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ORIGINAL PAPER

Characterization of Toll-like receptor gene expression and the pathogen agonist response in the antarctic bullhead notothen *Notothenia coriiceps*

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Abstract *Notothenia coriiceps*, a typical Antarctic notothenioid teleost, has evolved to adapt to the extreme Antarctic marine environment. We previously reported an extensive analysis of the Antarctic notothenioid transcriptome. In this study, we focused on a key component of the innate immune system, the Toll-like receptors (TLRs). We cloned the full-length sequence of 12 TLRs of *N. coriiceps*. The *N. coriiceps* transcriptome for TLR homologue (ncTLR) genes encode a typical TLR structure, with multiple extracellular leucine-rich regions and an intracellular Toll/IL-1 receptor (TIR) domain. Using phylogenetic analysis, we established that all of the cloned ncTLR genes could be classified into the same orthologous clade with other teleost TLRs. ncTLRs were widely expressed in various organs, with the highest expression levels observed in immune-related tissues, such as the skin, spleen, and kidney. A subset of the ncTLR genes was expressed at higher levels in fish exposed to pathogen-mimicking agonists, heat-killed *Escherichia coli*, and polyinosinic-polycytidylic acid (poly(I:C)). However, the mechanism involved in the upregulation of TLR expression following pathogen exposure in fish is currently unknown. Further research is required to elucidate these mechanisms and to thereby increase our understanding of vertebrate immune system evolution.

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Introduction

The innate immune system, an evolutionarily conserved defense mechanism found in all organisms other than prokaryotes, provides immediate defense against infectious pathogens such as viruses, bacteria, fungi, or protozoa (Medzhitov 2001). Innate immune cells express various pattern-recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), double-stranded RNA, and unmethylated cytosine–guanine (CpG) motifs. The Toll-like receptors (TLR) are the most-studied PRRs; they trigger immune signaling pathways that ultimately leads to elimination of invading pathogens. TLRs are transmembrane proteins that recognize conserved non-self molecules via an N-terminal domain. The extracellular N-terminal domain consists of approximately 16–28 leucine-rich repeats (LRRs), each consisting of 20–30 amino acids with the conserved motif “LxxLxLxxN.” The LRR region functions as a pathogen-recognition domain that binds to several PAMPs (Bell et al. 2003). Binding of a ligand to this domain triggers an intracellular signaling cascade. TLRs possess another key structure, located on the intracellular C-terminus domain: the Toll/IL-1 receptor (TIR) domain. The cytoplasmic TIR domain harbors conserved amino acids that have been shown to be involved in both TLR signaling and localization (Funami et al. 2004; Slack et al. 2000). The TIR domain relays the pathogenic invasive signal to intracellular immune-responsive components via adapter molecules such as myeloid differentiation primary response protein 88 (MyD88) or TIR domain-containing adapter-inducing interferon-β (TRIF). This leads to the activation of inducible transcription factors, such as NF-κB, activator protein 1

(AP-1), and interferon regulatory factor (IRF), which in turn leads to the production of inflammatory cytokines, chemokines, and type I interferon (Kawai and Akira 2010; Iwasaki and Medzhitov 2004).

To date, 26 distinct TLR gene types have been identified from various animal species (Boudinot et al. 2014; Kasamatsu et al. 2010; Quiniou et al. 2013; Roach et al. 2005; Temperley et al. 2008). Two major TLR subfamilies are found in humans. The first includes TLRs 1, 2, 4, 5, 6, and 10 and mainly senses microbial metabolites and/or products such as lipids, sugars, and proteins (Hajjar et al. 2001; Hayashi et al. 2001; Hoshino et al. 1999; Takeuchi et al. 2001; Werts et al. 2001). The second group includes TLRs 3, 7, 8, and 9 and senses nucleotide derivatives of viral or bacterial origin (Alexopoulou et al. 2001; Diebold et al. 2004; Gibbard et al. 2006; Gorden et al. 2005; Latz et al. 2004). Bony fishes form the largest and most diverse group of vertebrates, accounting for roughly half of extant vertebrate species (Volff 2004). They are thought to have a primitive immune system, considering their diverse species pool. Therefore, great scientific interest has been expressed in comparing the innate and adaptive defense mechanisms of bony fish with those of mammals. The first piscine member of the interleukin-1/TLR superfamily was isolated from rainbow trout (Sangrador–Vegas et al. 2000). Since then, 19 TLR types (TLRs 1, 2, 3, 4, 5, 5S, 7, 8, 9, 13, 14, 18, 19, 20, 21, 22, 23, 25, and 26) have been identified in more than a dozen teleost species (Boudinot et al. 2014; Jault et al. 2004; Kongchum et al. 2009; Meijer et al. 2004; Ortega-Villaizan et al. 2009; Oshiumi et al. 2003; Palti 2011; Palti et al. 2006; Quiniou et al. 2013; Rebl et al. 2007; Rodriguez et al. 2005). However, the number of TLRs might differ among teleostean species (Palti 2011; Rebl et al. 2010). The piscine TLR repertoire includes fish-specific TLRs, such as TLRs 18–26. The fact that many different TLRs, in addition to factors involved in TLR signaling, are conserved between fish and mammals suggests that regulation of the immune response might be also similar in these two classes of animals (Bricknell and Dalmo 2005). However, fish TLRs also exhibit very distinct features and great diversity, which is likely a result of their expansive evolutionary history (Palti 2011). Moreover, cold-adaptive evolution in fish has generated some unique immune system features. For example, Atlantic cod (*Gadus morhua*), a cold-adapted teleost, has a highly expanded and unique TLR repertoire. Antarctic fish have undergone extraordinary evolutionary episodes since the onset of widespread glaciation in Antarctica approximately 34 Ma ago when the Southern Ocean cooled to the freezing point of seawater (−1.9 °C) (Eastman et al. 1993). The Antarctic fish fauna is dominated by the perciform suborder Notothenioidei, which has survived in the subzero waters of the continental shelf and may have experienced a unique type of adaptive radiation known as species flock (Eastman 2000; Eastman and Clarke 1998). The notothenioids have undergone resistant and compensatory adaptations to the extreme Antarctic marine environment, as well as regressive evolutionary changes. Thus, they are considered an attractive model species for evolutionary and physiological studies (Eastman 2000; Maher 2009).

