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Review

Ligand specificities of Toll-like receptors in fish: Indications from infection studies

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ABSTRACT

Toll like receptors (TLRs) are present in many different fish families from several different orders, including cyprinid, salmonid, perciform, pleuronectiform and gadiform representatives, with at least some conserved properties among these species. However, low conservation of the leucine-rich repeat ectodomain hinders predictions of ligand specificities of fish TLRs based on sequence information only. We review the presence of a TLR genes, and changes in their gene expression profiles as result of infection, in the context of different fish orders and fish families. The application of RT-qPCR and availability of increasing numbers of fish genomes has led to numerous gene expression studies, including studies on TLR gene expression, providing the most complete dataset to date. Induced changes of gene expression may provide (in)direct evidence for the involvement of a particular TLR in the reaction to a pathogen. Especially when findings are consistent across different studies on the same fish species or consistent across different fish species, up-regulation of TLR gene expression could be a first indication of functional relevance. We discuss TLR1, TLR2, TLR4, TLR5 and TLR9 as presumed sensors of bacterial ligands and discuss as presumed sensors of viral ligands TLR3 and TLR22, TLR7 and TLR8. More functional studies are needed before conclusions on ligands specific to (groups of) fish TLRs can be drawn, certainly true for studies on non-mammalian TLRs. Future studies on the conservation of function of accessory molecules, in conjunction with TLR molecules, may bring new insight into the function of fish TLRs.

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1. Introduction to Toll-like receptors

Once a pathogen has breached physical barriers such as the skin or mucosal tissue, recognition by receptors on dendritic cells, phagocytes, B cells, endothelial cells and other cell types can trigger a series of reactions aimed at the final removal of the pathogen. Macrophages and dendritic cells, generally assigned to the innate immune system, not only have an important regulatory role in the early recognition of pathogens but also are crucial instructors of adaptive immunity. Activation of these cell types can be triggered by the recognition of pathogens by germ line-encoded receptors that recognize conserved patterns of pathogens (Janeway, 1989). These Pattern Recognition Receptors, or PRRs, come in distinct classes that together are able to recognize a large array of ligands, also named Pathogen Associated Molecular Patterns (PAMPs). Toll-Like Receptors (TLRs) are one group of well-known PRRs, each TLR binding to its own set of preferred ligands (Akira et al., 2006; Beutler, 2009; Takeda et al., 2003). Thereby TLRs trigger a rapid inflammatory response *and* prime adaptive immunity (Foster et al., 2007; Iwasaki and Medzhitov, 2010; Takeuchi and Akira, 2010). The Toll receptor itself was first described in the fruit fly and was initially characterized for its developmental function; only later recognition of fungal pathogens was ascribed to the same receptor (Horng and Medzhitov, 2001; Lemaitre et al., 1996). To date, Toll-*like* receptors have been described in virtually every class of the animal kingdom including fish from several different orders, among which cyprinid, salmonid, perciform, pleuronectiform and gadiform representatives.

Toll-like receptors are type-I 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. TLR specificity is determined by variation in the sequence and number of LRR motifs that can interact with ligands as diverse as lipids, carbohydrates, proteins and nucleic acids. When a ligand binds to the concave side of the extracellular domain of a TLR, conformational changes initiate receptor homo- or heterodimerization. Owing to receptor dimerization two intracellular Toll/interleukin-1 receptor homology (TIR) signaling domains are brought close together initiating the recruitment of adaptor molecules. In contrast to the extracellular LRR motifs, the cytoplasmic TIR domain is highly conserved; not only between different TLRs of one species but also between different animal species, and has a central role in recruiting adaptor molecules (Werling et al., 2009). The TIR domain-containing adaptor proteins MyD88, MAL, TRIF, TRAM and SARM can trigger one of two main signaling pathways (O'Neill and Bowie, 2007). One pathway leads to the activation of the transcription factor nuclear factor-κB (NF-κB) whereas the other pathway leads to the activation of activator protein-1 (AP-1). But both pathways trigger transcription of pro-inflammatory cytokines such as interleukin-1, interleukin-6, or tumor necrosis factor alpha. Several reviews with detailed descriptions of the intracellular routes of activation following receptor-ligand interaction in fish have been published (Collet and Secombes, 2002; Rebl et al., 2010; Zhang and Gui, 2012) and these routes of activation will not be subject of the present review. Since the LRR ectodomains of TLRs are not very well conserved, predictions of ligand specificities may be unreliable when based primarily on sequence information, thus additional, functional studies are required to determine ligand specificities of TLRs in fish.

