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Molecular and functional characterization of Toll-like receptor (Tlr)1 and Tlr2 in common carp (*Cyprinus carpio*)

Inge R. Fink ^a, Danilo Pietretti ^a, Carlos G.P. Voogdt ^b, Adrie H. Westphal ^c, Huub F.J. Savelkoul ^a, Maria Forlenza ^a, Geert F. Wiegertjes ^{a,*}

^a Cell Biology and Immunology Group, Department of Animal Sciences, Wageningen University, PO Box 338, 6700 AH, Wageningen, The Netherlands

^b Department of Infectious Diseases and Immunology, Utrecht University, Yalelaan 1, 3584 CL, Utrecht, The Netherlands

^c Laboratory of Biochemistry, Wageningen University, PO Box 8128, 6700 ET, Wageningen, The Netherlands

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ABSTRACT

Toll-like receptors (TLRs) are fundamental components of innate immunity that play significant roles in the defence against pathogen invasion. In this study, we present the molecular characterization of the full-length coding sequence of *tlr1*, *tlr2a* and *tlr2b* from common carp (*Cyprinus carpio*). Each is encoded within a single exon and contains a conserved number of leucine-rich repeats, a transmembrane region and an intracellular TIR domain for signalling. Indeed, sequence, phylogenetic and synteny analysis of carp *tlr1*, *tlr2a* and *tlr2b* support that these genes are orthologues of mammalian TLR1 and TLR2. The *tlr* genes are expressed in various immune organs and cell types. Furthermore, the carp sequences exhibited a good three-dimensional fit with the heterodimer structure of human TLR1-TLR2, including the potential to bind to the ligand Pam₃CSK₄. This supports the possible formation of carp Tlr1-Tlr2 heterodimers. However, we were unable to demonstrate Tlr1/Tlr2-mediated ligand binding in transfected cell lines through NF-κB activation, despite showing the expression and co-localization of Tlr1 and Tlr2. We discuss possible limitations when studying ligand-specific activation of NF-κB after expression of Tlr1 and/or Tlr2 in human but also fish cell lines and we propose alternative future strategies for studying ligand-binding properties of fish Tlrs.

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1. Introduction

Pattern recognition receptors recognize widely-conserved motifs of pathogens and are crucial for initiating immune responses against invading microorganisms. Toll-like receptors (TLRs) are a family of germline-encoded pattern recognition receptors and known to activate rapid inflammatory responses upon detection of

their cognate ligands [1]. TLRs are type-I transmembrane proteins with numerous extracellular leucine-rich repeat (LRR) motifs which collectively form a horseshoe-shaped solenoid, which is responsible for ligand binding. The cytoplasmic part of each TLR contains a Toll/interleukin-1 receptor homology (TIR) domain which, upon dimerization of two TLRs, initiates a signalling cascade leading to activation of transcription factors such as NF-κB or AP-1, and subsequently to production of pro-inflammatory cytokines [2].

Most vertebrate genomes are recognized to have at least one gene representing each of the six major TLR families (TLR1, TLR3, TLR4, TLR5, TLR7 and TLR11) [3]. Also within the modern bony fish (Teleostei) the number of Tlr families generally is consistent with what is found for most (higher) vertebrates, although it is not unusual to find duplicated *tlr* genes due to several fish-specific whole-genome duplication events [4–8]. In addition to these duplications, some novel Tlrs seem to be “fish-specific”, such as a soluble form of Tlr5, and Tlrs 18–27 [9,10], indicating that an expansion of Tlrs has occurred during the evolution of teleosts. To date, little is known about the ligand specificities of individual Tlrs in fish, since the LRR

Abbreviations: BSA, bovine serum albumin; GFP, green fluorescent protein; IRAK1, interleukin-1 receptor-associated kinase 1; LRR, leucine-rich repeat; LTA, lipoteichoic acid; MYD88, myeloid differentiation primary response 88; NF-κB, nuclear factor kappa B; PAMP, pathogen-associated molecular pattern; Pam₃CSK₄, N-palmitoyl-S-(2,3-bis(palmitoyloxy)-(2R5)-propyl)-(R)-Cys-(S)-Ser-(S)-Lys₄; PBL, peripheral blood leukocytes; PBS, phosphate buffered saline; PE, phycoerythrin; PGN, peptidoglycan; RLU, relative light units; RT-qPCR, real-time quantitative polymerase chain reaction; TIR, toll/interleukin-1 receptor; TLR, Toll-like receptor; TRAF6, TNF receptor-associated factor 6; UTR, untranslated region; WGD, whole genome duplication.

* Corresponding author.

E-mail address: geert.wiegertjes@wur.nl (G.F. Wiegertjes).

ectodomains of Tlrs are not always highly conserved and sequence information alone cannot infer functional properties [10]. In apparent contrast to the variation in the ectodomains, intracellular TIR domains of fish Tlrs appear highly conserved and downstream signalling via well-described molecules such as MyD88, Irak1 and Traf6 identified in several fish species, suggest a conserved mechanism of innate immune signalling could exist [11]. Yet, studies into ligand-binding properties of fish Tlrs are essential to characterize their exact function within the immune system of fish.

The mammalian TLR1 family consists of TLR1, 2, 6, and also includes TLR10. TLR2 recognizes a variety of microbial components including lipoproteins/lipopeptides, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, parasite glycosylphosphatidylinositol anchors, and fungal zymosan (reviewed by Takeda et al. [1]). TLR2 functions as a heterodimer with either TLR1 or TLR6; the TLR2/TLR1 heterodimer recognizes a variety of triacylated lipoproteins [12], whereas the TLR2/TLR6 recognizes mycoplasma-derived diacylated lipoproteins [13]. TLR6 and TLR10 seem to have arisen as paralogs of TLR1 in the mammalian lineage, with TLR10 found in humans. Neither TLR6 nor TLR10 have been identified in genomes of any lower vertebrate, including teleosts. In fish, Tlr1 and Tlr2 were first identified in fugu [14] and zebrafish [15,16]. Subsequently, Tlr1 and/or Tlr2 have been described in several fish species; Japanese flounder (*Paralichthys olivaceus*) [17], channel catfish (*Ictalurus punctatus*) [18,19], rainbow trout (*Oncorhynchus mykiss*) [20,21], Tetraodon (*Tetraodon nigroviridis*) [22], orange-spotted grouper (*Epinephelus coioides*) [23], large yellow croaker (*Larimichthys crocea*) [24–26], and rohu (*Labeo rohita*) [27]. However, studies into ligand-binding properties of fish Tlr1 and/or Tlr2 molecules have been scarce.

We previously identified and characterized common carp (*Cyprinus carpio*) Tlr2 [28,29]. Transfection of human HEK293 cells with carp *tlr2* suggested the ability to bind the prototypical TLR2 ligands LTA, PGN and Pam₃CSK₄. Stimulation of carp macrophages with PGN induced *tlr2* gene expression, MAPK-p38 phosphorylation and led to an increased production of nitrogen and oxygen radicals. Here, we present the identification of Tlr1 and molecular characterization of the mRNA and genomic structure of both *tlr1* and *tlr2* from common carp. We compare the gene expression of *tlr1* and *tlr2* in the same tissue samples and purified cell populations and describe our efforts to characterize the function of putative Tlr1/TLR2 heterodimers by studying subcellular localization and ligand-binding properties. We discuss possible limitations when studying ligand-specific activation of NF- κ B after overexpression of Tlr1 and/or Tlr2 in human but also fish cell lines and propose alternative future strategies for studying ligand-binding properties of fish Tlrs.

2. Materials and methods

2.1. Animals

European common carp (*Cyprinus carpio* L.) were reared in the central fish facility Carus, at Wageningen University, Wageningen, The Netherlands. Fish were kept at 23 °C in recirculating UV-treated tap water and fed pelleted dry food (Sniff, Soest, Germany) daily. R3×R8 carp are the hybrid offspring of a cross between fish of Polish origin (R3 strain) and Hungarian origin (R8 strain) [30]. Carp were between 9 and 11 months old at the start of the experiments. All studies were performed with approval from the local animal welfare committee (DEC) of Wageningen University.

2.2. Organ isolation

Carp were euthanized with 0.3 g/L tricaine methane sulfonate

(TMS, Crescent Research Chemicals, Phoenix, AZ, USA) buffered with 0.6 g/L NaHCO₃. Carp were bled from the caudal vein using a needle and syringe containing cRPMI medium (RPMI 1640 with 25 mM HEPES (Lonza, Basel, Switzerland) adjusted to an osmolality of 280 mOsm/kg with sterile water) containing 50 U/mL heparin (Leo Pharma, Ballerup, Denmark), 50 U/mL penicillin G (Sigma-Aldrich, St. Louis, MO, USA), and 50 µg/mL streptomycin sulphate (Sigma-Aldrich). For isolation of peripheral blood leukocytes (PBL), the heparinized blood was centrifuged at 100g for 5 min at 4 °C and then another 5 min at 300g. The buffy coat was collected, carefully layered on Ficoll-Paque PLUS (GE Healthcare, Little Chalfont, UK) and centrifuged at 800g for 25 min at 4 °C without brake. The leukocyte layer was collected and washed twice with cRPMI. The obtained PBL were stored at –80 °C until used for RNA isolation. After bleeding the fish, the organs of interest were aseptically removed and immediately frozen in liquid nitrogen and stored at –80 °C until used for RNA isolation.

2.3. Isolation of leukocyte subtypes

Carp leukocyte subtypes were isolated by density gradient separation and/or magnetic cell sorting using specific antibodies as described before for thrombocytes [31], granulocytes [32], B cells [59,60], and macrophages [33]. In short, PBL or single-cell suspensions derived from carp organs were incubated with primary mouse monoclonal antibody: WCL-6 for thrombocytes (from blood), TCL-BE8 for neutrophils (from mid kidney), WCI-12 for B cells (from blood), and WCL-15 for monocytes/macrophages (from spleen). After incubation and washing, cells were stained with phycoerythrin (PE)-conjugated goat anti-mouse secondary antibody. After washing and counting of cells, magnetic beads (anti-PE MicroBeads, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were added and allowed to bind, before washing and magnetic separation on LS Midi Columns using a MidiMACS Separator (Miltenyi Biotec). Head kidney-derived macrophages were isolated and cultured as described by Joerink et al. [34].

