Toll-like receptors in teleosts

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ABSTRACT

Toll-like receptors (TLR) recognize the pathogen-associated molecular patterns (PAMPs) and regulate the subsequent immune responses in mammals. We have so far succeeded in finding the 11 types of TLR genes from Japanese flounder (*Paralichthys olivaceus*). TLR genes have also been found in the genomes of fugu (*Takifugu rubripes*) and zebrafish (*Danio rerio*). However, only limited information is known about the function of TLRs in fish. The fragmentary information of the fish TLR gene expression, immune response following PAMPs stimulation and intracellular signaling are available in fish. In addition, the phylogenetic analysis in vertebrate TLR genes and comparison of TLR family members between teleost fish species revealed that specific divergence of TLR genes between mammals and fish as well as between fish species. Here, the possible functions of teleost fish TLR and its importance are discussed.

Key words: immune response; pathogen molecular pattern (PAMP); toll-like receptor (TLR).

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INTRODUCTION

The innate immune system is responsible for the first line of defense in host protection against invading microbial pathogens and it is relatively evolutionarily conserved among mammals (Akira *et al*., 2006). The pathogen-associated molecular patterns (PAMPs) initiate the host innate immune responses of animals including insects and vertebrates. *Drosophila* Toll, which was initially identified as a receptor essential for dorso-ventral polarity during development. This Toll was first shown to participate in innate immune responses against fungal infections in 1996 (Lemaire *et al*., 1996). Subsequently, numerous homologues of Toll, termed Toll-like receptors (TLRs), were identified in mammals. These receptors were also demonstrated to recognize PAMPs to trigger the networks of innate immune responses (Akira *et al*., 2006). Mammalian TLR signaling pathways which are triggered by different TLRs and consequent immune responses are highly distinct and diverged. To date, two major TLR signaling pathways of MyD88-dependent and -independent pathways were known. The MyD88-dependent pathway leads to activation of AP-1 transcription factors through activation of MAP kinases and NF-κB activation through activation of the IKK complex. Activation of these transcription factors triggers inflammatory cytokine production (Takeda and Akira, 2005). The MyD88-independent pathway is transduced from TLR3 and TLR4. This pathway activates interferon (IFN) regulatory factor 3 (IRF3) with consequent production of IFN-β (Takeda and Akira, 2005). The complex of these signaling pathways modulate the proper immune response against pathogens..

The first report of fish TLR gene was found in gold fish, *Carassius auratus auratus* (Stafford *et al*., 2003). The conservation of essential domain structures in vertebrate TLRs, including leucine-rich repeats (LRRs) in the extracellular portion and a Toll/IL-1 receptor homology (TIR) domain were confirmed in goldfish TLR. In a same year, TLR genes have been globally surveyed from the draft whole genome sequence of fugu (*Takifugu rubripes*) and unique TLR genes were reported (Oshiumi *et al*., 2003). In addition, zebrafish (*Danio rerio*) genome database was also surveyed to discover the all of predictable TLR genes and reported in 2004 (Jault *et al*., 2004; Meijer *et al*., 2004). In our study Japanese flounder (*Paralichthys olivaceus*), we have identified 11 types of TLRs homologues. These results were reported at the 7th Symposium on Diseases in Asian Aquaculture. To date, the involvement of teleost fish TLRs in the innate immune system have been uncovered by virtue of TLR gene cloning and their fundamental studies. In addition, several novel TLR genes were discovered in teleost fish. Hence, the immune response which is introduced in the PAMPs recognition through the fish TLRs will be an intrigue topic for the comparative immunology. Here, to clarify the future direction of study in teleost fish TLRs, the present information of teleost fish TLRs are briefly summarized and their possible functions are discussed.

