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Toll-like receptors in maraena whitefish: Evolutionary relationship among salmonid fishes and patterns of response to *Aeromonas salmonicida*

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ABSTRACT

Toll-like receptors (TLRs) interact directly with particular pathogenic structures and are thus highly important to innate immunity. The present manuscript characterises a suite of 14 TLRs in maraena whitefish (*Coregonus maraena*), a salmonid species with increasing importance for aquaculture. Whitefish TLRs were structurally and evolutionary analysed. The results revealed a close relationship with TLRs from salmonid fish species rainbow trout and Atlantic salmon. Profiling the baseline expression of TLR genes in whitefish indicated that mainly members of the TLR11 family were highly expressed across all investigated tissues. A stimulation model with inactivated *Aeromonas salmonicida* was used to induce inflammation in the peritoneal cavity of whitefish. This bacterial challenge induced the expression of pro-inflammatory cytokine genes and evoked a strong influx of granulated cells of myeloid origin into the peritoneal cavity. As a likely consequence, the abundance of TLR-encoding transcripts increased moderately in peritoneal cells, with the highest levels of transcripts encoding non-mammalian TLR22a and a soluble TLR5 variant. In the course of inflammation, the proportion of granulated cells increased in peripheral blood accompanied by elevated TLR copy numbers in spleen and simultaneously reduced TLR copy numbers in head kidney at day 3 post-stimulation. Altogether, the present study provides *in-vivo* evidence for relatively modest TLR response patterns, but marked trafficking of myeloid cells as an immunophysiological consequence of *A. salmonicida* inflammation in whitefish. The present results contribute to improved understanding of the host-pathogen interaction in salmonid fish.

1. Introduction

The distinction between self and non-self structures is a crucial feature of living beings and represents a prerequisite for defence against pathogenic invaders, especially bacteria, viruses and fungi [1]. Conserved pathogenic structures such as bacterial lipopolysaccharides, viral RNA or fungal zymosan are known as pathogen-associated molecular patterns (PAMPs) [2]. Toll-like receptors (TLR) are predominantly expressed by particular host cells which are the first to encounter these PAMPs. Stimulated TLRs then activate a signalling cascade that will ultimately trigger the expression of relevant effector genes, contributing to the clearance of pathogens and the maturation of several immune cell populations [3]. In addition, endogenous danger-associated molecular patterns (DAMPs) have been demonstrated to activate TLR signalling routes [4].

Abbreviations: cDNA, complementary DNA; DAMP, danger-associated molecular pattern; FC, fold change; LRR, leucine-rich repeats; IL, interleukin; PAMP, pathogen-associated molecular pattern; PBL, peripheral blood leukocytes; PBS, phosphate-buffered saline; RT-qPCR, quantitative real-time reverse transcription polymerase chain reaction; TIR, Toll/interleukin-1 receptor homology domain; TLR, toll-like receptor; TMD, transmembrane domain; TNF, tumour necrosis factor (alpha).

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The molecular architecture of TLRs has been largely preserved during evolution including a variable number of extracellular leucine-rich repeats (LRR domain), a transmembrane domain (TMD), and an intracellular Toll/interleukin-1 receptor homology domain (TIR) [5,6]. Genomes of all vertebrate species encode a panel of TLRs, each with a slightly different structure affecting its specificity for certain ligands. Six major TLR families are present in vertebrates (TLR1, TLR3, TLR4, TLR5, TLR7 and TLR11), though the number of encoded TLRs is species-specific [7,8]. Ten TLRs were found in humans (TLR1 to −10), while two more TLRs are present in mice (TLR1 to −9 and TLR11 to −13) [9]. Further (non-mammalian) TLRs have been identified in birds (TLR15, TLR16, TLR21) [10,11] and amphibians (TLR14) [12]. Certainly, teleost fishes possess the broadest repertoire of TLRs among vertebrates [13–15]. These receptors comprise orthologs of mammalian TLR1 to −5 and TLR7 to −9 as well as a rich panel of non-mammalian receptors from TLR14 to TLR27 [16–19]. Furthermore, the TLR repertoire differs from fish species to fish species. Cyprinids, for instance, are the only teleost family that features TLR4 [20–22], which is presumably the TLR in humans that has been best investigated. Atlantic cod lacks TLR4 as well as TLR1, -2, and -5. On the other hand, cod has multiple variants of TLR7, -8, -9 and -22 [23]. This pattern may be a representative example for the TLR panel in many other fish species that were analysed and profiled previously, such as zebrafish *Danio rerio* [20,22], pufferfish *Fugu rubripes* [24], channel catfish *Ictalurus punctatus* [17], Atlantic salmon *Salmo salar* [25], and rainbow trout *Oncorhynchus mykiss* [26]. All these reports showed that several well-investigated mammalian TLR orthologs might be absent, while all fish species have varyingly high numbers of poorly investigated non-mammalian or even fish-specific TLRs.

