Evolution of Recognition of Ligands from Gram-Positive Bacteria: Similarities and Differences in the TLR2-Mediated Response between Mammalian Vertebrates and Teleost Fish

Carla M. S. Ribeiro, Trudi Hermsen, Anja J. Taverne-Thiele, Huub F. J. Savelkoul and Geert F. Wiegertjes

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Evolution of Recognition of Ligands from Gram-Positive Bacteria: Similarities and Differences in the TLR2-Mediated Response between Mammalian Vertebrates and Teleost Fish

Carla M. S. Ribeiro, Trudi Hermsen, Anja J. Taverne-Thiele, Huub F. J. Savelkoul, and Geert F. Wiegertjes

We investigated the role of the TLR2 receptor in the recognition of ligands from Gram-positive bacteria in fish. Comparative sequence analysis showed a highly conserved Toll/IL-1 receptor domain. Although the leucine-rich repeat domain was less conserved, the position of the critical peptidoglycan (PGN)-binding residues in the leucine-rich repeat domain of carp TLR2 were conserved. Transfection of human embryonic kidney 293 cells with TLR2 corroborated the ability of carp TLR2 to bind the prototypical mammalian vertebrate TLR2 ligands lipoteichoic acid (LTA) and PGN from Staphylococcus aureus. The synthetic triacylated lipopeptide N-palmitoyl-S-(2,3-bis(palmitoyloxy)-(2RS)-propyl)-(R)-Cys-(S)-Ser-(S)-Lys4 trifluorohydrochloride (Pam3CSK4) but not the diacylated lipopeptide macrophage-activating lipopeptide-2 (MALP-2) also activated TLR2 transfected human cells. We identified clear differences between the mammalian vertebrates and carp TLR2-mediated response. The use of the same ligands on carp macrophages indicated that fish cells require high concentrations of ligands from Gram-positive bacteria (LTA, PGN) for activation and signal transduction, react less strongly (Pam3CSK4) or do not react at all (MALP-2). Overexpression of TLR2 in carp macrophages indicated that fish cells require high concentrations of ligands from Gram-positive bacteria (LTA, PGN) for activation and signal transduction, react less strongly (Pam3CSK4) or do not react at all (MALP-2). Overexpression of TLR2 in carp macrophages confirmed TLR2 reactivity of the response to LTA and PGN, low-responsiveness to Pam3CSK4 and non-responsiveness to MALP-2. A putative relation with the apparent absence of accessory proteins such as CD14 from the fish TLR2-containing receptor complex is discussed. Moreover, activation of carp macrophages by PGN resulted in increased TLR2 gene expression and enhanced TLR2 mRNA stability, MAPK-p38 phosphorylation and increased radical production. Finally, we could show that NADPH oxidase-derived radicals and MAPK-p38 activation cooperatively determine the level of PGN-induced TLR2 gene expression. We propose that the H2O2-MAPK-p38-dependent axis is crucial for regulation of TLR2 gene expression in fish macrophages.

The innate immune system of vertebrates uses pattern recognition receptors (PRRs) to sense invading pathogens. TLRs act as PRRs and, as such, are able to recognize pathogen-associated molecular patterns (PAMPs) that are conserved molecular structures and unique of a given microbial class (bacteria, viruses, fungi, and protozoa), thereby allowing sufficient responses to limit or eradicate invading microbes. TLRs are type I transmembrane proteins with an ectodomain containing interspersed leucine-rich repeat (LRR) motifs involved in recognition of PAMPs. The cytoplasmic domain is characterized by a Toll/IL-1 receptor (TIR) motif that is involved in signal transduction (1, 2). In mammalian vertebrates, TLR2 senses bacterial lipopeptides (3–5) and lipoteichoic acids (LTAs) (6, 7) as well as GPI anchors from parasites (8, 9). TLR2 recognizes microbial patterns as homodimer (10) and as heterodimers with TLR1 or TLR6 (11, 12). Expression of TLR2 in humans is highest in myeloid cells (monocytes, macrophages, dendritic cells, and granulocytes), whereas in mice, TLR2 expression was also found in T cells (13–15). Investigations of TLR-mediated immune recognition in nonmammalian vertebrates, such as teleost (modern bony) fish, can provide new insights into the evolution of immunity (16). Research on teleost fish TLR2 has been mostly restricted to the sequence identification of the TLR2 gene in a few fish species (zebrafish, Japanese flounder, fugu, and catfish) with limited studies on the basal and inducible expression levels of TLR2 transcripts (17–20). Although these studies demonstrate the presence of a TLR2 ortholog, the role of the TLR2 receptor in the recognition of ligands from Gram-positive bacteria has not been studied in fish.