1. Structural conservation of TLRs between teleost fish and mammal

TLRs are single-pass transmembrane proteins composed of an N-terminal extracellular LRR domain and C-terminal intracellular domain of TIR domain. This basic structure is highly conserved in TLRs of animal. The LRRs are responsible for recognition of respective ligand

of pathogen components. The extracellular domains of TLRs contain about 20 to 30 LRRs sandwiched between the LRR N-terminal (LRRNT) domain and LRR C-terminal (LRRCT) domain modules (Matsushima *et al*., 2007) to shield the bydrophobic core of the first LRR at the N-treminal or last LRR at the C-terminal (Matsushima *et al*., 2005; Kajava, 1998). A LRR is consisted of 20 to 33 amino acid residues. The 'LXXLXLXXN' elements are conserved in the LRR (Jin and Lee, 2008; Matsushima *et al*., 2007). Bell *et al*. (2003) 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. A LRR is known to form a arc-shape structure, then juxtaposition of LRR loops produces a solenoid-like structure. Today, the variation of the solenoid-like structure in extracellular domain which is generated from the different LRR conformations is considered to be related to the specific PAMPs recognition of TLRs (Bell *et al*., 2003). Interestingly, a certain level of conservation in LRR sequence between the same class of TLRs in teleost and mammal are reported, including a class of TLR5 and a class of TLR9 (Takano *et al*., 2007; Tsoi *et al*., 2006). Hence, these structural conservation may indicate the recognition of the common PAMP between fish and mammalian TLRs.

Intracellular TIR domain of TLR initiates the signal transduction to associate with another TIR domain of adaptor proteins. The TIR domain is composed of roughly 200 amino acid residues in length. The three functionally important regions box 1, box 2 and box 3 are conserved in mammalian TLRs (O'Neill, 2000; Rock *et al*., 1998). The involvement of these three boxes in signal transduction were confirmed by mutagenesis studies of human, *Homo sapiens* (Slack *et al*., 2000). The conservation of these boxes have also been reported from teleost fish TLRs (Jault *et al*., 2004; Takano *et al*., 2007). The assessment of fugu, zebrafish and mammalian TIR domain evolution (Meijer *et al*. 2004) 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 (Roach *et al*. 2005). Favor interpretation for this phenomena is the conservation of similar TLR signaling cascade in fish. In fact, the conserved homologues of four of the TIR domain adaptor proteins (MyD88, MAL, TRIF and SARM) from zebrafish and Fugu have been reported (Meijer *et al*., 2004). These reports clearly show that the TLR system has an important role in fish immunity.

2. TLR repertoire

Today, a total of 10 TLRs numbered 1-10 in humans and a total of 13 TLRs in mouse (*Mus musculus*) have been identified (Verstak *et al*., 2007; Akira, 2004). TLR1-9 are conserved in humans and mice. TLR10 is functinal in humans but mouseTLR10 gene is disrupted by two retroelements. On the other hand, human TLR genes corresponding mouse TLR11-13 are represented only by a pseudogene (Roach *et al*., 2005).

At the beginning of this decade, the TLR family are surveyed from draft genome sequence of teleost fish, such as fugu (Oshiumi *et al*., 2003) and zebrafish (Jault *et al*., 2004). Surprisingly, higher numbers of diverged TLR than mammalian TLR genes are discovered from these fish including novel TLR genes. TLR1, -2, -3, -5, -7, -8 and -9 genes

were conserved in the both fish as well as mammal, while mammalian TLR4 homologue was only seen in zebrafish genome. In addition, TLR1 family of TLR6 and TLR10 genes are missing in the teleost fish (Roach *et al*., 2005). For the novel TLR genes, TLR14, 21 (also found from aves), 22 and 23 including soluble form TLR5 (also found from amphibian) were identified from the fugu, while TLR18 (orthologue of fugu TLR14), 19, 20, 21 and 22 gene have been identified from zebrafish (Oshiumi *et al*., 2003; Jault *et al*., 2004; Meijer *et al*., 2004; Roach *et al*., 2005). The duplication of zebrafish TLR4, 5, 8 and 20 gene were reported by Meijer *et al*. (2003). From the comparison of TLR gene loci between fugu and zebrafish using the ensemble genome browser (http://oct2007.archive.ensembl.org/index.html), the tandem duplication of zebrafish TLR19 (gene ENSDARG00000026663 and ENSDARG00000070392) was also evident. However, it is seems that the tandem gene duplication of many TLR genes have been happened only in zebrafish genome but not in fugu genome. Further, interspecific specific TLR gene duplication was reported from rainbow trout (*Onchorhynchus mykiss*) TLR22a and TLR22b showing 95.6 % homology in nucleotide sequence level (Rebl *et al*., 2007).