2.4. RNA isolation

Total RNA from carp organs and leukocytes was extracted using the RNeasy Mini kit according to the manufacturer's protocol (Qiagen, Venlo, The Netherlands) including on-column DNase treatment with the RNase-free DNase set (Qiagen). Final elution was performed with 30 µL nuclease-free water. The integrity of the RNA was determined by agarose gel electrophoresis and the RNA quality and concentrations were assessed spectrophotometrically by measuring the absorbance at 260 nm and 280 nm (Nanodrop, Thermo Scientific, Waltham, MA, USA). RNA was stored at –80 °C until use.

2.5. cDNA synthesis

Prior to cDNA synthesis, 500 ng–1 µg of total RNA was subjected to an additional DNase treatment by using DNase I Amplification Grade (Invitrogen, Carlsbad, CA, USA). Synthesis of cDNA was performed with Invitrogen's SuperScript III Reverse Transcriptase, according to the manufacturer's instructions. As control for genomic contamination, for each sample a reaction without SuperScript III Reverse Transcriptase was performed. cDNA samples were diluted 25 times in nuclease-free water before use as templates in real-time quantitative PCR experiments.

2.6. Real-time quantitative PCR

Real-time quantitative PCR (RT-qPCR) was performed in a Rotor-

Table 1
List of primers used in this study. Restriction sites are italicized, Kozak sequence is shown in bold, sequence encoding a FLAG-tag is single underlined, and His-tag is bolded and italicized.

Oligo name	Sequence 5' → 3'	Purpose
CycaTLR1_FL_FW1	ATCTACAGCAGACGGAAG	Cloning of full-length <i>tlr1</i>
CycaTLR1_FL_RV4	TCTTGAAGCCCTGTGAAAG	Cloning of full-length <i>tlr1</i>
TLR1_pcDNA3_FW1	ACACGTCCTCCATTCTGGCCGATTACAAGGATGACGATGACAAGATTAAGAGGATCATAGTGAA	Cloning of full-length <i>tlr1</i> with FLAG-tag
TLR1_pcDNA3_FW2	ACGATGATCA AACATGG AGCCGTTGGACTGGTGGCTG	Cloning of full-length <i>tlr1</i> with FLAG-tag
TLR1_pcDNA3_FLAG	ACGATGATCA AACATGG AGCCGTTGGACTGGTGGCTGCTGTTTGTATGTCACATGTTCCAC ACGTCTCCCATTTCTGGCCGATTACAAGGATGACGATGACAAGATTAAGAGGATCATAGTGAA	Cloning of full-length <i>tlr1</i> with FLAG-tag
TLR1_pcDNA3_RV1	CAGTCTCGAGTTCTTCTCTCTGGGGAC	Cloning of full-length <i>tlr1</i> with FLAG-tag
TLR2_pcDNA3_FW1	GCTTCACTACTCCAGACACATCATCACCATCACCATTGTGATTGTGACCAGCAATA	Cloning of full-length <i>tlr2a</i> with His-tag
TLR2_pcDNA3_FW2	ACGAGGATCCA AACATGG AATTCTTGGGAAGAGAGCGCTCCATAATTATTTCATATTAATTTTG	Cloning of full-length <i>tlr2a</i> with His-tag
TLR2_pcDNA3_His	ACGAGGATCCA AACATGG AATTCTTGGGAAGAGAGCGCTCCATAATTATTTCATATTAATTTTG CCCAAGGCTTCACTACTCCAGGACACATCATCACCATCACCATTGTGATTGTGACCAGCAATA	Cloning of full-length <i>tlr2a</i> with His-tag
TLR2_pcDNA3_RV1	CATGCTCGAGACATTCATCTCTCTAGAGCAGC	Cloning of full-length <i>tlr2a</i> with His-tag
CycaTLR1_qFW1	AAAAGCGACCTTGACATTGC	RT-qPCR of <i>tlr1</i>
CycaTLR1_qRV1	GCTAACGGTGGCTAGGATTTC	RT-qPCR of <i>tlr1</i>
TLR2_qFW	TCAACA+CTCTTAATG+TGAGCCA ^a	RT-qPCR of <i>tlr2</i>
TLR2_qRV	TGTG+CTGGAAA+GGTTCAGAAA ^a	RT-qPCR of <i>tlr2</i>
40S_FW	CCGTGGGTGACATCGTTACA	RT-qPCR of carp 40S
40S_RV	TCAGGACATTGAACCTCACTGTCT	RT-qPCR of carp 40S

^a + in front of a nucleotide indicates that it contains an LNA modification.

Gene 6000 with a 72-well rotor (Corbett Research, Qiagen) with the Absolute QPCR SYBR Green Mix (Thermo Scientific) as detection chemistry as described previously [35]. All primers were from Eurogentec (Liège, Belgium). RT-qPCR data were analysed by Rotor-Gene 6000 Series Software 1.7. The melting temperature and profile of the melting curves were used to validate the specificity of the amplification. The gene expression was analysed using the average amplification efficiency for each primer pair and the take-off value of each sample (as derived from the Comparative Quantitation Analysis of the Rotor-Gene 6000 Series Software 1.7). The relative gene expression was calculated as the average amplification efficiency to the power of the take-off value. The gene expression of the housekeeping gene *40s ribosomal protein s11* was used to normalize the data. The sequences of primers used in this study are given in Table 1.

2.7. Cloning full-length *tlr1* from common carp

Carp head kidney leukocytes were obtained by density gradient separation. RNA was isolated as described above and then used as template in reverse transcription-PCR with degenerate primers designed on the basis of *tlr1* sequence information from other teleost fish. RACE (rapid amplification of cDNA ends) was then performed to obtain sequence at 5' and 3' ends. This allowed for design of carp primers in 5' and 3' untranslated regions (UTR), thus enabling the amplification of full-length carp *tlr1* sequence. For this, RNA from head kidney leukocytes was used as template in RT-PCR using the LongRange 2Step RT-PCR kit (Qiagen). Reverse transcription was done with an oligo-dT primer and the subsequent PCR was done with gene-specific primers located in 5' and 3' UTR (CycaTLR1_FL_FW1 and CycaTLR1_FL_RV4, see Table 1). The PCR product was subsequently cloned in pGEM-T Easy (Promega, Madison, WI, USA) and transformed into *Escherichia coli* JM109 competent cells (Promega). Clones were sequenced and sequence data were analysed with Sequencher version 4.10 (Gene Codes, Ann Arbor, MI, USA) and aligned with Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). A clone with the consensus sequence was used for further studies.

2.8. Bioinformatics

We obtained the coding sequence of the unique carp *tlr1* gene

after cloning (our sequence is identical to the recently deposited GenBank accession number LHQP01021877, Contig21898, cypCar_00044472, from whole genome shotgun sequencing). Cloning of *tlr2* was previously reported by our group [28]. Now referred to as *tlr2a*, the sequence has been updated in GenBank (accession number FJ858800) and the automatic annotation of the recently deposited LHQP01006764, Contig6769, cypCar_00005269 has been adjusted. A second *tlr2* sequence was identified in this study, predicted as genome sequence data (Bioproject PRJNA73579) [36], and partially confirmed as RNAseq data (unpublished data) and will be referred to as *tlr2b* (GenBank accession number LHQP01045997, Contig46054, cypCar_00039549 has been adjusted to reflect our manual annotation). Exon-intron structure was studied by multiple alignments and open reading frame predictions (FGENESH at <http://linux1.softberry.com/berry.phtml?topic=fgenes&group=programs&subgroup=gfind>, and GENSCAN at <http://genes.mit.edu/GENSCAN.html>). Nucleotide sequences were translated into protein sequence using the EMBL-EBI tool (<http://www.ebi.ac.uk/Tools/st/>) and these were aligned with Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). Molecular weights were calculated with ProtParam (<http://web.expasy.org/protparam/>). The protein sequences were examined for the presence of a signal peptide using SignalP (<http://www.cbs.dtu.dk/services/SignalP/>) and transmembrane regions were predicted using TMHMM Server v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>). Individual LRRs were identified by LRRfinder (<http://www.lrrfinder.com/>) and manually according to previous descriptions [37,38]. Synteny analysis was performed on the basis of sequence information from Ensembl release 82 (<http://www.ensembl.org/>). The multiple sequence alignment for the phylogenetic tree was made with ClustalX 2.1 [39] and the tree was constructed using the Neighbour Joining method with the number of bootstrap trials set to 10000. The phylogenetic tree was visualized with MEGA6.

2.9. *tlr1* and *tlr2* expression plasmids

Each of the genes encoding the fluorescent proteins GFP and mCherry were cloned into pcDNA3 using the *EcoRI* and *XbaI* sites. An *XhoI* site and a short linker sequence of 15 nucleotides encoding the amino acids GGSGG was placed upstream of the GFP and mCherry sequence. Subsequently, the full-length carp *tlr1* sequence

was modified from the above-mentioned pGEM-T Easy construct to include sequence to encode a FLAG-tag at the N-terminus (after the leader peptide), and to remove the stop codon, using primers TLR1_pcDNA3_FW1, TLR1_pcDNA3_FW2, TLR1_pcDNA3_FLAG and TLR1_pcDNA3_RV1 (see Table 1). The modified *tlr1* was then cloned between the *Bam*HI site and the newly created *Xho*I site in pcDNA3, thus creating a fusion of *tlr1*-GFP and *tlr1*-mCherry each in pcDNA3.

Given the high sequence similarity between *tlr2a* and *tlr2b*, for functional analysis, we proceeded with the previously characterized *tlr2a* construct [28]. This gene was sub-cloned into mCherry-pcDNA3 using primers TLR2_pcDNA3_FW1, TLR2_pcDNA3_FW2, TLR2_pcDNA3_His and TLR2_pcDNA3_RV1 (see Table 1) thereby creating a His-tagged fusion of *tlr2a*-mCherry. Furthermore, *tlr1* was sub-cloned into pBI-CMV1, a plasmid with two multiple cloning sites (MCS), where *tlr1* was cloned in MCS1 between *Bam*HI and *Pvu*II sites, and the GFP was no longer fused to the *tlr* sequence but subcloned in the MCS2 between *Eco*RI and *Xba*I sites. A similar construct was made for *tlr2a*. Isolation of transfection-grade plasmid DNA was performed with the S.N.A.P. MidiPrep Kit (Invitrogen) according to the manufacturer's instructions.