We previously reported an extensive analysis of Antarctic notothenioid transcriptomes (Shin et al. 2012). From this transcriptome information, we have selected genetic sequences associates with the innate immune system, specifically TLRs. As Antarctic fish possess a primitive immune system, analyzing their transcriptomes is important in studying the evolution of the vertebrate immune system. The major aims of this investigation were to characterize the TLR expression pattern and TLR agonist response profile of Antarctic fish. These data allow us to define orthologs in other species and to characterize variation within and among vertebrate lineages, providing insight into immune system evolution.

Materials and methods

Gene identification and sequencing

Twelve *Notothenia coriiceps* TLRs were identified from previously analyzed transcriptome data (Shin et al. 2012). To obtain full-length complementary (cDNA) of the 12 TLR genes, 3′- and 5′-ends were amplified using the CapFishing Full-Length cDNA Kit (Seegene, Seoul, Korea) according to the manufacturer's instructions. Gene-specific primers are detailed in Table S1. Rapid Amplification of cDNA End (RACE) polymerase chain reaction (PCR) products were sequenced from both the 5′- and 3′-ends. Complete sequences were analyzed by searching for similarities using the BLASTX search program of the National Center for Biotechnology Information (NCBI).

Fish sample collection and TLR agonist challenge experiments

N. coriiceps (length about 35 cm) were collected from depths of 20–30 m in Marian Cove, near King Sejong Station, on the northern Antarctic Peninsula (62° 14′ S, 58° 47′ W) in January 2012 using the hook-and-line method. After capture, the fish were maintained in flow-through aquaria at ambient seawater temperatures (−1.5 °C) for 48 h, with a constant air supply. After acclimation for 2 days, *N. coriiceps* were transferred to 100-L temperature-controlled aquaria, filled with filtered (<0.2 μm) natural seawater. Each aquarium contained nine animals and the water was exchanged every 24 h. Pathogen-mimicking agonist-exposed groups were treated with heat-killed *Escherichia coli 011:B4* (HKEB; InvivoGen, San Diego, CA) at a final concentration of 1×10^7/mL and poly(I:C)-LMW (synthetic analog of double-stranded (ds)

RNA, InvivoGen) at a final concentration of 10 μg/mL. The seawater was aerated and the water temperature was maintained at 1.0±0.1 °C throughout the experimental period. After 0, 6, and 12 h, three specimens were randomly selected and removed from each of the three aquaria (i.e., $n=3$ per treatment) to isolate total RNA. All experimental procedures were conducted according to the law on activities and environmental protection to Antarctic approved by the Minister of Foreign Affairs and Trade of the Republic of Korea (Shin et al. 2012).

Tissue expression analysis using reverse transcription-PCR

Specific primers for 12 TLRs were designed for reverse transcription (RT)-PCR, based on sequencing results (Table S2). Equal amounts of RNA from various tissues (liver, stomach, kidney, intestine, spleen, muscle, skin, brain, blood, and egg) of *N. coriiceps* were used for RT-PCR, which was performed using Quick Taq HS DyeMix (Toyobo, Osaka, Japan). *N. coriiceps* β-actin was used as a control. PCR products were visualized on a UV-transilluminator after electrophoresis on a 1.5 % agarose gel containing GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA).

Quantification of TLR messenger RNA expression by real-time quantitative RT-PCR

The messenger RNA (mRNA) levels of *N. coriiceps* were measured by real-time quantitative RT-PCR. PCR amplifications were performed in 20-μL reactions with 1 μL of a 1:20 dilution of cDNA generated from 5 μg of the original RNA template, 2 μM each of the gene-specific RT-PCR primers, and 10 L of QuantiFast SYBR Green PCR kit (Qiagen, Hilden, Germany). Amplified signals were monitored continuously with the Mx3000P qPCR System (Stratagene, La Jolla, CA). The amplification protocol was as follows: 5 min of denaturation and enzyme activation at 95 °C, followed by 40 cycles at 95 °C for 5 s, 55 °C for 20 s, and 72 °C for 15 s. The β-actin gene of *N. coriiceps* was used as a reference to normalize the expression levels among samples. All data are expressed relative to β-actin to compensate for any differences in reverse transcriptase efficiency. The product sizes for the PCRs were verified on agarose gels and a dissociation curve analysis was performed to verify that a single PCR product was generated. All experiments were performed in triplicate. The relative gene expression fold change was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). All the data represent means±standard deviation and were subjected to one-way analysis of variance (ANOVA), followed by Dunnett's two-tailed t test (Dunnett's test), using SPSS version 18.0.0 (PASW Statistics 18; SPSS Inc., Chicago, IL). Differences were considered statistically significant at $p<0.05$.

Phylogenetic analysis

Phylogenetic trees were constructed using the amino acid sequences from various organisms, including vertebrates and invertebrates. The amino acid sequences were aligned using ClustalW (version 1.81) (Thompson et al. 1997), and molecular phylogenetic trees were constructed using the MEGA6 software (version 6.06) applying the neighbor-joining method and default parameters option. The evolutionary distances were computed using the JTT+G distances with complete deletion option and are in the units of the number of amino acid substitutions per site. Bootstrap values based on 1,000 replicates with percentages indicated on each branch (Tamura et al. 2013).