Teleost fish possess orthologs of the different mammalian TLR families and a further two additional fish-specific TLR family members (17, 21). So far, only for the orthologs of TLR3 in zebrafish and rainbow trout (22, 23) and TLR5 in rainbow trout (24) proof for functional analogy to the mammalian counterparts has been provided. In fact, clear differences between fish and mammalian vertebrates in the recognition and response to certain PAMPs have been observed. Cells from teleost fish require much...
higher doses (micrograms/milliliter) of LPS from Gram-negative bacteria than human cells (nanograms/milliliter) for activation (25, 26). In teleost fish, LPS signals via a TLR4-independent manner (27) and, in fact, it was hypothesized that the TLR4 genes in zebrafish are paralogous rather than orthologous to human TLR4 (28). The activation of fish cells by a prototypical TLR ligand such as LPS thus provides no evidence for a direct relationship between ligand, the corresponding receptor, and the resulting immune response and classification of TLR proteins solely based on sequence homology may lead to false presumptions on functional conservation (28). In addition, although in mammalian vertebrates cytokine and radical production have been extensively used as markers of TLR2 activation (29, 30), differences in the activity of certain cytokines in fish, such as TNF-α (31, 32), indicate that TLR2 activation and subsequent cytokine induction do not necessarily have homologous functions in mammalian and teleost vertebrates. This led us to carefully examine the ligands for TLR2 and activation of cytokines and nitrogen and oxygen radicals downstream of TLR2 activation in fish.

In mammalian vertebrates, TLR2-mediated recognition of PAMPs from Gram-positive bacteria such as LTA or peptidoglycan (PGN) results in recruitment of a set of TIR domain-containing adaptor proteins such as MyD88. These interactions trigger downstream cascades leading to the activation of NF-κB and MAPKs, which control induction of inflammatory genes like IL-1β, TNF-α, and inducible NO synthase (iNOS) and control the activation of antimicrobial host defense mechanisms such as production of reactive oxygen species (ROS) and nitrogen species (29, 30, 33, 34). In mammalian vertebrates, TLR2-mediated recognition of PAMPs from Gram-positive bacteria such as LTA or peptidoglycan (PGN) signals via a TLR4-independent manner (29, 30, 33, 34). In general, in teleost fish, the intracellular components acting downstream of TLR receptors are highly conserved, with orthologous sets of genes in mammalian vertebrates and teleosts (35). The extracellular components of receptors such as TLRs seem to be more divergent (36). We analyzed the TLR2 receptor sequence of common carp (Teleostei, Cyprinidae) and found a high degree of conservation of the position of leucine residues critical for ligand recognition. Transfection of human embryonic kidney (HEK) 293 cells and carp macrophages with the carp TLR2 receptor confirmed activation by the prototypical ligands from Gram-positive bacteria LTA, PGN, and N-palmitoyl-S-(2,3-bis(palmitoyloxy)-(2RS)-propyl)-(1RS)-(3S)-Cys-(3S)-Ser-(3S)-lys trihydrochloride (Pam3CSK4), but not macrophage-activating lipopeptide-2 (MALP-2). Activation of carp macrophages indicated that fish cells require high concentrations of ligands from Gram-positive bacteria (LTA, PGN) for activation and signal transduction, react less strongly (Pam3CSK4) or do not react at all (MALP-2). However, LTA and PGN from Staphylococcus aureus did regulate carp TLR2 gene expression and enhanced TLR2 mRNA stability. Furthermore, our results suggest that H2O2 radicals via MAPK-p38 activation play an indispensable role in the regulation of TLR2 gene expression in carp macrophages. Further studies may confirm that our observations also apply to other teleost fish species.

Materials and Methods

Animals

European common carp (Cyprinus carpio carpio L.) were reared in the central fish facility at Wageningen University, The Netherlands, at 23°C in recirculating UV-treated tap water and fed pelleted dry food (Trouvit, Nutreco, The Netherlands) daily. R3xR8 carp are the hybrid offspring of a cross between fish of Hungarian origin (R8 strain) and of Polish origin (R3 strain) (37). Carp were between 9 and 11 mo old. All studies were performed with approval from the animal experimental committee of Wageningen University.

Stimuli and inhibitors

Purified LTA from S. aureus (tlr-psLTA), soluble secreted PGN from S. aureus (tlr-ssPGN), synthetic triptamiloylated lipopeptide Pam3CSK4 (tlr-1pms) and ultrapure LPS from Porphyromonas gingivalis (tlr-pgpLPS) were purchased from InvivoGen (Cayla SAS, France). Synthetic MALP-2, S-12-3-bis(Palmitoylxylo)-(2R)-propyl-cysteinyl-冈NDESISIFKEK) was purchased from Alexis Biochemicals (Axzorra, Germany). LPS from Escherichia coli (L2880), muramyl dipeptide, and polyinosinic polycytidylic acid were purchased from Sigma-Aldrich (St. Louis, MO). NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC), xanthine oxidase inhibitor allopurinol, mitochondrial chain 1 inhibitor rotenone, NADPH oxidase inhibitor diphenyleniodonium chloride (DPI), superoxide dismutase (SOD), and catalase were purchased from Sigma-Aldrich. MAK-p38 inhibitor SB203580 was purchased from Calbiochem (Merck Chemicals, Nottingham, U.K.). Phosphatidylinositol-specific phospholipase C (PI-PLC) from Bacillus cereus was purchased from Sigma-Aldrich.