2.10. Subcellular localization of carp *Tlr1*, and co-localization with *Tlr2*

Human embryonic kidney 293 (HEK) cells were cultured at 37 °C at 5% CO₂ in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (Gibco), 2 mM L-glutamine (Merck, Darmstadt, Germany), 50 U/mL penicillin G (Sigma-Aldrich), and 50 µg/mL streptomycin sulphate (Sigma-Aldrich). HEK cells were seeded on untreated glass cover slips placed in 6-well plates, 4.5×10^4 cells each. The following day, cells were transfected with 0.5 µg *tlr1*-mCherry-pcDNA3, *tlr2*-mCherry-pcDNA3, mCherry-pcDNA3, or a combination of *tlr1*-GFP-pcDNA3 with *tlr2*-mCherry-pcDNA3 using jetPRIME (Polyplus Transfection, Illkirch, France) according to the manufacturer's instructions at a 1:3 ratio of plasmid:transfection reagent. Three days after transfection, cover slips were carefully washed with HBSS, cells were fixed for 20 min at room temperature with 4% paraformaldehyde, washed with HBSS and stained with wheat germ agglutinin-Alexa Fluor 488 conjugate (Molecular Probes, Invitrogen) for 15 min at room temperature, before final washing with HBSS and mounting with Vectashield (Vector Laboratories, Burlingame, CA, USA).

The Epithelioma papulosum cyprini (EPC) cell line, which is an adherent cell type derived from fathead minnow, was cultured at 27 °C at 5% CO₂ in RPMI 1640 (Lonza) supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 50 U/mL penicillin G, and 50 µg/mL streptomycin sulphate. EPC cells were seeded in 6-well plates, 8×10^5 cells/well, and allowed to adhere. EPC cells were transfected the following day with 2 µg *tlr1*-GFP-pcDNA3 using FuGENE HD Transfection Reagent (Promega) according to the manufacturer's instructions at a 1:3.5 ratio of plasmid:transfection reagent. Three days after transfection, EPC cells were detached with cold medium, fixed with 4% paraformaldehyde and washed with 1% BSA in PBS. Some samples were permeabilized with 0.1% Triton X-100 in 1%BSA/PBS while others were not permeabilized. Antibody staining was performed with mouse anti-FLAG primary antibody (1:200 dilution, Sigma-Aldrich) followed by Cy3-labelled donkey anti-mouse secondary antibody (1:100 dilution, Jackson ImmunoResearch, West Grove, PA, USA). Cells were mounted with Vectashield.

Cells were visualized with a Zeiss LSM-510 (Zeiss, Oberkochen, Germany) confocal laser scanning microscope with a Plan-Apochromat 63×/1.4 oil immersion objective. Green fluorescent signal (GFP or Alexa Fluor 488) was excited with a 488 nm argon laser and detected using a band-pass filter (505–550 nm). Red

fluorescence (mCherry protein or Cy3) was excited with a 543 nm helium-neon laser and detected using a long-pass filter (585 nm). Image processing was performed with ImageJ (<http://imagej.nih.gov/ij/>).

2.11. Structural model of heterodimer formation

The sequences of carp *Tlr1*, carp *Tlr2a/b*, human TLR1 and human TLR2 were used for sequence alignment using ClustalX [40]. Using these alignments and the available structure of the human TLR1/TLR2 complex, including a tri-acylated lipopeptide moiety (Pam₃CSK₄) and all sugar chains and water molecules (PDB-id: 2z7x) as templates, structural models were obtained for the carp *Tlr1*-*Tlr2a/b* complexes using the Modeller program (version 9.12) [41]. Thirty comparative models were generated, after which the model with lowest corresponding DOPE score [41] was selected for image generation.

2.12. Ligand binding

HeLa-57a cells (stably transfected with NF-κB luciferase reporter [42]) were cultured in DMEM with 5% foetal bovine serum (Bodinco, Alkmaar, The Netherlands) at 37 °C in 10% CO₂ atmosphere. Cells were transfected in 6-well plates with a combination of carp *tlr1* and *tlr2* plasmids, or human *tlr1* and *tlr2* [43] as positive control. All plasmids were transfected using FuGENE HD (Roche, Basel, Switzerland) in a DNA:FuGENE ratio of 1:3. A total of 2 µg plasmid DNA was transfected; when multiple plasmids were combined, equal amounts of each plasmid were used. To all combinations (except empty vector) a plasmid encoding human CD14 [43] was added. Twenty-four hours after transfection cells were redistributed from 6-well plates to 48-well plates and left to attach for 24 h. Cells were then stimulated with the following ligands: Pam₃CSK₄ (100 ng/mL), peptidoglycan (PGN, 10 µg/mL), and lipoteichoic acid (LTA, 1 µg/mL) which were purchased from Invivogen, San Diego, CA, USA. Stimulation was performed for 5 h, after which the cells were washed twice with PBS and then lysed with 100 µL reporter lysis buffer (Promega) and frozen at –80 °C. After 1 h cells were thawed at room temperature and 20 µL cell lysate was mixed with 50 µL Luciferase-6-reagent (Promega). Luminescence was measured with a Turner Designs TD20/20 luminometer. Results are expressed as relative light units (RLU).

3. Results

3.1. Identification and characterization of carp *tlr1* and *tlr2* genes

Information on *tlr1* and *tlr2* genes is available for some fish species but *tlr1* orthologues had not yet been identified in carp. In the process of identifying new genes in carp, it is often useful to compare with the closely related zebrafish, a species which has a very well annotated genome. Usually, the presence of two genes in carp versus a single gene in the genome of zebrafish is expected based on an additional whole genome duplication (WGD) event that has taken place in carp [36]. Thus, although we previously reported on the presence of a *tlr2* orthologue in carp, given the additional WGD event in the carp lineage, a second copy of the *tlr2* gene was expected.

Making use of a conventional cloning approach combined with information from the recently annotated carp genome we identified a single complete *tlr1* gene (Fig. 1) and an additional, but truncated, *tlr1* sequence in the carp genome (not shown). The full-length carp *tlr1* is composed of a single exon, similar to channel catfish, Tetraodon and fugu *tlr1* genes. Human and mouse *TLR1* are composed of four exons, but the entire coding region is contained in

[illegible]

Fig. 1. Alignment of Tlr1 protein sequences. The sequences from human, channel catfish, zebrafish and carp were aligned to show the conserved features and domains. The signal peptide is underlined, LRRs are highlighted in grey, transmembrane domain is indicated by a double underline, and the TIR domain is highlighted in black. Furthermore, LRRC2 cysteines are indicated with arrows.

a single exon. Also for zebrafish, *thr1* is divided over multiple (two) exons with the coding region contained in a single exon. The full-length coding sequence of carp *thr1* is 2394 bp and translates into a Thr1 protein of 797 aa with predicted molecular weight of 91 kDa. Thr1 has 20 leucine-rich repeats (LRRs) and a C-terminal LRR

(LRRCT), a transmembrane region and a highly conserved Toll/IL-1R (TIR) domain. Similar to other Tlr1 molecules [19], there is no N-terminal LRR (LRRNT).

We previously described a carp *tlr2* gene [28]. Upon further investigation of the carp genome we now identified an additional

tlr2 gene in the carp genome (Fig. 2). Both carp *tlr2* genes are composed of a single exon, similar to the human, zebrafish and channel catfish *tlr2* genes, whereas in some other fish species *tlr2* genes are encoded by multiple exons, e.g. Tetraodon and fugu *tlr2* each are comprised of 11 exons, confirming that the distribution and number of introns/exons among TLRs do not seem conserved across species [19]. The full-length sequences of carp *tlr2a* and *tlr2b* are 2367 and 2358 bp encoding for Tlr2 proteins of 788 and 785 aa with predicted molecular weights of 91 and 90 kDa, respectively. They share 88% amino acid identity. Both Tlr2 molecules have 20 LRRs, and in addition an N-terminal (LRRNT) and a C-terminal LRR (LRRCT), a transmembrane region and a highly conserved TIR domain.

Phylogenetic analyses on amino acid sequences of multiple Tlr1 and Tlr2 sequences (Fig. 3), using carp Tlr3 as an outgroup to root the phylogenetic tree, showed an overall topology indicating clusters of Tlr sequences consistent with evolutionary distance between different fish families. Sequences for both Tlr1 and Tlr2, from Cyprinids (carp, zebrafish) and Siluriforms (catfish), which are their closest living relatives, clustered together with very high bootstrap values, away from other fish species belonging to the Salmonids, Tetraodontiforms and Perciforms. All fish Tlr1 as well as Tlr2 sequences form common clusters separate from mammalian TLR1 and TLR2 sequences.

In the human genome, *TLR1*, *TLR6* and *TLR10* are organised as a conserved scaffold of genes. Clearly, in all fish genomes investigated so far, both *tlr6* and *tlr10* are missing, indicating a recent duplication of *TLR1* in the mammalian but not the teleost lineage. Conservation of synteny of teleost *tlr1* and *tlr2* was investigated by comparing the genomic regions immediately up- and down-stream of human *TLR1* (Fig. 4) and human *TLR2* (Fig. 5), with the genomic regions up- and down-stream of the corresponding homologue in the annotated genome of several teleost fish species, including zebrafish and common carp. For *tlr1*, the genomes of fish appear to have in common a block of 10 genes fairly conserved in the investigated fish species. For carp, although the scaffolds are limited in length, the analysis does confirm conservation of synteny with *ints10* downstream of *tlr1*, and *k1b* upstream of the putative *tlr1* pseudogene. The fact that the carp *tlr1* pseudogene shows conservation of synteny, supports the whole genome duplication event from zebrafish to common carp, with loss of function associated with the second *tlr1* gene in carp. For *TLR2*, in the human genome a block of 8 genes fairly conserved in the investigated fish species could be identified. However, there is a gap in zebrafish between the *tlr2* region and the *mf175/trim2* gene cluster further downstream. For carp, although the scaffolds are limited in length, the analysis does confirm conservation of synteny with zebrafish.

Altogether, sequence, phylogenetic and synteny analysis of carp *tlr1* and *tlr2* support that these genes are indeed orthologues of mammalian *TLR1* and *TLR2*.