Taken together, TLR repertoire, such as TLR1, 2, 3, 5, 7, 8 and 9, between teleost fish and other vertebrates seems to be highly conserved. Because, it is almost certainly that microbes cannot mutate basic structure of their PAMPs (Roach *et al*. 2005), and these PAMPs have became the essential ligands for the TLRs. On the other hand, the novel TLR gene in fish and species specific TLR gene divergence is becoming clear. This difference is favorably interpreted as a result of different selection pressure of exposed pathogens in distinct environments (Oshiumi *et al*. 2003). Therefore, comparison of immune responses which are mediated through different repertoire TLRs between human and teleost fish species are interesting as well as comparing between teleost fish species.

3. Toll-like receptor of teleost fish and immune response against PAMPs

The ligands for mammalian TLRs are reviewed by Kawai and Akira (2007). The study on specific ligands determination for the teleost TLRs are limited. However, as mention above, there is conservation of LRR structure between teleost fish and mammalian TLR. Therefore, teleost fish TLR may be able to recognize the similar PAMPs with mammalian TLRs. Here, the immune responses against PAMPs stimulation and possible corresponding TLRs of teleost fish is discussed.

3.1. TLR1 and TLR2

TLR1 and TLR2 gene are commonly existed in the teleost fish and it may indicate functional importance for immune modulation. In mammal, TLR1 and TLR6 is able to associate with TLR2 to form a heterophilic dimer and discriminate the lipid structures between diacylated and triacylated lipoproteins, respectively. (Takeda and Akira, 2005). As mention above, TLR1 family of TLR6 and TLR10 does not existed in teleost fish. Therefore, it is intrigue that whether teleost fish are able to discriminate the two types of lipopeptide. Purcell and his colleague conducted the diacylated lipoprotein (Pam2CSK4) and triacylated lipopeptide

(Pam3CSK4) stimulation of rainbow trout leukocytes (Purcell *et al*., 2006). Interestingly, the transcription level of IFN-α1 and IL-1β 1 were increased in the both diacylated and triacylated lipoprotein stimulated leukocytes. But, it is still unclear whether both lipoproteins are recognized by TLR1/TLR2 heterophilic dimer or functional substitute molecules in teleost fish.

Mammalian TLR2 is also able to recognizes Gram-positive bacteria cell wall component of peptidoglycan (PG). The PG is also known as a strong inducer of innate immune response in teleost fish. We demonstrated the TLR2 gene up-regulation and increased number of TLR2 expressing leukocytes in Japanese flounder following PG stimulation. The PG induces significant production of cytokines such as IL6-cytokine subfamily that are involving cellular development, inflammatory function, and acute phase and immune responses have been reported in teleost fish (Hwang *et al*., 2007; Castellana *et al*., 2008). These reports may support the functional involvement of TLR2 to the PG recognition of fish immune system as well as mammalian TLR2.

3.2. TLR3

In mammal, pathogen derived double stranded RNA such as RNA virus genome are recognized by TLR3. The double stranded RNA (dsRNA) analogue of poly I:C have been used as antiviral immune response mediator in teleost fish. The injection of poly I:C to Atlantic salmon (*Salmo salar* L.) resulted gene up-regulation of type I interferon (IFN) and antiviral Mx gene in spleen and kidney (Strandskog *et al*., 2008). Further, the gene expression of rainbow trout IFN regulatory factor (IRF) 3 having crucial role in TLR3 signaling were up-regulated following poly I:C stimulation in leukocytes (Holland *et al*., 2008). Therefore, TLR3 mediated immune responses in teleost fish may have an important role in viral infection as well as that of mammals. Recently, two intracellular dsRNA receptor of retinoid-inducible gene 1 (RIG-1) and melanoma differentiation-associated gene 5 (MDA-5) were reported in teleost fish (Robertsen, 2008). The functional corporation of TLR3 and these intracellular dsRNA receptor may also be important for type I IFN production in teleost fish.