Results

Identification of TLR sequences in the transcriptome of *N. coriiceps*

We screened the *N. coriiceps* transcriptome for TLR homologues (denoted ncTLRs) and designed gene-specific primers for each TLR identified. The full coding regions of ncTLRs were identified by 3′ and 5′ RACE-PCR. We found 12 ncTLR full sequences, including three fish-specific TLRs: ncTLR21, ncTLR22, and ncTLR23. Detailed information of the 12 full-length transcripts and GenBank accession numbers are listed in Table 1, and the alignment of deduced amino acid sequences of ncTLRs is shown in Supplemental Figure S1. The predicted amino acid sequence of ncTLRs contains features common to TLRs, namely, a transmembrane domain, an intracellular domain, and multiple extracellular leucine-rich regions (LRRs). The domain structures of ncTLRs were predicted using the Web-based software program Simple Modular Architectural Research Tool (SMART), and a sequence alignment of TIR domains is shown in Fig. 1. Additionally, LRRfinder (http://www.lrrfinder.com/) and TMHMM (http://www.cbs.dtu.dk/services/ TMHMM/) were used to predict LRRs and transmembrane region. This analysis revealed well-conserved groupings of multiple LRRs that are known to be important for ligand recognition (Table 1). Other domains with high interspecies conservation included the N- and C-terminal cysteine-flanked LRRs, the transmembrane domain, and the TIR domain phosphorylation sites. Eleven of the 12 genes encode typical TLR structures (Fig. 1), but the ncTLR5S gene has only LRR repeats.

Phylogenetic analysis

To analyze the 12 ncTLRs in the large context of vertebrate TLRs, we conducted phylogenetic analysis using the

Table 1 Structural features of *Notothenia coriiceps* TLR genes

Structural feature[a]	ncTLR1	ncTLR2	ncTLR3	ncTLR5S	ncTLR5	ncTLR7	ncTLR8	ncTLR9	ncTLR14	ncTLR21	ncTLR22	ncTLR23
Nucleotide length (bp)	2,965	2,541	3,392	2,371	3,299	3,421	3,544	3,876	2,862	3,498	2,874	3,586
Amino acid residues (AA)	749	790	918	588	873	1,055	1,027	1,055	760	973	756	948
Number of LRRs	10	10	16	14	16	23	23	24	12	17	17	14
Length of TIR (AA)	101	146	144	–	133	147	147	151	95	150	41	144
Signal peptide	1–22	1–22	1–36	–	1–51	1–21	1–25	1–19	1–17	1–23	–	1–26
LRR-NT	–	–	–	–	50–81	29–65	–	–	–	–	–	31–62
LRR-CT	528–587	530–584	658–711	–	619–672	789–840	758–809	783–834	553–607	685–731	600–651	682–733
Transmembrane	599–621	586–608	715–737	–	685–707	846–878	818–840	836–858	610–632	739–761	657–679	734–777
TIR domain	649–749	637–782	770–913	–	736–868	896–1,042	865–1,011	891–1,041	665–759	790–939	710–750	789–932
Predicted MW (kDa)[b]	84.06	89.69	103.78	65.9	98.64	121.48	118.01	120.36	87.97	112.2	86.83	108.46
GenBank accession no.	KJ531970	KJ531971	KJ531972	KJ531973	KJ531974	KJ531975	KJ531976	KJ531977	KJ531978	KJ531979	KJ531980	KJ531981

[a] Each domain is predicted by SMART (http://smart.embl-heidelberg.de), LRRfinder (http://www.lrrfinder.com/), TMHMM (http://www.cbs.dtu.dk/services/ TMHMM/), and NCBI Conserved Domains (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml)

[b] Protein molecular weight was predicted using the Bioinformatics molecular weight tool (http://www.bioinformatics.org/sms/prot_mw.html)

neighbor-joining method. The alignment of amino acid sequences used for phylogenetic inference is shown in Supplemental Figure S2. All of the ncTLR genes were located in the same ortholog clade (Fig. 2). ncTLR21, ncTLR22, and ncTLR23 fell into a major clade containing a group similar to murine TLRs 11–12. The sequence identity between *ncTLR* and corresponding fish TLR nucleotide sequences is shown in Table 2. Translation of the ncTLR nucleotide sequences and alignment with other fish TLR protein shows 30–80 % amino acid sequence conservation. ncTLRs have particularly high homology (38–80 %) with takifugu (*Takifugu rubripes*) TLRs.

Tissue-specific expression patterns of the 12 ncTLRs

The mRNA expression patterns of the 12 ncTLR genes were measured in 10 tissues by RT-PCR (Fig. 3). β-Actin was used as an internal control gene. ncTLR1, 2, 3, 5, 8, 9, 14, 21, 22, and 23 were widely expressed in each organ, and ncTLR1 and ncTLR3 transcripts were detected in all examined tissues. ncTLR5S and 7, however, were expressed in restricted organs. The kidney shared expression of 12 ncTLR transcripts, whereas the other tissues expressed tissue-specific TLR homologues.