3.2. Differential expression of *tlr1* and *tlr2* genes

To investigate the relative gene expression of the newly identified carp *tlr1* and to determine whether the newly identified *tlr2b* would present an expression pattern similar to the previously reported *tlr2a* sequence, we measured the constitutive gene expression of *tlr1*, *tlr2a* and *tlr2b*, in organs of four healthy carp and in sorted leukocytes. The results from 15 organs are shown in Fig. 6A. Significant differences were found between organs for all *tlrs* (one-way ANOVA, $P < 0.05$). Typically, highest *tlr* gene expression was found in head kidney and mid kidney, peripheral blood leukocytes, spleen and gut. The relatively high gene expression in immune organs, and low to non-detectable expression in other tissues was confirmed by transcriptome analysis of the same organs from carp

(unpublished data).

Detection of *tlr1*- and *tlr2*-specific gene expression in various immune cell types from carp (Fig. 6B), showed significant differences between cell types (one-way ANOVA, $P < 0.05$). In general, among immune cell types B cells, in particular, express high levels of *tlr* genes. Of interest, granulocytes express *tlr1* > *tlr2*, whereas head kidney-derived macrophages express *tlr2a* > *tlr1*, suggesting high expression of *tlr1* and *tlr2* is not always restricted to the same immune cell type.

3.3. Structural model of potential Tlr1/Tlr2 heterodimer

Mammalian TLR1 and TLR2 have been shown to heterodimerize and the crystal structure of the extracellular portion of human TLR1-TLR2 heterodimer with Pam₃CSK₄ as a ligand is known (PDB-id: 2z7x). Using the known human model, we set to investigate whether the identified carp Tlr1 and Tlr2 could potentially interact. In the human heterodimeric complex of TLR1-TLR2, the receptor multimer is arranged in a symmetrical manner. Three-dimensional modelling shows the typical horseshoe shape of each TLR with a well-positioned pocket to accommodate the tri-acylated lipopeptide ligand. Having noted that the number of LRRs is conserved between the human and carp molecules it was considered appropriate to model the carp Tlrs on their human counterparts. Thus, carp Tlr1 was modelled together with either carp Tlr2a (Fig. 7) or Tlr2b (data not shown). Both Tlr1-Tlr2 combinations exhibited a good three-dimensional fit with the heterodimer structure of human TLR1-TLR2, including the potential to bind to Pam₃CSK₄. This three-dimensional fit supports the possible formation of carp Tlr1-Tlr2 heterodimers.

3.4. Ligand stimulation of Tlr1 and Tlr2 does not lead to NF-κB activation

To study the biological activity and ligand binding properties of possible Tlr1-Tlr2 heterodimers we first created human HEK and cyprinid EPC cells stably transfected with a NF-κB luciferase reporter [44]. Both cell lines were subsequently transiently transfected with carp Tlr1 and Tlr2a, individually and combined. Each Tlr was cloned as a fusion to GFP/mCherry to monitor transfection efficiency. Stimulation with classical Tlr1-Tlr2 ligands including Pam₃CSK₄, LTA and PGN, however, did not lead to luciferase reporter activity (data not shown). Alternatively, we used HeLa-57A cells transfected with various *tlr1/tlr2* expression plasmids, which however gave a similar outcome (Fig. 8): none of the tested ligands led to a significant increase in NF-κB activation via overexpressed carp Tlr1-Tlr2. To rule out any inhibitory effect of fusing GFP to the C-terminal end of the Tlr molecules, we also used pBI-CMV1 constructs in which GFP was placed in a cloning site separate from the Tlr. This still allowed for detection of transfection levels by GFP, whilst leaving the structure and function of the Tlr unaffected, but did not alter the negative outcome. Furthermore, in order to rule out an inhibitory effect of GFP itself (whether fused or not to the Tlrs), mCherry constructs were used instead of GFP. Again, no increase in NF-κB activity after ligand stimulation could be measured. Since the positive control; HeLa-57A cells transfected with human TLR1 and TLR2, did lead to high responses to Pam₃CSK₄, LTA and PGN (see Fig. 8), we conclude that both the reporter system itself and the ligands used, were functional.

3.5. Carp Tlr1 and Tlr2 are expressed and co-localize

Since we could not confirm NF-κB activation by over-expressed carp Tlr1 and Tlr2 stimulated with ligands, we next investigated whether this could be due to erroneous expression of these

		↓ ↓	
<i>H. sapiens</i>	-----MPHTLWMVWVGLVVISLSKESSNQASLSCDRNGICKSGSSGLNSIPGLTEAV		54
<i>I. punctatus</i>	MKLSLSVVICLSLAWTR-----AQTSDRPNCECEDHFCNSAKTLHRVPIV-PASV		52
<i>D. rerio</i>	MRLVGTMTAILIMFIL-----AGGLECSQTCCKDQMYFNCSSNNLHQVPTV-PSDV		52
<i>C. carpio a</i>	MQFLGREASIIIFILIL-----AQGFHYSTRCTCDQQYFNCSSNNLHQVQPKV-PANA		52
<i>C. carpio b</i>	MRPLEAKKSVILFLLIL-----AQGFYSWTNCNQDQYFNCSSNNLHQVQPKV-PAND		52
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<i>H. sapiens</i>	KSLDLSNNRITYISNSDLQRCVNLQALVLTSGINTIEEDSFSSLSGLEHLDLSYNYLSN		114
<i>I. punctatus</i>	LSLDVSFNEIESVTKKDLAAYTALRTKLQENELSAHKDAFHSQSKLEELDLSFNKLEN		112
<i>D. rerio</i>	LGLDLSFNQIESINMTDLSYNYELIILNLHKNKLRIHHRDAFHSQHNLEVLDSLNNLN		112
<i>C. carpio a</i>	LGLDLSFNQIESIDMSDLSYSELKTLNLHKNKLKFIHKDAFHSQHKLEVLDSLNNLN		112
<i>C. carpio b</i>	LCLDLPFNQIEAIDMNDLSYSKLTKTLNLHKNKLKFIHKAFKSHKLEVLDSLNNLEK		112
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<i>H. sapiens</i>	LSSSWFKPLSSLTFLNLLGNPYKLTGETSLFSHLT--KLQILRVGNMDFTKIQRKDFAG		172
<i>I. punctatus</i>	ISSLWFSSLRSLKHLNIGNRYTTLGAVGLFQFVEDPALRTLRFGNP-SIEDVRRNMLNK		171
<i>D. rerio</i>	LSPSWFHKLSLQQLNLVGNPYTVGPAPIFSSL--VNLRTLHLGSP-SLRLEHKNGLDV		169
<i>C. carpio a</i>	LSSSWFQELNSLQQLNLVGNPYTVGPAPIFQSL--LNLRLRFGSP-SLREVKNGLDG		169
<i>C. carpio b</i>	LSSSWFHELSLQQLNLVGNPYTVGPAPIFQSL--LNLRLRFGSP-SLREVKNGLDG		169
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<i>H. sapiens</i>	LTFLEELIDASDIQSYEPKSLKSIQNVSHLILHMKQ----HILLEIFVDVTSSVECL		227
<i>I. punctatus</i>	IRQLDELTFVGGKLSYESSGFKTAQPIRVVSLSLQGLFQDDPALVSKILRDVSHPETSL		231
<i>D. rerio</i>	LTHLDEMTFFGNSLRYSYENGSLKAARPTGSVLSLQNLFESDPFLVSKVLQDVSHPETLL		229
<i>C. carpio a</i>	LTHLDEMTFVGSNLRYSYENGSLKAARPTGLVLSLQNLFQNNPELVSKVLQDVSHPETLL		229
<i>C. carpio b</i>	LTHLDEMTFVGSNLRYSYENGSLKAARPTSLVLSLQNLFQNDPELVSKVLQDVSHPETLL		229
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<i>H. sapiens</i>	ELRDTDLDTFHFSELSTGETNSLIKKFTFRNVKITDES LFQVMKLNQISGLLEFDDC		287
<i>I. punctatus</i>	TVRDVSLTREPISQFKEVREGCTRLTFQNASTTDEGVTHLLEVLGD-SPMSYLGLEDI		290
<i>D. rerio</i>	IIKDVMTKNTSTEPFKMVEGKTSLTQNSSTTDQALTSFLEFMDG-SPLSFIGLEDI		288
<i>C. carpio a</i>	IIKDVMTKNTSTEPFKSAREGGTKSLTQNSSTTDEAMTSLMVMGD-SLSYIGLEDV		288
<i>C. carpio b</i>	TIKDVMTKNTSTESFKATREGGTSLSLHNCITDEALTSFLMVMGD-SLSYIGLEEV		288
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<i>H. sapiens</i>	TINGVGNFRASDNDRIIDPGKVELTIRRLHIFRYLFYDLSTLYSLTERVKRITVENS		347
<i>I. punctatus</i>	HLVGTGSWEKARW---THFENLHTELRNVEIQGFREFSSMIQLAFLLKHLTKISVNNAT		347
<i>D. rerio</i>	QFVGIGEWQKAKY---THHDSLRATYLRNIEIEGFFGFSMIELGFLKHLHNVSVINAT		345
<i>C. carpio a</i>	HLIGQGWQKASY---THYESLHTAYLRNIDIEGFFFRFNSMMQLGFLVHLHKVSVINGT		345
<i>C. carpio b</i>	HLIGQGWQKASY---THYENLHTAYI---DIQGFREFSSMKQLGFLVHLHKVSVINGT		342
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<i>H. sapiens</i>	VFLVPCSLLSQHLKSEYLDLSENLMVEEYLNKNSACE--DAWPSLQTLILRQNHLSLEKT		405
<i>I. punctatus</i>	VFLVPCSTTYFLQKVEYLDLSONLLSDITIQESLNCNGDKMMDHLTNVSHNSLSLQVL		407
<i>D. rerio</i>	VFLVPIKETTFLLKNLEYMDLSONLLTDLTITQPTLYTGSGAYQNLNMLNVSQNVLSGLGM		405
<i>C. carpio a</i>	VFLVPIRETTPLLKNLEYMDLSONLLSDLTIEPTLYTGFGAYQNLNMLNVSQNVLSGLGM		405
<i>C. carpio b</i>	VFLVPIPTTVLLKKLYLDLSONLLSDLTITAPTLYTGFGAYQNLNMLNVSQNVLSGLGM		402
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<i>H. sapiens</i>	GETLLTLKNLTNIDISKNSFHSMPETCQWPEKMKYLNLSSTRIHVSVTGCIPTLEILDVS		465
<i>I. punctatus</i>	SRIVTRLRLHRLTSLDVSHNDPLKMPESCWPASLRFMNLATKLHRTPLPVSITVLDLS		467
<i>D. rerio</i>	SRIVTNLKLKLYLDLSYNSFVSMPEKCSWPVTLRFLNLSSTKSLTTPCLPSSLTVDLS		465
<i>C. carpio a</i>	SRLATKLKSLTYLDLSHNSFVSMPEKCSWPATLRLFLNLSSTKRLKLTPLPSSLTALDLS		465
<i>C. carpio b</i>	SQLATNLKSLTYLDLSHNSFVSMPEKCSWPATLRLFLNLSSTKRLKMTPLPSSLTVDLS		462
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<i>H. sapiens</i>	NNNLNLSNLPQLKELYISRNKMLTPDASLLPMLLVLKISRNAITTSKEQLDSFHTL		525
<i>I. punctatus</i>	QNYLTFEHLQHLPSLMLWLTGNRFISLPEGGWPSLRTLLIQSNTLMFNKSDLMFAQSL		527
<i>D. rerio</i>	ENDLKAFKQRFPHLTTLTILGNRLMKLPDGLFPLSNTLLIQNALRMFNQSSLSRKFCTL		525
<i>C. carpio a</i>	ENDLMVFENQRFPLTTLTILGNRFMKLPQGLFPRLQTLTIQNALRMFNQNDLRKFCTL		525
<i>C. carpio b</i>	ESDLMVFENQRFPLTTLTILGNRFKLPQGLFPRLQTLTIQNALRMFNRSNDRNFKNL		522
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<i>H. sapiens</i>	KTLEAGNNFICSGEFLSFTQEQALAKVLIDWPANYLDCSPSHVRGQQVQDVRLSVSEC		585
<i>I. punctatus</i>	QVLEAGNNFVCSQDFVEFFTGRIIDLITLRDGHRSYVCDSPFTLRGAVDNARLSVFEC		587
<i>D. rerio</i>	LYLEAGANNFVCSQKFVSFFKQDVEDLITLQDGRQNYVCDTPFTLRGNAIDSVRLSVFEC		585
<i>C. carpio a</i>	QVLEAGNNFVCSQEFVSFFRHVDHFTITRDNRRNYVCDTPFTLRGDAVDSVRLSVFEC		585
<i>C. carpio b</i>	QVLEAGNNFVCSQKFVSFFKQDVLFITLRDSRRNYVCDTPFTLRGDSIDSVRLSVFEC		582
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<i>H. sapiens</i>	HRTALVSGMCCALFLLILLTGVLCHRFHGLWYMKMMWAWLQAKRKPRA-PSRNICYDAF		644
<i>I. punctatus</i>	HMILSVSLCSAIVLVLTAGVTCYKHLVLYLQMTLAWLRAKSPAVRSGGADLYDAF		647
<i>D. rerio</i>	YMIPAVSVLCFGITITAGLVLTCHKLHVLYLQMTKAWIQAQRKPAVGRLEPELYDAF		645
<i>C. carpio a</i>	YMIPAVSVLCGLIITVLGLIVVTCYKFIHWLYLHMTKAWIQAQRKPAVGRLEPELYDAF		645
<i>C. carpio b</i>	YMIPAVSVLCVITITAGLVVTCYKHLHVLYLQMTKAWIQAQRKPAVGRLEPELYDAF		642
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<i>H. sapiens</i>	VSYSERDAYVENVLMQVLENFNPPFKLCLHKRDFIPGKWIIDNIIDSIEKSHKTVFVLS		704
<i>I. punctatus</i>	VSYSQHDAEWVEEILVPKLESSEPPVTLCLHKRDFLPGRWIVDNIIESIESSHRTLFVLS		707
<i>D. rerio</i>	VSYSQHDAEWVEEILVALEDTQPSFSLCLHKRDFRPGRWIVDNIIDSIEKSHRTLFVLS		705
<i>C. carpio a</i>	VSYSQHDAEWVEEILVPELSAHPFPALCLHKRDFQPGRWIVDNIIDSIEKSHRTLFVLS		705
<i>C. carpio b</i>	VSYSQHDAEWVEEILVPELSAHPFPALCLHKRDFQPGRWIVDNIIDSIEKSHRTLFVLS		702
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<i>H. sapiens</i>	ENFVKSEWCKYELDFSHFRIVDEHNDSAVLVLEPIKETIPKRECKLRKIMNSRTYLEW		764
<i>I. punctatus</i>	ENFVTSEWCKYELDFSHFRIVDEHNDSAVLVLEPIKETIPKRECKLRKIMNSRTYLEW		767
<i>D. rerio</i>	EHFVSEWCKYELDFSHFRIMDEHNDSAVLVLEPIKETIPKRECKLRKIMNSRTYLEW		765
<i>C. carpio a</i>	EHFVTSEWCKYELDFSHFRIVDEHNDSAVLVLEPIKETIPKRECKLRKIMNSRTYLEW		765
<i>C. carpio b</i>	EHFVTSEWCKYELDFSHFRIVDEHNDSAVLVLEPIKETIPKRECKLRKIMNSRTYLEW		762
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<i>H. sapiens</i>	PMDEAQREGFWNLRAAIKS--- 784		
<i>I. punctatus</i>	PHDEEKREVFQNLRAALKREHR 790		
<i>D. rerio</i>	PEDEKREDFWNLRAALQORDEC 788		
<i>C. carpio a</i>	PEDEKREDFWGLRAALQORDEC 788		
<i>C. carpio b</i>	PEDEKREDFWGLRAALQORDEC 785		
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Fig. 2. Alignment of Tlr2 protein sequences. The sequences from human, channel catfish and zebrafish were aligned with both carp Tlr2 sequences to show the conserved features and domains. The signal peptide is underlined, LRRs are highlighted in grey, transmembrane domain is indicated by a double underline, and the TIR domain is highlighted in black. Furthermore, LRRNT and LRRCT cysteines are indicated with arrows.