3.3. TLR4

TLR4 is known as a receptor for bacterial lipopolysaccharide (LPS) (Kawai and Akira, 2007). Zebrafish has two TLR4 genes, but there are no report of TLR4 molecules from other fish species. LPS have been widely used as immunostimulant of teleost fish and numbers of study demonstrated the potential of LPS for mediating pro-inflammatory cytokine production, chemokine production, nitric oxide production and macrophage activation (Neumann *et al*., 1995; Stafford *et al*., 1999; Corripio-Miyar *et al*., 2007; Darawiroj *et al*., 2008). It is highly possible that there are substitution of TLR4 in teleost fish for sensing LPS. Whilst, the possibility of the immunostimulation effect by the contaminant in the crude LPS preparation was mentioned from the recent study of rainbow trout (Purcell *et al*. 2006). However, there are no other available studies to elucidate these discrepancy.

3.4. TLR5

TLR5 is responsible for the bacterial flagellin recognition. The unique point of teleost fish TLR system as well as amphibian is existence of both membrane type TLR5 (TLR5M) and soluble form TLR5 (TLR5S) which do not contain C-terminal intracellular domain of TIR and transmembrane domain (Tsujita *et al*., 2005). The both rainbow trout TLR5 are able to recognize the flagellin of *Vibrio anguillarum*. Rainbow trout TLR5S gene is predominantly expressed in the liver and highly up-regulated following *V. anguillarum* stimulation in the rainbow trout hepatoma cell line, similar as acute phase proteins produced from the liver. The activity of NF-κB occurring from TLR5M signaling was potentiated by the combination of rainbow trout TLR5M and TLR5S. From this observation, Tsujita *et al*. (2005) hypothesized that the combination of TLR5M and induced circulatory TLR5S systemically provoke robust activation of NF-B, which leads to full response to flagellin in the whole body. To date, TLR5S genes were found from several fish species (Oshiumi *et al*., 2003; Baoprasertkul *et al*., 2007). This unique flagellin recognition system is seems to be conserved in the wide range of the teleost fish. On the other hand, TLR5S have not been found form zebrafish, so comparison of sensitivity against flagellin between zebrafish and other teleost fish TLR5 is interesting topic for the further study.

3.5. TLR7, 8 and 9

Mammalian TLR7, 8 and 9 detect single stranded nucleic acids derived from pathogens in the endosome. Especially, TLR7 recognizes single stranded RNA derived from various viruses as well as synthetic imidazoquinolines. The human TLR8 also participates in the recognition of ssRNA and imidazoquinolines, whereas the function of mouse TLR8 remains unclear (Kawai and Akira, 2007). Immidazoquinolines are also efficient immune modulator in teleost fish. Imidazoquinolines such as S-27609 and R848 are reported as antiviral immune mediator of salmon, although he corresponding receptor either TLR7 or TLR8 have not been defied (Purcell *et al*., 2006; Kileng *et al*., 2008; Sun *et al*., 2008). The drastic gene up-regulation of IFN-α1, IL-1β IL-8, TNF-α1 and α2 were occurred following R848 stimulation in rainbow trout leukocytes, whereas the leukocytes did not respond to another compound of imidazoquinoline of loxoribine (Purcell *et al*., 2006). IFNa (two genes), IFNb (four genes) and IFNc (five genes) were found in Atlantic salmon (Sun *et al*., 2008). To study whether these different salmon IFN subtypes are associated with imidazoquinoline specific IFN induction pathways, the expression pattern of these genes were profiled following S-27609 (Sun *et al*, 2008). Interestingly, only IFNb genes were strongly up-regulated by S-27607, and weakly up-regulated by poly I:C in Atlantic salmon. From this observation, the author speculated that the IFNb genes are mainly induced through the TLR7 or TLR8 pathway whilst other IFN genes are induced through the TLR3, RIG-I and/or MDA-5 pathway. Taken together, it is likely that the salmonid TLRs responsible for single stranded RNA is able to discriminate the structure of imidazoquinolines and activate specific signaling pathway to produce particular cytokines such as Atlantic salmon IFNb genes.