Expression analysis of mRNA following stimulation with TLR agonists

To analyze the modulation of expression of 12 ncTLR genes in *N. coriiceps* tissues after pathogen-mimicking agonist stimulation, real-time quantitative RT-PCR was conducted on mRNA from liver, kidney, and spleen tissues. Heat-killed *E. coli* 011:B4 (HKEB) and poly(I:C) [polyinosinic-polycytidylic acid; a low-molecular-weight synthetic analog of double-stranded (ds) RNA] were used to mimic bacterial and viral infection, respectively. All data are expressed relative to β-actin to compensate for any differences in reverse transcriptase efficiency. In the case of stimulation mimicking bacterial infection (HKEB agonist) (Fig. 4), the mRNA expression response of each of the TLRs differed depending on the organ. In the liver, ncTLR2 and ncTLR3 were upregulated approximately threefold, whereas in the spleen, ncTLR1, ncTLR2, ncTLR3, ncTLR5S, ncTLR5, ncTLR14, ncTLR21, and ncTLR23 were all instantly upregulated two- to eightfold. The greatest level of upregulation following HKEB stimulation was that of ncTLR23 in the spleen, where an eightfold change in expression was observed. During stimulation mimicking viral infection (poly(I:C) agonist) (Fig. 5), ncTLR5S and ncTLR8 were instantly upregulated threefold at 6 h of exposure in the kidney. ncTLR7 was upregulated sevenfold at 6 h of exposure in liver tissue. At 12 h of exposure, the expression levels of ncTLRs

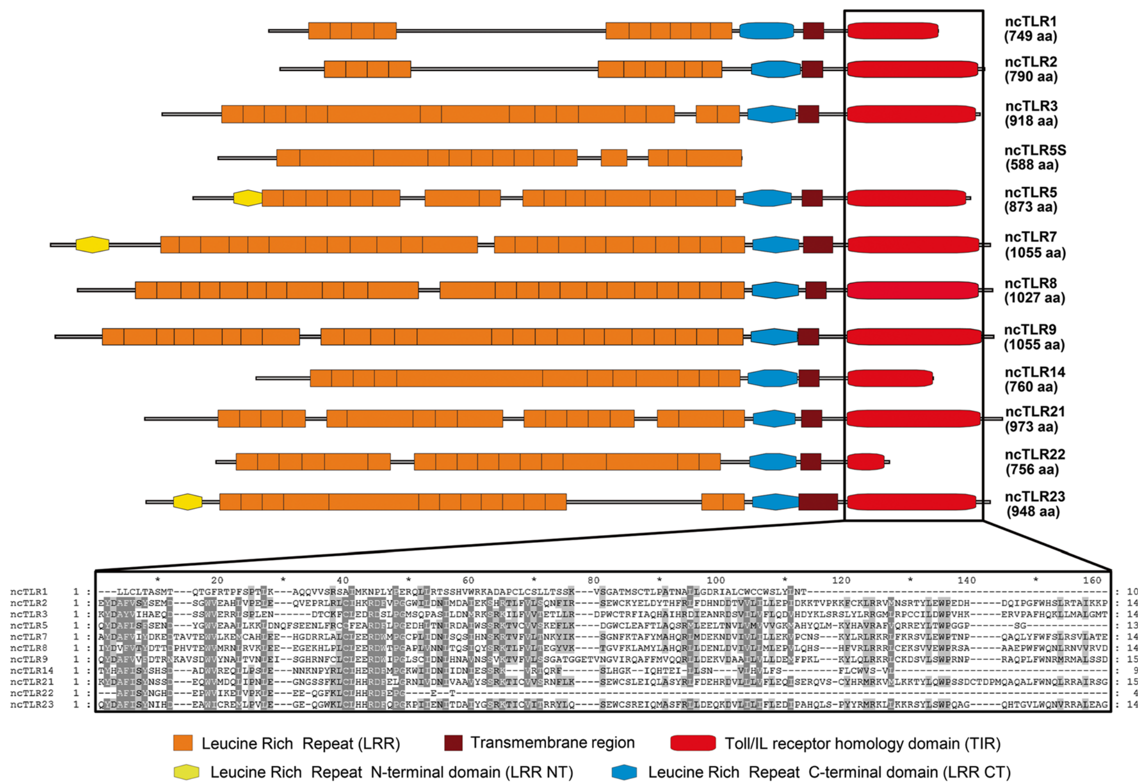

Fig. 1 Schematic representation of the TLR domain structure showing amino acid alignment of *Notothenia coriiceps* TIR domains. Each domain was predicted using the SMART, LRRfinder, and TMHMM programs. Like all reported mammalian TLR molecules, all *N. coriiceps* TLR molecules (except ncTLR5S) have LRR repeats in the N-terminal region, followed by a transmembrane region and a C-terminal TIR domain. ncTLR5S has only LRR repeats

were upregulated over twofold in the following tissues: ncTLR2, 5S, 7, 9, 14, and 21 in liver tissue; ncTLR5S, 5, 7, 8, 9, and 21 in kidney tissue; ncTLR1, 7, 8, 9, and 23 in spleen tissue. The greatest level of upregulation following poly(I:C) stimulation was as in the following : ncTLR7 was upregulated sevenfold in liver tissue, ncTLR5S was upregulated 4.5-fold in kidney tissue, and ncTLR23 was upregulated fivefold in spleen tissue. The expression levels of other ncTLRs did not significantly change. ncTLR23 was responsive to both bacterial and viral pathogen ligands in spleen tissues.