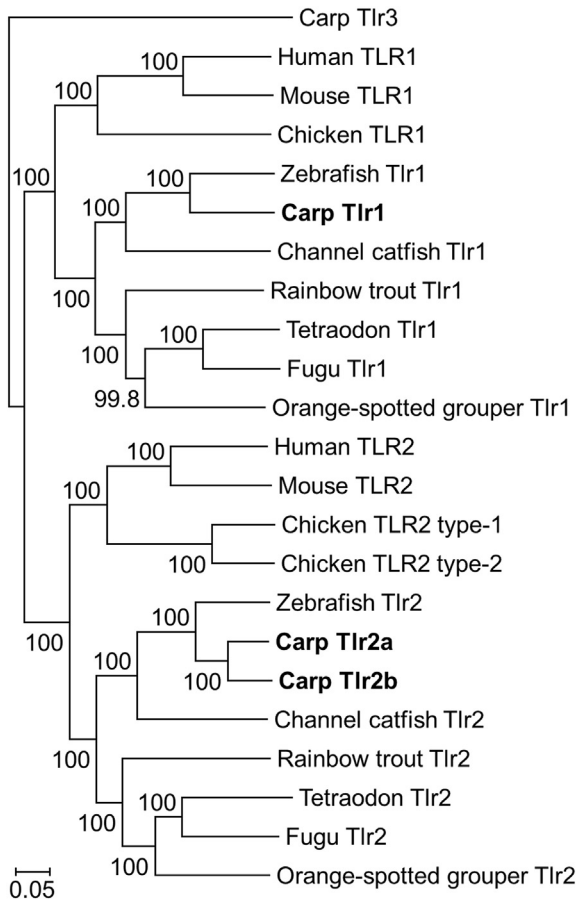


Fig. 3. Phylogenetic tree of Tlr1 and Tlr2 protein sequences. The phylogenetic tree shows the evolutionary relationship between Tlr1 and Tlr2 proteins of various species. All branches have high bootstrap values. The sequence of carp Tlr3 was used as an outgroup to root the tree. Amino acid sequences used for Tlr1: Human (*Homo sapiens* NP_003254.2), mouse (*Mus musculus* NP_109607.1), chicken (*Gallus gallus* NP_001007489.4), zebrafish (*Danio rerio* AAI63271.1), carp (*Cyprinus carpio* cypCar_00044472), channel catfish (*Ictalurus punctatus* AEI59662.1), Tetraodon (*Tetraodon nigroviridis* ABO15772.1), fugu (*Takifugu rubripes* XP_003970412.2), orange-spotted grouper (*Epinephelus coioides* AEB32452.1), rainbow trout (*Oncorhynchus mykiss* ACV92064.1). Amino acid sequences used for Tlr2: Human (*Homo sapiens* AAH33756.1), mouse (*Mus musculus* NP_036035.3), chicken type 1 (*Gallus gallus* NP_989609.1), chicken type 2 (*Gallus gallus* NP_001155122.1), zebrafish (*Danio rerio* NP_997977.1), carp a (*Cyprinus carpio* cypCar_00005269), carp b (*Cyprinus carpio* cypCar_00039549), channel catfish (*Ictalurus punctatus* AEI59663.1), Tetraodon (*Tetraodon nigroviridis* ENSTNIP00000005681), fugu (*Takifugu rubripes* XP_003976919.1), orange-spotted grouper (*Epinephelus coioides* AEB32453.1), rainbow trout (*Oncorhynchus mykiss* CCK73195.1). Amino acid sequence of carp Tlr3 (*Cyprinus carpio* AHE74141.1).

molecules. In a previous study, carp Tlr2 was successfully transfected into human HEK cells, so we continued with first transfecting this cell line with constructs expressing each carp Tlr fused to the fluorescent protein mCherry. The cell membrane was visualized by staining with wheat germ agglutinin-Alexa 488 conjugate which exhibits green fluorescence. Confocal microscopy confirmed expression of Tlr1, and Tlr2a, at least in the cytoplasm of the cells mostly concentrated in discrete speckles (Fig. 9). For comparison, mCherry protein alone was expressed without being fused to a Tlr and red fluorescence was evenly distributed throughout the cells, indicating that the staining pattern observed when fused to a Tlr is determined by the Tlr itself. Upon further examination of subcellular co-localization of Tlr1 and Tlr2, using carp Tlr1 fused to GFP and carp Tlr2a fused to mCherry, it became clear that both carp Tlr1 and Tlr2a co-localized in the cytoplasm of HEK cells (Fig. 10). To

confirm the suspected cytoplasmic localization, carp Tlr1 fused to GFP was detected using anti-FLAG-specific antibody. Only in permeabilized cells, carp Tlr1 could be detected with antibody staining, confirming that Tlr1 was not expressed on the surface of HEK cells overexpressing Tlr1 (Fig. 11). To exclude that the observed expression pattern was not an artefact due to the use of mammalian cells grown at 37 °C, we performed the same experiment using various fish cell lines grown at lower temperature. Similar results were obtained with EPC and CLC cells transfected with the same Tlr constructs (data not shown). Altogether, these data demonstrate that carp Tlr1 and Tlr2 can be both successfully (over)expressed *in vitro* in homologous and heterologous cell lines, and they appear to be present in the same subcellular compartments of these cells.