Another nucleic acids receptor of TLR9 recognizes unmethylated CpG DNA motifs present in bacterial and viral genomes. The CpG oligodeoxynucleotides (ODN) mimicking unmethylated CpG DNA have been used for the studies on vertebrate TLR9 studies including

teleost fish. Takano *et al*. (2007) demonstrated specific recognition of CpG ODN by TLR9 and activation of TNF-α gene promoter following CpG ODN stimulation in Japanese flounder. Further, more than twenty kind of CpG ODNs were assessed to confirm optical motif for immunostimulation of Atlantic salmon by measuring kidney leukocytes IFN-like activity, and the the highest IFN-like activity were observed following CpG ODN 1681 stimulation (Jørgensen *et al*., 2003). In addition, the uptake of CpG ODN by head kidney leukocytes (macrophages) and enhancement of the type I IFN and Mx production following CpG ODN 1681 were confirmed in Atlantic salmon (Pedersen *et al*., 2006). Hence, teleost fish TLR9 have pivotal role in the CpG DNA recognition and there is a optical CpG DNA motif for the antiviral activity induction such as IFN production.

3.6. Novel TLRs

The TLR14 subfamily expanded in teleost fish and amphibians. For the function of TLR14, the favorable hypothesis was mentioned by Roach *et al*. (2005) based on the analysis of TLR evolution in vertebrates. The phylogenetic analysis of TLR genes revealed assortative evolution of TLR14 and the lipopeptides specific TLRs including vertebrates TLR1, -2, -6 and -10. The TLR6 and TLR10 homologue in teleost fish have not been found, while TLR14 have not been reported from mammals. Therefore, teleost fish TLR14 was speculated as functional substitute for mammalian TLR6 and TLR10. Recently, the functional involvement for the long double stranded RNA recognition by fugu TLR22 was reported. Hence, the fugu TLR22 is said as a functional substitute of human cell-surface TLR3 (Matsuo *et al*., 2008). Meanwhile, the functional information of TLR19, -20, -21 and -23 are completely lacking and further studies on these novel TLRs are eagerly awaited.

4. Future perspectives

4.1 Functional comparison and characterization of TLR systems in teleost fish

It is evident that numerous molecules involved in biodefense system are resemble and are conserved in mammals and teleost fish. Here, the high level of conservation of TLRs between fish and mammals have been discussed and it is almost certain that PAMPs recognition by TLR is one of the crucial mechanisms for innate immunity in fish. On the other hand, there are novel TLRs in teleost fish although their functions have not been identified. Interspecific TLR diversity have also been observed in the fish genome. Especially, it is said that zebrafish experienced the relativity recent TLR gene duplications (Meijer *et al*. 2004). Therefore, comparison of immune modulation from the different repertoire of TLR in different species are important. Furthermore, some of novel TLRs in teleost fish are hypothesized to compensate to the function of mammalian TLRs such as TLR4, 6 and TLR10 which have not been found in teleost fish (Oshiumi *et al*. 2003; Roach *et al*. 2005). Therefore, functional analysis in the novel fish TLRs are also interesting subject for the comparative immunology between mammals.

4.2. Analysis of immunomodulation by TLR-expressing cells

To survey for infectious agents particularly at the epithelial tissues including mucosal surfaces are interlaced with resident innate leukocytes such as dendritic cells (DCs),

macrophages and mast cells. In mammal, antigen-presenting cells (APCs) such as DCs play central role for orchestrating immune response. Especially, TLR signaling are important for the modulation of DCs activity. DCs are involved in the generation of multiple effector cell types, including TH1, cytotoxic T cell (CTL) and B cell responses (Kaisho and Akira, 2006; Iwasaki and Medzhitov, 2004). Therefore, the studies on mammalian TLR-expressing cells are prosperously conducted for the therapeutic purpose such as vaccine and its adjuvant development.

On the other hand, fish leukocytes expressing TLRs have not been classified. Transcription of fish TLR genes were highly occurring in the leukocytes (Hirono *et al*., 2004; Purcell *et al*., 2006; Takano *et al*., 2007; Holland *et al*., 2008). Further, we demonstrated the proliferation and infiltration of Japanese flounder TLR9-expressing cells at the site of bacterial infection (Takano *et al*., 2007). Thus, the fish leukocytes may also important in the modulation of immune responses. Therefore, the classification of teleost fish TLR-expressing cells and analysis of their role in immunomodulation followed by TLR signaling are also essential for the development of efficient therapeutic techniques for aquaculture.

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