Discussion

Structural analysis of 12 ncTLR genes reveals that they all possess the typical domain features of Toll-like receptors. In the extracellular TLR domain, all of the ncTLRs have a common structure characterized by 10–24 multiple extracellular LRRs (Fig. 1). LRR domains of TLRs are often flanked by N-terminal and C-terminal cysteine-rich domains. The LRRs are capped by the LRR-NT and LRR-CT domains, each containing a cysteine cluster. The N-terminal cap, known as the LRR-NT domain, has functions related to the binding of metal ions such as Ca^{2+}. Crystal structure analysis of internalin B (InlB) predicts that the LRR-NT domain acts as a bridge between the pathogen and the mammalian cell surface receptor during pathogenic invasion (Freiberg et al. 2004). LRR-NT domains vary among vertebrate TLRs (Quiniou et al. 2013). In *N. coriiceps*, LRR-NT domains were detected in ncTLR5, ncTLR7, and ncTLR23. LRR-CT regions, however, contain a highly conserved consensus sequence common to all vertebrate TLRs and *Drosophila melanogaster* Toll (Bell et al. 2003; Kobe and Deisenhofer 1994). The LRR-CT domain is very close to the transmembrane region (1–13 residues in ncTLRs) (Bell et al. 2003; Bella et al. 2008). Our structural analysis revealed that LRR-CT regions are conserved in 11 ncTLRs, except ncTLR5S (Fig. 1). Other common conserved domains are the transmembrane domain and the Toll/IL-1 receptor domain (TIR) found in 11 of the 12 ncTLR genes. These domains were not, however, detected in the ncTLR5S gene. Amphibians and teleosts have a soluble short form of the TLR5 gene (TLR5S) that diverged from the LRR domains of TLR5. In structural classification, it is not strictly a TLR, because it lacks a TIR domain, yet it is closely related to TLR5 and is often considered in conjunction with true TLRs in

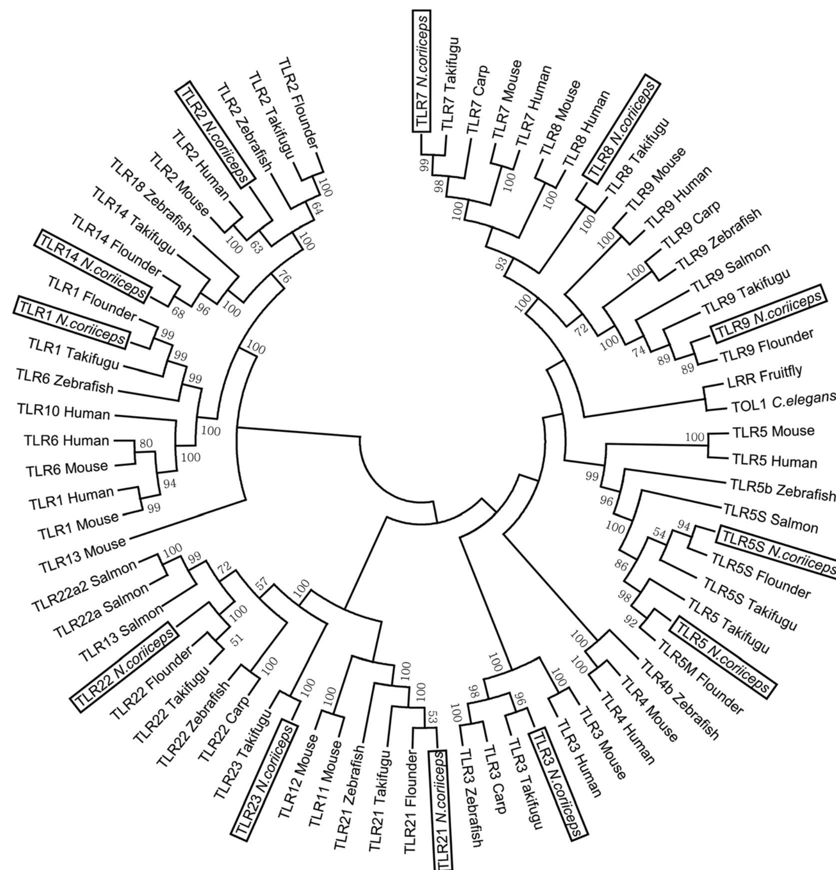

Fig. 2 The phylogenetic tree of TLRs, including vertebrates, teleosts, and *Notothenia coriiceps*. The phylogenetic tree depicts the evolutionary history relationship between teleost TLRs. The alignment of the amino acid sequences was performed using ClustalW software (version 1.81). The phylogenetic tree was constructed using the MEGA6 software (version 6.06) applying the neighbor-joining method and default parameters option. The phylogenetic tree shown is topology-based. The evolutionary distances were computed using the JTT+G distances with complete deletion option and are in the units of the number of amino acid substitutions per site. Bootstrap values based on 1,000 replicates with percentages indicated on each branch and only shown 50 % and above cutoff. The TLRs of *N. coriiceps* are denoted by a *square*. The reference sequences were derived from *Caenorhabditis elegans* (TOL-1, AAK37544), *Drosophila melanogaster* (fruitfly LRR, AAB33383), *Takifugu rubripes* (takifugu TLR1, AAW69368; TLR2, AAW69370; TLR3, AAW69373; TLR5, AAW69374; TLR5S, AAW69378; TLR7, AAW69375; TLR8, AAW69376; TLR9, AAW69377; TLR14, AAW69369; TLR21, AAW69371; TLR22, AAW69372; TLR23, AAW70378), *Salmo salar* (salmon TLR5S, AAV35178; TLR9, NP_001117125; TLR13, NP_001133860; TLR22a, CAJ80696; TLR22a2, CAR62394), *Cyprinus carpio* (carp TLR3, ABL11473; TLR7, BAJ19518; TLR9, ADC45018; TLR22, ADR66025), *Danio rerio* (zebrafish TLR2, NP_997977; TLR3, NP_001013287; TLR4b, NP_997978; TLR5b, NP_001124067; TLR6, NP_001124065; TLR9, NP_001124066; TLR18, NP_001082819; TLR21, NP_001186264; TLR22, NP_001122147), *Paralichthys olivaceus* (flounder TLR1, AFW04264; TLR2, BAD01044; TLR5M, BAJ16367; TLR5S, BAJ16369; TLR9, BAE80690; TLR14, BAJ78226; TLR21, AFW042630; TLR22, BAD01045), *Mus musculus* (mouse TLR1, NP_109607; TLR2, NP_036035; TLR3, NP_569054; TLR4, NP_067272; TLR5, Q9JLF7; TLR6, Q9EPW9; TLR7, NP_573474; TLR8, NP_573475; TLR9, NP_112455; TLR11, NP_991388; TLR12, NP_991392; TLR13, NP_991389), *Homo sapiens* (human TLR1, NP_003254; TLR2, NP_003255; TLR3, NP_003256; TLR4, NP_612564; TLR5, NP_003259; TLR6, NP_006059; TLR7, NP_057646; TLR8, NP_619542; TLR9, NP_059138; TLR10, AF296673)