4. Discussion

Ever since TLRs were first described as important pattern recognition receptors for mice and humans, they have been studied extensively in an ever increasing number of animal species, including invertebrates such as sea urchin, where a great expansion of *tlr* genes has occurred [45]. The abundance of Tlrs in sea urchin suggests this class of receptors plays an important role in the innate immune defence also in lower animals. Often, the conserved nature of Tlrs is emphasized, with particular emphasis on the extracellular leucine rich repeat regions and the intracellular TIR domain. In this study, we present the molecular characterization of *tlr1* and *tlr2* from common carp and discuss our attempts to determine the ligand-binding properties of putative Tlr1/Tlr2 heterodimers.

Sometimes, after a WGD, gene duplication allows for partitioning of function or acquiring of new function, whereas in other cases the duplicated gene can lose its function. A single full-length carp *tlr1* gene could be identified; a second *tlr1* hit was truncated with a partial TIR domain only. Many of the orthologous relationships of TLRs can be confirmed by observations of conserved synteny, i.e. preservation between species of the order and orientation of orthologous genes [3]. Based on the conserved order of the *klb* gene neighbouring the second *tlr1* hit we consider the truncated *tlr1* a true orthologue of *TLR1*, but one which has lost its function. In rainbow trout a similar situation exists; a single full-length *tlr1* gene is present on chromosome 14 and a *tlr1* pseudogene is located on chromosome 25 [20]. Thus, it appears that only a single full-length *tlr1* gene remained in the genomes of rainbow trout and common carp. In contrast, two full-length genes for carp *tlr2* could be identified, both of which give rise to detectable transcripts. Also rainbow trout express two *tlr2* genes, one of which is contained within a single exon but the other segmented into multiple exons [21]. Of interest, a similar situation has recently been reported for yellow croaker [26], suggesting more fish species might have *tlr2* genes with quite different intron-exon organisations. Zebrafish *tlr2* and also channel catfish [19], similar to human *TLR2*, is intronless. Likewise, we have not found evidence for the presence of a segmented *tlr2* gene in carp.

High expression of *tlr1* and both *tlr2* genes was found in immune organs such as head kidney and mid kidney, peripheral blood leukocytes, spleen and gut. Of the two *tlr2* genes present in carp, *tlr2a* was always higher expressed than *tlr2b* in all the investigated organs and cell types. Leukocytes of both myeloid and lymphoid origin expressed *tlr1* and *tlr2*; B cells, granulocytes, and head kidney-derived macrophages had the highest expression levels. Also rainbow trout *tlr2* is expressed in mononuclear cells/macrophages at levels similar to that in B cells [21], *tlr1* gene expression was not studied in rainbow trout. In mammals, B cells as well as cells of myeloid origin express TLRs [46,47], suggesting a conserved expression pattern for carp *tlr1* and *tlr2* on phagocytes and B cells.

Our three-dimensional modelling of the carp Tlr1 and Tlr2

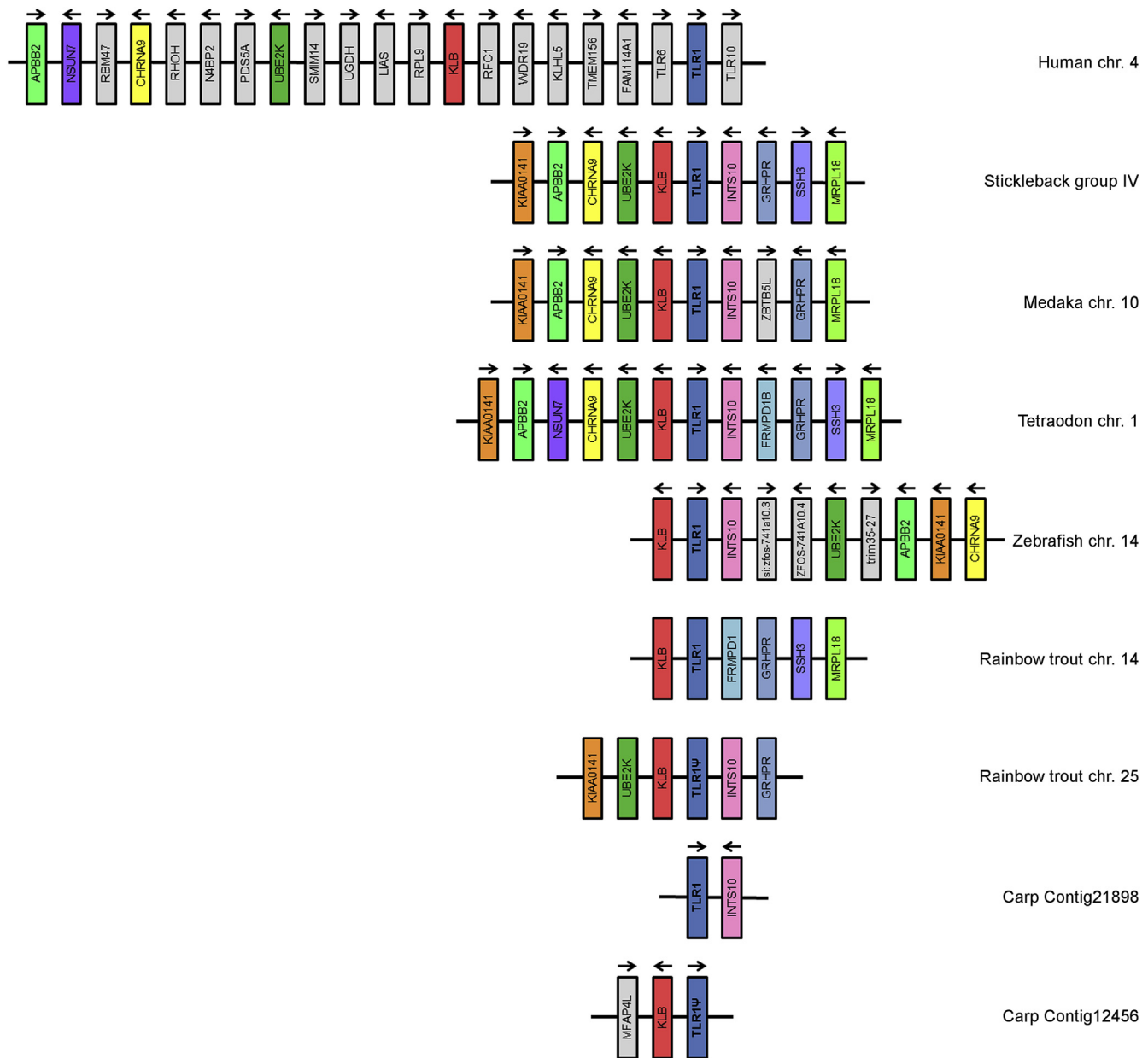
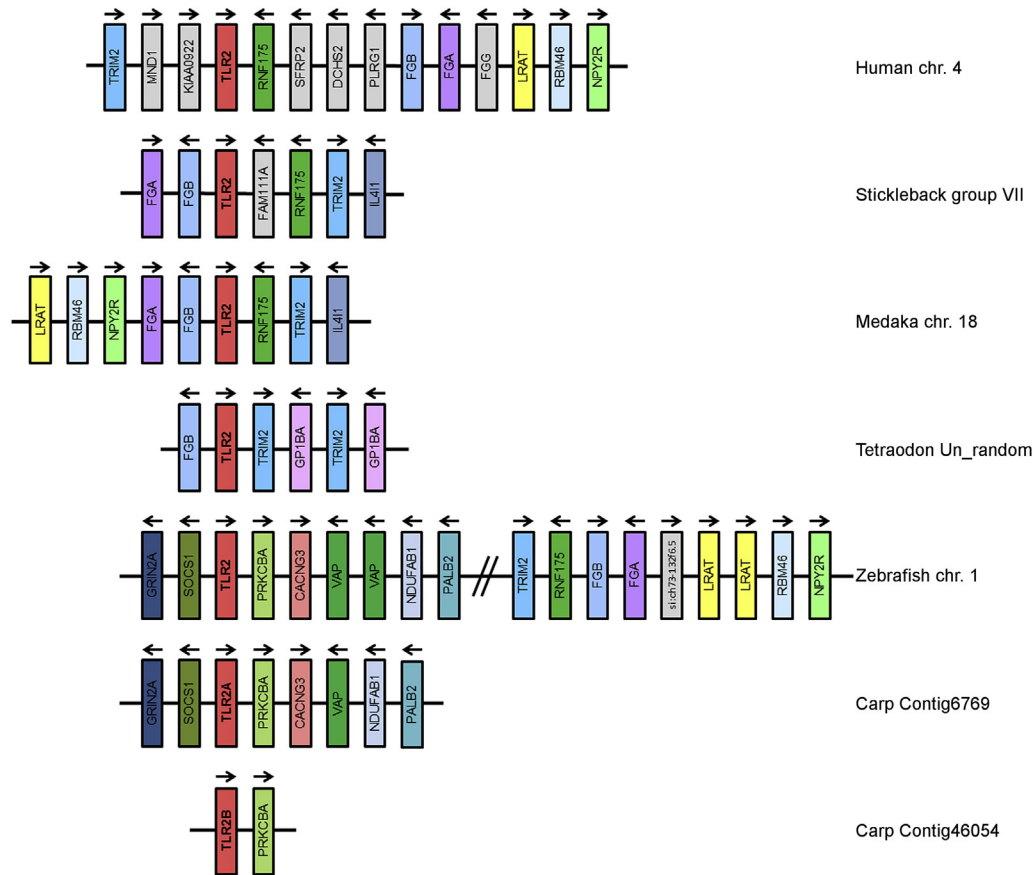