2. Evolution of Toll-like receptor families and genes

Some of the building blocks of TLRs go far back in evolution, for example, LRRs have been identified as important motifs in disease resistance proteins in plants (Rairdan and Moffett, 2007). The first combination between vertebrate-type TIR and LRR domain may have occurred after the divergence of Cnidaria and Bilateria. Subsequently, a recombination of both domains possibly occurred before or during the evolution of primitive vertebrates, leading to the generation of vertebrate TLR molecules (Wu et al., 2011). The ascidian sea squirt *Ciona intestinalis* and the nematode *Caenorhabditis elegans* seem to have only one or two TLR genes (Sasaki et al., 2009; Satake and Sekiguchi, 2012). In contrast, sea urchin (*Strongylocentrotus purpuratus*) and amphioxus (*Branchiostoma lanceolatum*) possess a (very) large number of TLRs of more than two-hundred in the case of sea urchin (Hibino et al., 2006; Holland et al., 2008; Huang et al., 2008; Pancer and Cooper, 2006; Pujol et al., 2001; Rast et al., 2006). Japanese lamprey (*Lethenteron japonicum*) represent a very ancient lineage of jawless vertebrates, and have not many more than 16 TLR genes (Kasamatsu et al., 2010), close to the number of TLR genes found in higher vertebrates. In general, since most vertebrate genomes are recognized to have at least one gene representing each of the six major TLR1, TLR3, TLR4, TLR5, TLR7 and TLR11 families (Roach et al., 2005) this suggests (but does not demonstrate) conservation of vertebrate TLRs.

Although within the modern bony fish (Teleostei) the number of TLR families generally is consistent with what is found for most vertebrates, it is not unusual to find duplicated TLR genes in fish. First postulated by Ohno, two rounds of whole genome duplication (WGD) have occurred during early vertebrate evolution (Ohno, 1970), whereas in teleosts a third, fish-specific genome duplication (FSGD) occurred later in a basal teleost (Jaillon et al., 2004; Kasahara et al., 2007; Ohno, 1999). To complicate matters, some 25–100 million years ago (MYA) in salmonids and more recently (11–21 MYA) also in (some) cyprinids, a fourth WGD event took place (Allendorf and Thorgaard, 1984; Allendorf and Utter, 1973; David et al., 2003; Larhammar and Risinger, 1994; Ohno, 1970). Fish-specific gene duplications as a result of WGDs may lead to the appearance of paralogues with partitioned functions of the ancestral gene (subfunctionalization) (Cresko et al., 2003; Lynch and Force, 2000), or may lead to the development of new functions (neofunctionalization) (Force et al., 1999). Evolution of sub- or neofunctionalization of TLRs can be particularly well studied in tetraploid species such as common carp (*Cyprinus carpio*) in comparison with a related diploid species such as zebrafish (*Danio rerio*). A comparative study into the additional genome duplication event that occurred in the common carp lineage but not in zebrafish showed an almost complete synteny of genes (Henkel et al., 2012).

With respect to partitioning of functions of duplicated genes it is of interest to mention the TLR repertoire of Atlantic cod (*Gadus morhua*), a cold-adapted teleost. Besides a highly expanded number of MHC class I genes, Atlantic cod express a unique composition of TLR families; most TLR genes seem absent from the genome but instead, a single *tlr21*, two *tlr23* and 12 *tlr22*-related genes have been found (Star et al., 2011). The large number of *tlr22* genes seems a result of positive selection pressure, supporting the hypothesis that the *tlr22* genes in cod are undergoing neofunctionalization (Sundaram et al., 2012b). In general, positive selection pressure is often taken as an indication of a history of host-pathogen interactions, which would confirm a role for TLR(22) genes in the recognition of pathogens.

3. Conservation of Toll-like receptors

Molecular analyses can provide information on the molecular structure of TLRs *per se* thereby providing the most 'clean' indication of TLR conservation, which is often displayed in a phylogenetic tree. Sometimes, phylogenetic trees may be good predictors of function. A good example is TLR7, one of the TLR molecules with a remarkably high sequence conservation among vertebrates and