phylogenetic analyses (Roach et al. 2005). Phylogenetic analysis places ncTLR5S close to flounder TLR5S and takifugu TLR5S and is predicted to be a putative soluble short form of ncTLR5. The other 11 ncTLRs were located in the same ortholog, suggesting that they are all phylogenetically close to the TLRs of takifugu (Fig. 2, Table 2).

The expression of the 12 ncTLRs was investigated at the transcriptional level in various tissues of *N. coriiceps* using RT-PCR. The kidney contained detectable levels of the majority of the 12 ncTLR transcripts, whereas other tissues showed tissue-specific TLR expression. This suggests that the kidney is the major, but not the only, innate immune organ of *N. coriiceps*. Immune organs vary among teleost fish species. The major immune tissues of bony fish include the kidney (especially the anterior kidney), thymus, spleen, and scattered immune areas within mucosal tissues (e.g., in the skin, gills, gut, and gonads) (Chilmonczyk 1992; Hoar et al. 1997). Different organs are known to possess tissue-specific ncTLRs.

Table 2 Nucleotide and amino acid sequence identity between ncTLRs and other species TLRs

Notothenia coriiceps	*Takifugu rubripes*		*Danio rerio*		*Salmo salar*		*Homo sapiens*	
	Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid
ncTLR1	64	50	–	–	–	–	21	22
ncTLR2	28	38	28	42	–	–	24	41
ncTLR3	69	66	59	51	–	–	21	42
ncTLR5	64	58	20	38	–	–	25	34
ncTLR5S	66	61	–	–	21	46	–	–
ncTLR7	79	80	–	–	–	–	32	56
ncTLR8	71	62	–	–	–	–	35	42
ncTLR9	69	61	37	49	64	57	27	35
ncTLR14	65	60	–	–	–	–	–	–
ncTLR21	69	64	62	55	–	–	–	–
ncTLR22	51	44	22	30	47 (TLR22a) 50 (TLR22a2)	33 (TLR22a) 36 (TLR22a2)	–	–
ncTLR23	28	55	–	–	–	–	–	–

Values represent percentage of residues matched exactly between *N. coriiceps* and reference sequences. Matching percentage values were derived using GeneDoc (http://www.nrbsc.org/gfx/genedoc/)

Remarkably, ncTLR1 and ncTLR3 transcripts were detected in all examined tissues. TLR3 plays a critical role in the response to molecules associated with viral infection, such as dsRNA and poly(I:C), and is predicted to form a general antiviral immune component in the organs of *N. coriiceps* (Fig. 3).

The mRNA expression of ncTLR23 in the spleen was upregulated eightfold by bacterial infection (HKEB agonist) and fivefold by viral infection [poly (I:C) agonist] (Figs. 4 and 5). The expression levels of the other 11 ncTLR genes were also modulated by stimulation with HKEB or poly(I:C). After stimulation with HKEB, ncTLR2 and 3 were upregulated approximately threefold in liver tissue (Fig. 4). Similarly, after stimulation with poly(I:C), ncTLR7 was upregulated sevenfold in liver tissue and ncTLR5S was upregulated 4.5-fold in kidney tissue. The expression levels of ncTLRs were upregulated two- to threefold in the following tissues: ncTLR2, 5S, 9, 14, and 21 in liver tissue; ncTLR5, 7, 8, 9, and 21 in kidney tissue; and ncTLR1, 7, 8, and 9 in spleen tissue (Fig. 5). The expression levels of other ncTLRs did not significantly change. ncTLR23 was responsive to both bacterial and viral pathogen ligands in spleen tissues.

The function of the fish-specific TLR23 has not been clearly elucidated (Boudinot et al. 2014). TLR23 was first identified in *T. rubripes* and has also been found in *G. morhua*. TLR23 is suggested to participate in LPS recognition and may compensate for the loss of TLR4 in pufferfish and cod (Roach et al. 2005; Sundaram et al. 2012). The fish-specific TLR types might be under less purifying selective pressure than the other TLR families. Previously, teleosts were known to

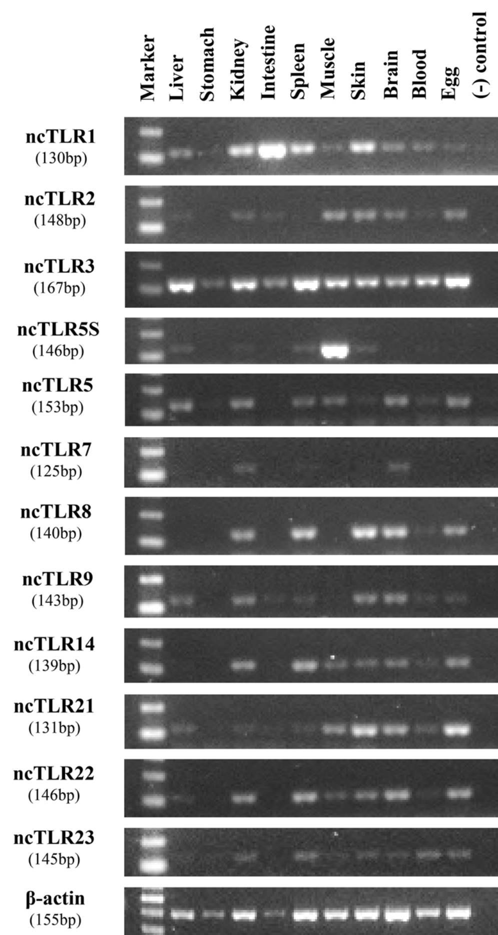

Fig. 3 Tissue-specific expression pattern of 12 ncTLRs as assessed by RT-PCR. Amplification of TLR cDNA fragments was performed using an equal number of PCR cycles for each sample