Fig. 4. Synteny analysis of *tlr1*. Comparative gene organization map of the regions where human, stickleback, medaka, Tetraodon, zebrafish, rainbow trout and carp *tlr1* genes are found. Orthologous genes are indicated by the same colour, except unique genes which are all light grey. Gene direction is indicated with arrows. Rainbow trout and carp have a pseudogene (ψ) in addition to the full-length *tlr1* gene. The human genome assembly version GRCh38.p3, stickleback genome assembly BROAD S1, medaka genome assembly HdrR, Tetraodon genome assembly TETRAODON 8.0, zebrafish genome assembly GRCz10, and the carp genome [36] were used for this analysis. Information on rainbow trout genomic organization is from Palti et al. [20].

proteins suggests the possibility of formation of a heterodimer, with a pocket that can accommodate the tri-acylated lipopeptide ligand Pam₃CSK₄, similar to the heterodimer structure of human TLR1-TLR2 on which the model was built. Both Tlr2 proteins of carp appear structurally capable of heterodimer formation with carp Tlr1. The *in silico* modelling supports the potential formation of heterodimers of carp Tlr1-Tlr2 *in vitro* or *in vivo*. Indeed, in cell lines of human as well as fish origin overexpressing both proteins, confocal microscopy confirmed subcellular co-localization of carp Tlr1 and Tlr2. Further experimental evidence, such as co-immunoprecipitation or fluorescence resonance energy transfer (FRET) [48,49], would be needed to unequivocally prove molecular interaction between the two Tlr proteins. The work by Sandor et al.

[50] demonstrates that human TLR1 and TLR2 co-localize in double-transfected (TLR1-YFP + TLR2-CFP) HEK cells in a ligand-independent manner, similar to our observations for carp Tlr1 and Tlr2. The pre-assembled human TLR1-TLR2 heterodimers are present inside as well as on the surface of cells.

Possibly because over-expression affects the natural molecular distribution, or because only minute and therefore difficult to detect amounts of Tlrs are required at the cell surface, we were unable to pinpoint sub-cellular localization to the cell surface. This may also be true for human TLR1 when over-expressed in HEK cells, because TLR1 is hard to detect at the cell membrane and is found mainly localized inside the cells with a diffuse pattern of distribution [50]. Further, the endogenous cytoplasmic pool of TLR1 in HEK



cells can be detected in permeabilized cells only, and human TLR2 is not found exclusively at the cell membrane either [50]. Also chicken TLRs cannot easily be detected on the cell surface after transfection (unpublished data). Yet, both human and chicken TLRs, when over-expressed, can lead to NF- κ B activation upon stimulation with appropriate ligands. These collective findings suggest that, although it is not easy to detect TLR molecules at the cell surface after transfection, intracellular localization of carp Tlr1 and Tlr2 may not necessarily explain the absence of NF- κ B activation in our experiments.

In mammals, TLR2 does not only form heterodimers with TLR1, but also with TLR6 (and TLR10 in humans). In fish genomes, *tlr6* or *tlr10* genes are absent but instead a number of fish-specific Tlrs have been recognized as members of the Tlr1 family. It is interesting to speculate that Tlr2 in fish might heterodimerize with some of these members, including Tlr14, Tlr18, Tlr25 and Tlr27

In previous work, we have described ligand-specific activation of carp Tlr2 via measurement of increased phosphorylation levels of the MAP kinase p38 in human HEK cells by Western blot [28]. Instead of this semi-quantitative method, we now used a quantitative read-out system based on NF- κ B activation and subsequent luminescence measurements. We could not confirm our initial ligand binding studies based on phosphorylation of p38, neither using the human HEK or HeLa-57A cell lines, nor using a cyprinid fish cell line (EPC), from fathead minnow [54]. Of interest, the HeLa-57A cells have successfully been used to study ligand binding of several chicken TLRs [43] and even reptilian TLR5 [55], confirming that human cells can be used to study ligand binding by TLRs from non-mammalian species. There are several possible explanations for the inability of carp Tlr1 and/or Tlr2 to induce activation in our NF- κ B reporter assay; i) carp TLRs may not have been functioning optimally because they did not display the correct sub-cellular localization on the cell surface, e.g. due to over-expression, ii) carp TLRs were not properly folded e.g. due to high temperature conditions for human cells, although this would not be the case for the fish cell line grown at 27 °C, iii) the carp TIR domains may not

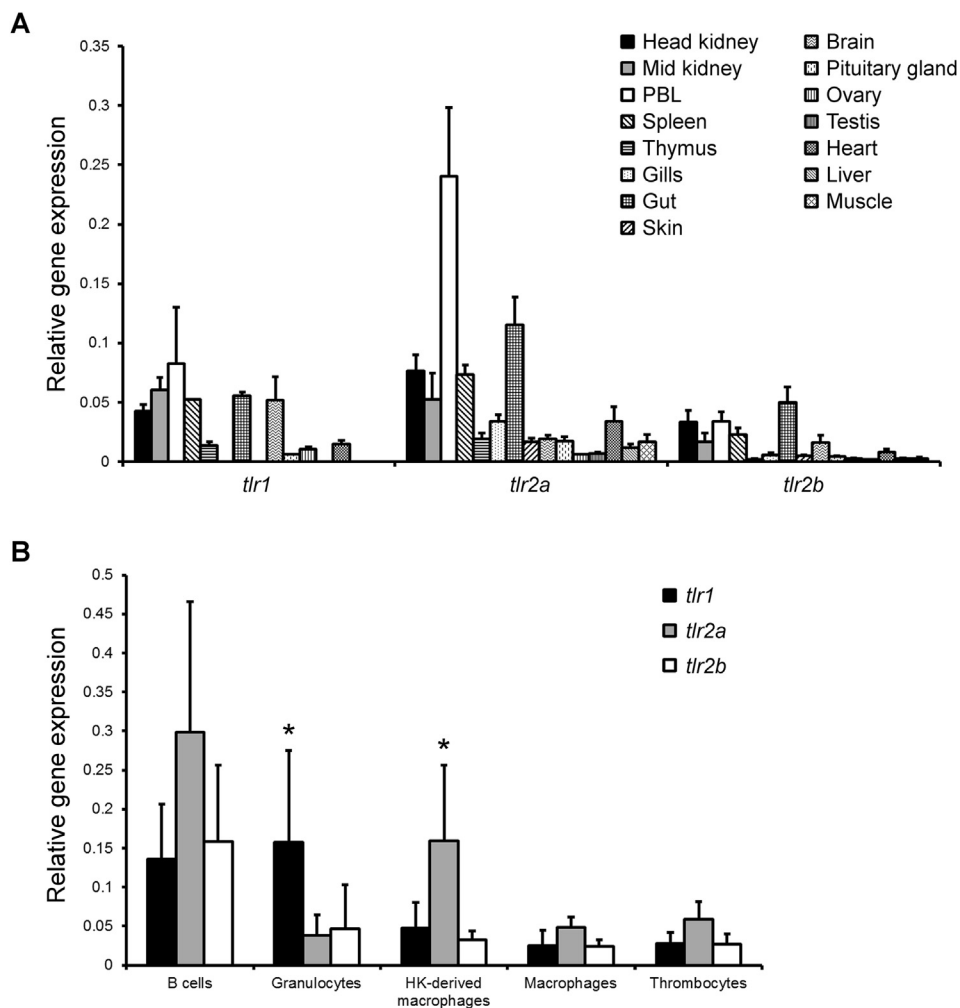


Fig. 6. Basal gene expression levels of *tlr1*, *tlr2a* and *tlr2b* in carp organs and immune cell types. Real-time quantitative PCR was performed using primers specific for carp *tlr1*, *tlr2a* and *tlr2b* on cDNA from (A) organs or (B) immune cell types isolated from healthy fish. Gene expression is normalized to the expression of the 40s ribosomal protein *s11* housekeeping gene. Bars indicate average gene expression of $n = 4$ adult fish and error bars indicate standard deviations. Asterisks denote significant differences in expression between *tlrs* within a cell type, $P < 0.05$. PBL = peripheral blood leukocytes. HK = head kidney.

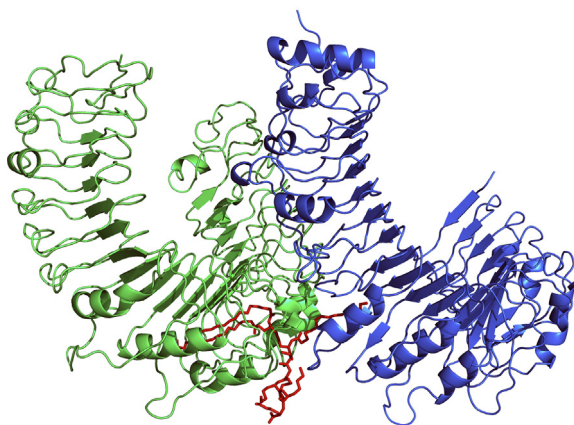


Fig. 7. Heterodimer of carp Tlr1-Tlr2(a), modelled on human TLR1-TLR2 with Pam₃CSK₄ as ligand. The extracellular part of the human TLR1-TLR2 heterodimer, crystallized with Pam₃CSK₄ as a ligand (PDB-id: 2z7x) was used to create models of carp Tlr1 and Tlr2a using the Modeller program (version 9.12). Thirty comparative models were generated, after which the model with lowest corresponding DOPE score was selected for image generation. Carp Tlr1 is shown in green, Tlr2a in blue, Pam₃CSK₄ in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

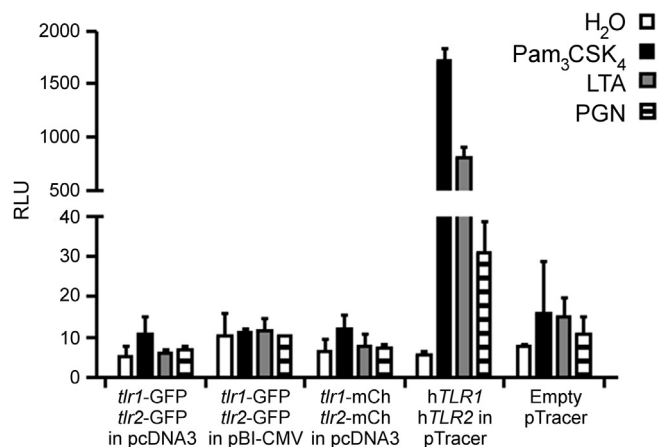


Fig. 8. Ligand stimulation of carp Tlr1 and Tlr2 in HeLa-57A cells does not lead to activation of NF- κ B. HeLa-57A cells stably transfected with NF- κ B luciferase reporter were transfected with carp or human TLR plasmids as indicated. Constructs in pcDNA3 encode for a fusion product of the Tlr and the fluorescent protein, whereas constructs in pBI-CMV express the two proteins separately. Cells were stimulated for 5 h with ligands Pam₃CSK₄ (100 ng/mL), LTA (1 μ g/mL) or PGN (10 μ g/mL), or water was added as negative control. Relative light units (RLU) are a measure of NF- κ B activity. Bars indicate mean of technical duplicates and error bars indicate standard deviation.