The present study characterises a panel of 14 TLRs from maraena whitefish (*Coregonus maraena*, synonym *Coregonus lavaretus*). Maraena whitefish is an anadromous freshwater fish that is distributed in the Baltic Sea and its adjacent rivers. Over recent decades, natural stocks of maraena whitefish have decreased strongly due to water pollution, the destruction of spawning habitats and consistently high catches. In order to protect this endangered species [27], e.g. in Finland and Germany, maraena whitefish have been kept in aquaculture in recent years [28,29]. The identification and characterisation of the TLR inventory in maraena whitefish supports the development of improved health management strategies for sustainable, eco-friendly aquaculture, as this approach provides us with knowledge that could allow using particular pathogen-derived ligands as potential adjuvants and fish vaccines.

2. Material and methods

2.1. Maraena whitefish, stimulation experiment and sampling procedure

Tissue samples for *de-novo* sequencing were collected from healthy maraena whitefish caught in the Peenestrom River, Germany (n = 4). Samples from adipose tissue, dorsal fin, gills, head kidney, heart, liver, posterior kidney and skin were sliced and immediately transferred to RNAlater solution (Qiagen GmbH, Hilden, Germany).

The stimulation experiment was performed in freshwater tanks at the Friedrich Loeffler Institute (Insel Riems, Germany). Maraena whitefish, aged 205 days post hatch, were randomly allocated and held under experimental conditions for up to 72 h. Water conditions were kept constant, and water quality was recorded regularly. Fish were fed once a day with standard pellet food.

Gram-negative bacteria *Aeromonas salmonicida* subsp. *salmonicida* (wild-type strain JF 2267) were cultivated at 15 °C in LB broth (Luria-Bertani Medium acc. to Miller; SIFIN Institut für Immunpräparate und Nährmedien GmbH, Berlin, Germany). After 72 h, bacteria were harvested and washed in sterile 0.9% sodium chloride solution by centrifugation at 5000 g for 10 min at 4 °C. Bacteria were then inactivated in 1.5% paraformaldehyde for 1 h, concentrated to 5×10^7 cells/ml in sterile PBS and stored at −20 °C. For stimulation, 1×10^7 bacteria were resuspended in 200 μl PBS. Eighteen fish were intraperitoneally injected with the bacterial suspension, while 17 control fish were administered 200 μl PBS only. Six fish per group were sampled at 24, 48 and 72 h post stimulation. The stimulation protocol and anaesthetisation (0.6 ml phenoxyethanol/litre water; Sigma-Aldrich, Munich, Germany) were conducted in compliance with the standards of the German Animal Welfare Act (§ 4 III TierSchG). Peripheral blood was drawn from a caudal vein using a heparinised syringe. Samples of gill, head kidney, liver, and spleen were collected, sliced and immediately frozen at −80 °C until RNA isolation.

The leukocytes in the peritoneal cavity, the site of stimulation, were retrieved through lavage with 5 ml ice-cold PBS (with 2 mM EDTA). The cell suspension was washed once with PBS-EDTA and resuspended in 1 ml PBS-EDTA. The 100-μl cell suspension was then diluted in 200 μl PBS-EDTA and used for flow cytometric analysis on a FACS Canto II (Becton Dickinson, Heidelberg, Germany). Samples were acquired for 20 s in "HIGH throughput" mode. The distribution of lymphoid (FSC-A^{lo}; SSC-A^{lo}) and large granulated myeloid cells (FSC-A^{hi}; SSC-A^{hi}) was evaluated using DIVA software (Becton Dickinson).

Ten microliters of blood was diluted in 300 μl PBS-EDTA and then submitted immediately for measurement using the FACS Canto II. The lymphocytes were identified using the FSC-W channel as described previously [30].

2.2. Nucleotide extraction and reverse transcription PCR

For RNA isolation, tissue samples were homogenised individually in 1 ml TRIzol Reagent (Invitrogen, Darmstadt, Germany) and purified using the RNeasy Mini Kit (Qiagen GmbH) with 30 min in-column DNase treatment. The concentration and quality of RNA were proven by gel electrophoresis and spectrophotometry (ND1000; Nano Drop technologies, USA). RNA was stored at −80 °C. Complementary DNA from tissue samples was synthesised from 1.5 μg total RNA using the SuperScriptII Reverse Transcriptase Kit (Invitrogen) according to manufacturer's protocol.

2.3. Identification of TLR sequences from maraena whitefish

Orthologous cDNA sequences from salmonid fish species (Atlantic salmon *S. salar* and rainbow trout *O. mykiss*) were extracted from the NCBI database, aligned (ClustalW Multiple Sequence Alignment [31]) and used for designing consensus oligonucleotide primers (Supplemental Table 1). All primers used in this study were obtained from Sigma-Aldrich. Amplification was performed with either OneTaq or Phusion High-Fidelity DNA Polymerase (both New England Biolabs GmbH, Frankfurt am Main, Germany) according to the supplier's protocol. The specific melting temperature for each primer pair was estimated using the Tm calculator v1.7.4 software (New England Biolabs; http://tmcalculator.neb.com/#!/) and adjusted experimentally.

Amplification was performed with consensus primers and hepatic cDNA from three maraena whitefish individuals. The amplified products were excised from agarose gel and purified using a High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Grenzach-Wyhlen, Germany). The purified fragments were cloned utilising the pGEM-T Easy Vector system (Promega GmbH, Mannheim, Germany). Each fragment was cloned up to three times.