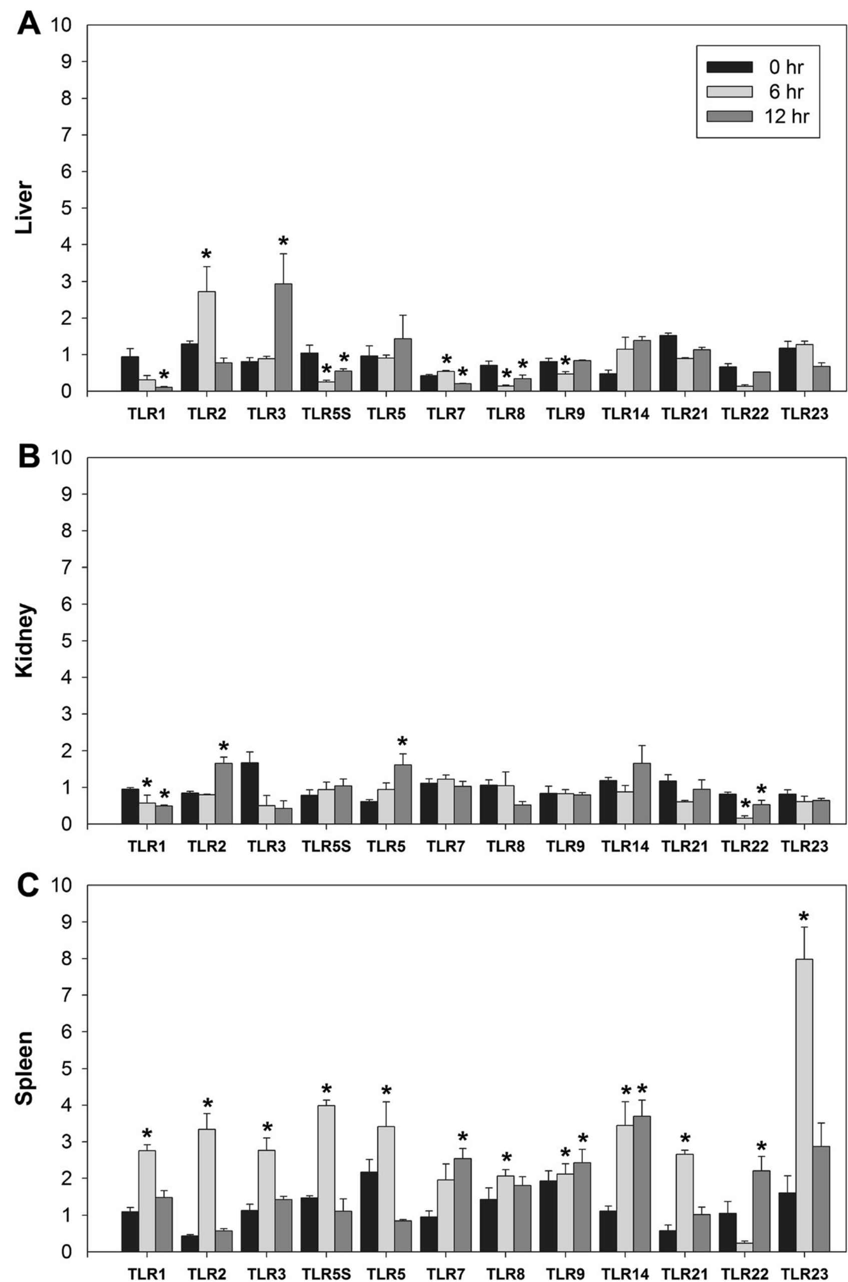

Fig. 4 Expression analysis of ncTLRs in different *Notothenia coriiceps* tissues following infection with a bacterial infection agonist. The **a** liver, **b** kidney, and **c** spleen of HKEB-exposed groups were sampled after 0, 6, and 12 h. Three specimens were selected for total RNA isolation for quantitative real-time RT-PCR (i.e., $n=3$ per treatment). The β-actin gene of *N. coriiceps* was used as a reference to normalize the expression levels among samples. An *asterisk* (*) indicates values significantly different from zero time point ($p<0.05$)

have 19 TLR types (TLR1, 2, 3, 4, 5, 5S, 7, 8, 9, 13, 14, 18, 19, 20, 21, 22, 23, 25, and 26). The fish-specific TLRs (TLR18–23, 25–26) were not found in any other vertebrate classes. Recently, teleosts were found to have more fish-specific TLRs. TLR25 and TLR26 were found in the channel catfish (*Ictalurus punctatus*) (Quiniou et al. 2013), although their function has not been clearly identified. Piscine TLRs, each specific for a broad range of PAMPs, are structurally conserved, suggesting similarities in regulation of the immune response between fish and mammals (Bricknell and Dalmo 2005).

