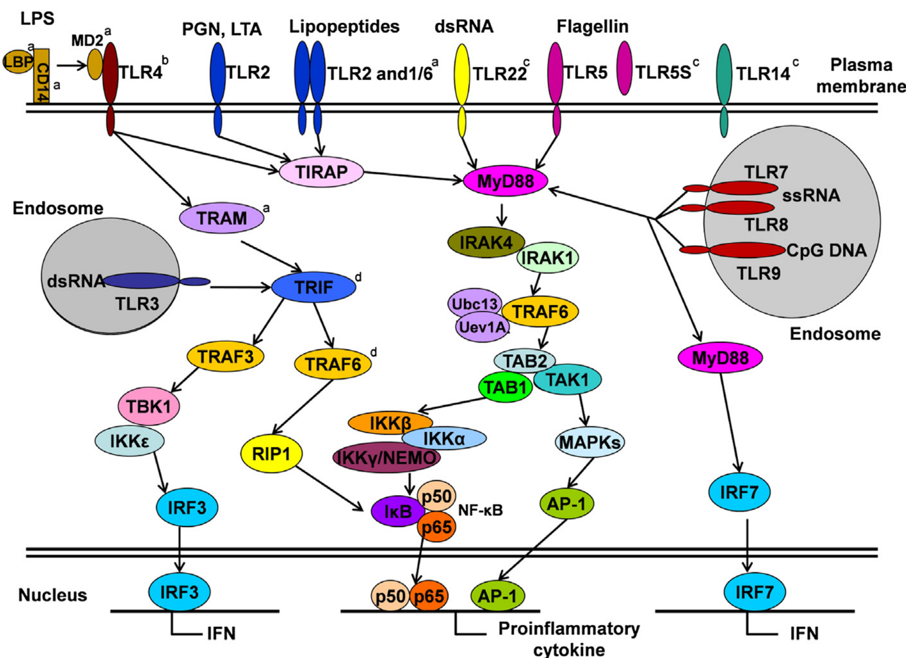

Fig. 2. TLR signaling pathways. a: molecules had not been found in fish (LBP, MD2, CD14, TRAM, TLR6). b: TLR4 was present in some but not all fish species. c: non-mammalian TLRs in fish (TLR5S, TLR14, TLR22). d: In mammals, TRIF interacts with TRAF6 and RIP1. However, TRIF fails to bind to TRAF6 in fish. Thus, NF-κB activation is dependent upon its interaction with RIP1 through an unknown mechanism. Modified from Refs. [1] [55], and.[93].

all mammalian TLRs, except TLR3, and activates the transcription factor NF-κB and mitogenactivated protein kinases (MAPKs), which results in the production of induced inflammatory cytokines [91,92].

In the MyD88-dependent pathway, MyD88 uses its death domain to interact with IL-1 receptor-associated protein kinase 4 (IRAK4), which is a serine/threonine kinase. Then, the MyD88-IRAK4 complex activates other IRAK family member IRAK2 or related IRAK1 [93,94]. To date, IRAK2 has not been identified in fish [1]. Subsequently, IRAK1 dissociates from MyD88 and interacts with an E3 ubiquitin ligase, TNFR-associated factor 6 (TRAF6). TRAF6 catalyzes the formation of a lysine 63 (K63)-linked polyubiquitin chain on TRAF6 itself and results in the autoubiquitination of TRAF6 with the help of an E2 ubiquitin-conjugating enzyme complex consisting of Ubc13 and Uev1A [3,95]. Following ubiquitination, TRAF6 interacts with a complex composed of TGF-β-activated kinase 1 (TAK1) and TAK1 binding proteins, TAB1 and TAB2 [96]. In this complex, TAB1 activates TAK1, and TAB2 functions as an adaptor that links TAK1 to TRAF6, thereby facilitating TAK1 activation [97]. Through phosphorylated IKKβ, the activated TAK1 activates the IκB kinase (IKK) complex, which comprises IKKα, IKKβ, and IKKγ/NF-κB essential modulator (NEMO) [98]. Then, IKKβ phosphorylates IκB proteins, which are bound to NF-κB subunits, and prevents their nuclear translocation, leading to the destruction of IκB and the subsequent translocation of NF-κB into the nucleus [1,12]. NF-κB subunits consisting of p50 and p65 participate in the transcription of genes for proinflammatory cytokines, such as TNF-α, IL-8, IL-6, and IL-12 [99]. In addition to NF-κB, the transcription factor complex AP-1, targeted cytokine genes, is also activated in the MyD88-dependent pathway through MAPKs phosphorylated [3]. Interestingly, TLR7, TLR8, and TLR9 signaling induce the production of type I IFNs in a MyD88-dependent manner, contrary to the aforementioned process [1,3,100]. In TLR2 and TLR4 signaling pathways, TIRAP (also called MAL) is necessary for TLR and MyD88 to interact [1].

In the MyD88-independent pathway, TRIF (also called TICAM1) is used by TLR3 and TLR4 that leads to the activation of the transcription factors IRF3 and NF-κB as well as the consequent induction of genes encoding for type I interferon and inflammatory cytokines [91]. TLR4 also requires the participation of TRAM (also called TICAM2), which acts as a bridging adaptor between TLR4 and TRIF in mammals [21,101]. However, TRAM has not been found in fish [94]. TRIF activates TRAF-family-member-associated NF-κB activator (TANK) binding kinase 1 (TBK1) through TRAF3 [102,103]. Then, TRAF3, TBK1, and IKKε phosphorylate IRF3. Activated IRF3 is dimerized and translocated into the nucleus, which induces the protein expression of type I IFNs. Additionally, mammalian TRIF also interacts with TRAF6 and receptor-interacting protein 1 (RIP1), which is responsible for the activation of NF-κB [93]. However, TRIF fails to bind to TRAF6 in zebrafish. Thus, NF-κB activation is dependent upon its interaction with RIP1 through an unknown mechanism [53].

4. Conclusions and perspectives

In this review, the ligand specificity and signal pathways of TLRs that recognize bacteria in fish are summarized. The TLRs, with diversity, can specifically recognize pathogenic microbes. At least 20 TLR types have been identified in fish. Of these TLRs, direct evidence of ligand specificity has only been shown for TLR2, TLR3, TLR5M, TLR5S, TLR9, TLR21, and TLR22. Some studies have suggested that TLR2, TLR5M, TLR5S, TLR9, and TLR21 could specifically recognize PAMPs from bacteria. In addition, other TLRs, including TLR1, TLR4, TLR14, TLR18, and TLR25, may also be sensors of bacteria. TLR signaling pathways in fish exhibit some particular features, despite the highly conserved structural and functional similarity of the key features of the fish TLRs and the factors involved in their signaling cascade to the mammalian TLR system have been observed. However, reports on the differences have been relatively few except TRIF in zebrafish. Therefore, further investigations are necessary to identify the ligand specificity of all fish TLRs, particularly non-mammalian TLRs because no clear mammalian references exist. Combining studies on accessory molecules, including mediators of ligand-delivery and ligand-recognition in fish, may elucidate the function of fish TLRs. In addition to innate immune response, TLRs also play an important role in adaptive immunity by activating antigen-producing cells. However, the role of TLRs in adaptive immune response is not well known and should be paid attention in future studies. The discovery of TLRs and their functions will contribute to the understanding of disease resistance mechanisms in fish and provide new insights for drug intervention in terms of manipulating immune responses. For example, inhibiting TLR signaling pathways may alleviate overactive innate responses. The effectiveness of the vaccines for different aquatic diseases can also be improved by increasing the adaptive immune response by supplementing TLR activators.

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