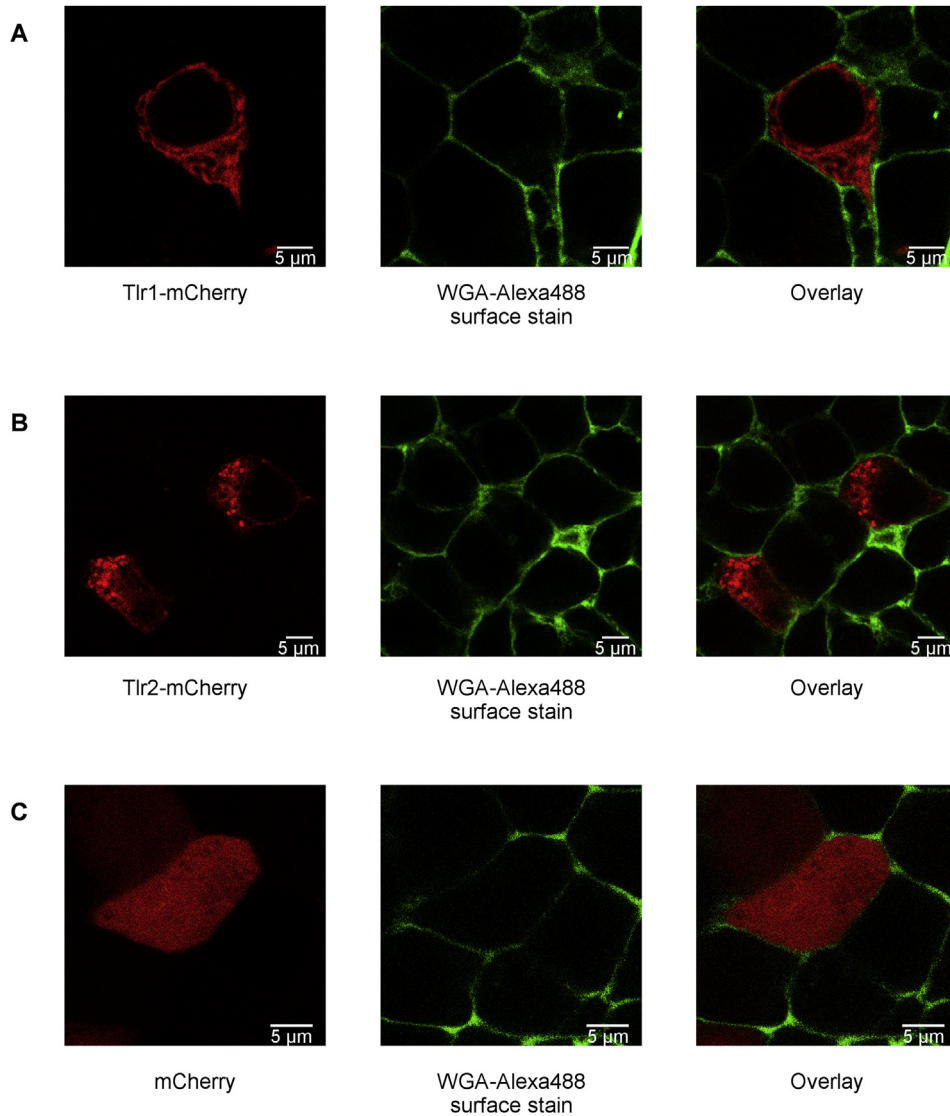


Fig. 9. Carp Tlr1 and Tlr2 localize intracellularly in HEK cells. HEK cells were transfected with (A) *tlr1*-mCherry-pcDNA3, (B) *tlr2*-mCherry-pcDNA3, or (C) mCherry-pcDNA3 (red) and analysed after 3 days by confocal laser scanning microscopy. Wheat-germ agglutinin conjugated to Alexa488 (green) was used to stain the cell surface. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

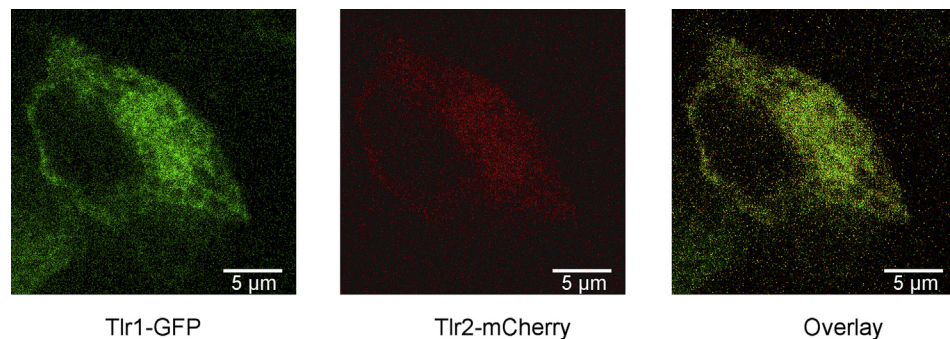


Fig. 10. Carp Tlr1 and Tlr2 co-localize intracellularly in HEK cells. HEK cells were co-transfected with *tlr1*-GFP-pcDNA3 (green) and *tlr2*-mCherry-pcDNA3 (red) and analysed after 3 days by confocal laser scanning microscopy. The overlay shows extensive co-localization of the green and red signal. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

have been able to interact with human MYD88 (at least in HEK and HeLa-57A cells), thus initiating no signalling cascade, iv) carp Tlrs may need a co-receptor or other co-factor [56], absent from the cell

lines we tested, v) different ligands are recognized by the carp Tlrs, vi) the present NF- κ B read-out system (although appropriate for mammalian, chicken and amphibian TLRs) is not suitable for

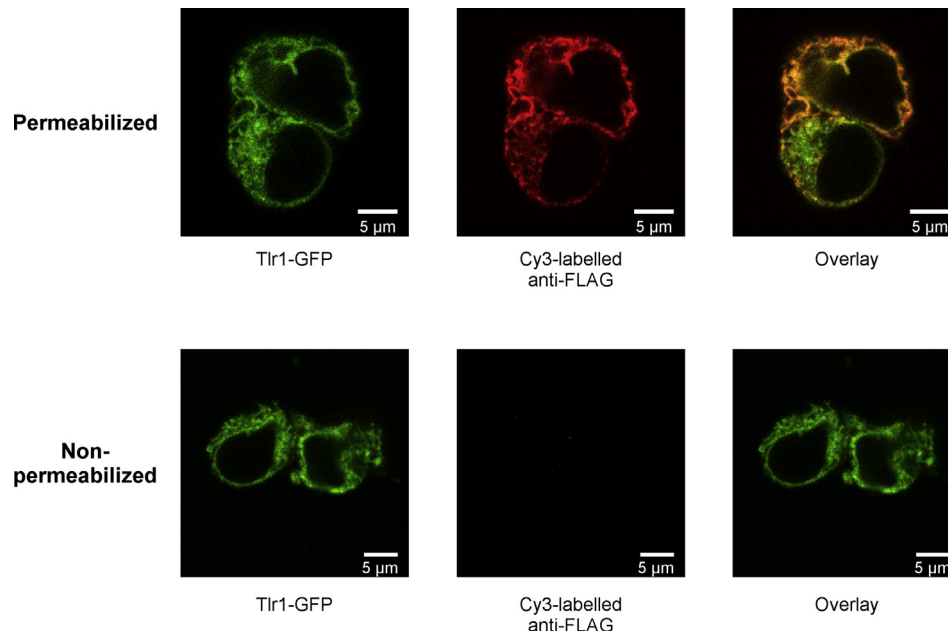


Fig. 11. Carp Tlr1 is not expressed on the membrane of HEK cells. HEK cells were transfected with FLAG-tagged Tlr1-GFP (*tlr1*-GFP-pcDNA3) (green) and analysed after 3 days by confocal laser scanning microscopy. Antibody staining was performed with Cy3-labelled anti-FLAG antibody (red) in permeabilized (top row) and non-permeabilized cells (bottom row). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

studying Tlrs of fish. Indeed, ligand-dependent NF- κ B activation mediated through fish-encoded Tlrs is not easily shown *in vitro* (as discussed in Ref. [21]). We do believe that bacterial PAMPs including lipoproteins could be potential activators of Tlr signalling in carp, as several of the components of the signalling pathway including MYD88 and TRAF6 [57], IRAK1 [58], and several accessory molecules for Tlrs [56] are all present. Indeed, carp macrophages respond to classical TLR2 ligands such as Pam₃CSK₄ and MALP-2 by altering gene expression of pro-inflammatory cytokines [28]. The latter study on carp Tlr2 using phosphorylation of p38 as a read-out is not in contradiction with the present study, the two studies rather suggest that several TLR signalling pathways and transcription factors should be scrutinized. Future studies could include measuring activation of transcription factors other than NF- κ B, such as AP-1 or IRF5, measuring caspase activity, or measuring downstream effects such as the production of pro-inflammatory cytokines. In other words, given the present difficulties detecting NF- κ B activation downstream of Tlr1-Tlr2 heterodimerization, it may be worthwhile in future work to refocus on signalling pathways other than NF- κ B as important activators of inflammatory cytokines.

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