We have confirmed that *N. coriiceps* has 12 TLRs, including three fish-specific TLRs: 21, 22, and 23. In teleosts, fish-specific TLR types show a tendency to compensate for other TLRs. For example, in mammals, TLR3 responds to dsRNA, whereas in the grass carp (*Ctenopharyngodon idella*), TLR22 works in conjunction with TLR3 to form a dual dsRNA recognition system (Lv et al. 2012). Our phylogenetic analysis places the fish-specific TLRs ncTLR21, 22, and 23 in close proximity, consistent with TLRs from other teleost fish (Fig. 2). The mammalian TLRs (TLR 11 and 12 from mice) are associated with the same branch/lineage as TLR 21, 22,

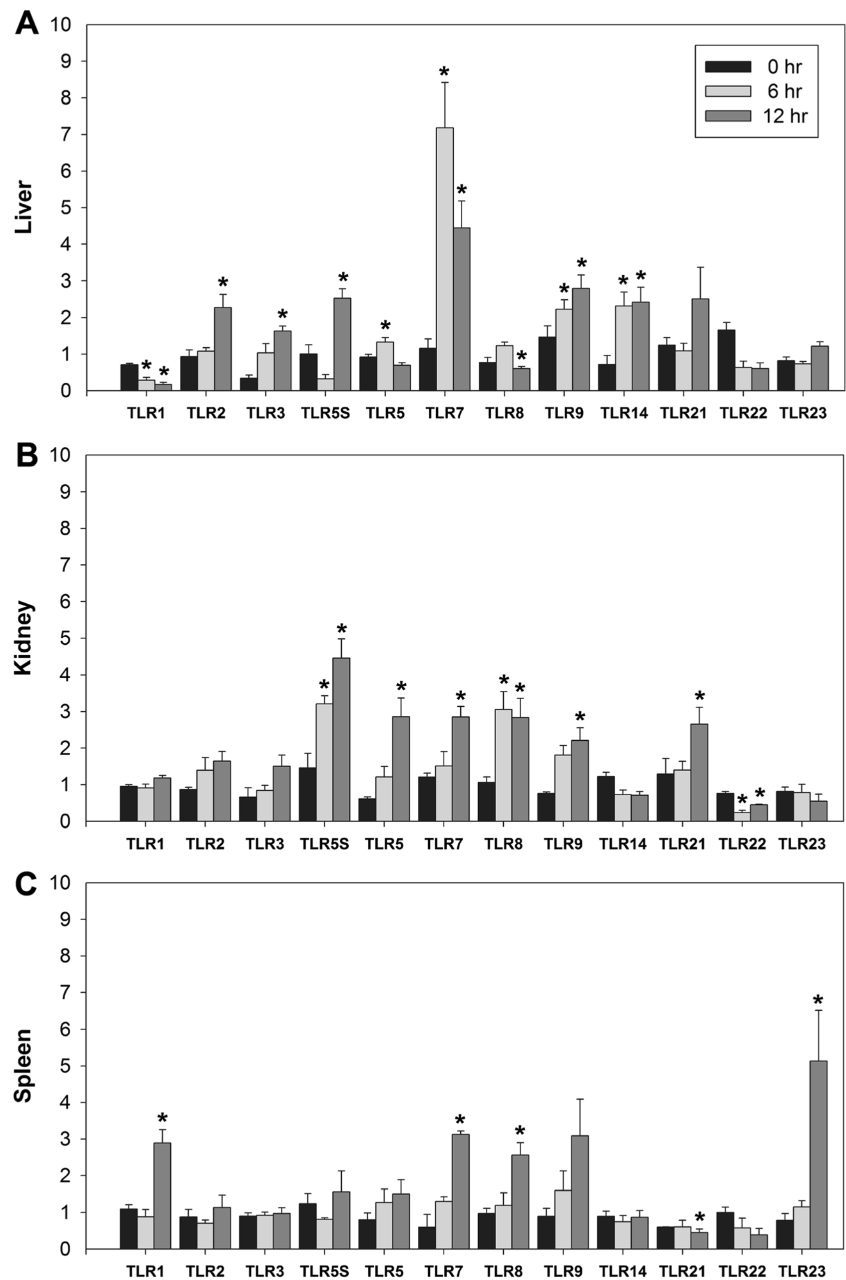

Fig. 5 Expression analysis of ncTLRs in different *Notothenia coriiceps* tissues following infection with a viral infection agonist. The **a** liver, **b** kidney, and **c** spleen of poly(I:C)-exposed groups were sampled after 0, 6, and 12 h progress. Three specimens were selected for total RNA isolation for quantitative real-time RT-PCR (i.e., $n=3$ per treatment). The β-actin gene of *N. coriiceps* was used as a reference to normalize the expression levels among samples. An *asterisk* (*) indicates values significantly different from zero time point ($p<0.05$)

and 23. This lineage's TLR families are perhaps under less purifying selective pressure than the other TLR families (Roach et al. 2005). ncTLR21, 22, and 23 have a close evolutionary relationship and may have diverged from the same ancestor. In this group, ncTLR23 was the most sensitive to pathogen ligand stimulation. They are predicted to diverged from the same lineage, but have different immune pathways. ncTLR23 might be associated with multiple PAMP-recognition cascades (Bricknell and Dalmo 2005).

N. coriiceps has evolved autonomously in the extreme Antarctic environment. The innate immune system is an evolutionary conserved defense mechanism in vertebrates, such as mammals and teleosts. Fish TLRs, however, exhibit distinct features and large diversity, likely a result of their diverse evolutionary history and the distinct environments that they occupy. In this study, we identified 12 TLRs in the Antarctic cod *N. coriiceps* and described their tissue-specific expression patterns before and after challenge with pathogen-mimicking agonists. However, further studies are required to fully elucidate the mechanisms regulating TLR signaling and to apply this information to increase our understanding of vertebrate immune system evolution.

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