Amplification of carp TRL2 wild type and TRL2ΔTIR cDNA

Oligonucleotide primers for carp TLR2 were designed based on known partial fish TLR2 sequences. cDNA from macrophages stimulated with LTA for 6 h was used as a template for PCR or nested PCR. The 5’ and 3’ ends of TLR2 were amplified using gene-specific primers (forward TLR2 and reverse TLR2, Table I) by 5’ and 3’ rapid amplification of cDNA ends (RACE) (38) using the Gene Racer Race Ready cDNA kit (Invitrogen, Breda, The Netherlands) according to the manufacturer’s protocol. Gene-specific primer (TLR2 Fw) was used in combination with the Gene Racer primers to amplify first strand of cDNA (Table I). A second round with gene-specific primers (TLR2Fw, TLR2Rv) was performed to obtain the wild carp type (WT) TLR2 (TLR2WT, 2367 bp) or TIR-domain truncated TLR2 (TLR2Fw, TLR2Rv nested) (TLR2ΔTIR, 2100 bp). PCR reactions were performed in Taq buffer, using 1 U Taq polymerase (Promega, Leiden, The Netherlands) supplemented with MgCl2 (1.5 mM), dNTPs (200 μM), and primers (400 nM each) in a total vol of 50 μl PCR and nested PCR were carried out under the following conditions: one cycle 4 min at 96°C; followed by 35 cycles of 30 s at 96°C, 30 s at 55°C, and 2 min at 72°C; and final extension for 7 min at 72°C, using a Gene Amp PCR system 9700 (PE Applied Biosystems, Foster City, CA). Products amplified by PCR, nested PCR or RACE-PCR were ligated and cloned in JM-109 cells using the pGEM-Teasy kit (Promega) according to the manufacturer’s protocol. From each product, both strands of eight clones were sequenced, using the ABI prismBigDye Terminator Cycle Sequencing Ready Reaction kit and analyzed using 3730 DNA analyzer.

Bioinformatics

The nucleotide sequence was translated using the ExPASy translate tool (http://us.expasy.org/tools/dna.html) and aligned with Clustal W (www.ebi.ac.uk/clustalw). The signal peptide cleavage site and the transmembrane region was predicted by using the SignalP 3.0 (www.cbs.dtu.dk/services/SignalP) and the TMHMM 2.0 (www.cbs.dtu.dk/services/TMHMM-2.0/) servers, respectively. Identification of LRRs within carp TLR2 were predicted using the method previously described (39). First, LRRs were predicted using PFAM (http://pfam.sanger.ac.uk/) and SMART (http://smart.embl-heidelberg.de/) servers. Second, LRRs candidates, that could not be recognized by PFAM, were identified by multiple sequence alignments with TLR2 from other species including human, mouse, and zebrafish. Third, protein secondary structure predictions of the LRR candidate were evaluated by Proteus (http://wks16338biology.ualberta.ca/proteus) server. Posttranslational modifications were predicted using the NetNGLyce 1.0 (www.cbs.dtu.dk/services/NetNGLyce/) server.

TLR2WT-GFP and TLR2ΔTIR-GFP expression plasmids

The vivid color pcDNA6.2/C-EmGF-GW/TOPO (Invitrogen, catalog no. K359-20) expression vector combined with TOPO cloning was used to fuse TLR2WT or TLR2ΔTIR to EmGF at the C-terminal end. Isolation of highly pure plasmid DNA suitable for transfection was performed using S.N.A.P. Midi Prep Kit (Invitrogen, catalog no. K1910-01) according to the manufacturer’s protocol. C-terminal fluorescent-tagged protein could be visualized using confocal microscopy.

Transient transfection of HEK 293 cells

HEK 293 cells were cultured in DMEM supplemented with 10% FBS (Invitrogen), 50 μM penicillin G (Sigma-Aldrich), and 50 μg/mL streptomycin sulfate (Sigma-Aldrich). Two days before transfection, HEK 293 cells were seeded into tissue culture flasks to reach 80–90% confluence at the day of transfection. For transient transfection, 2.5 μg carp TLR2WT-GFP or TLR2ΔTIR-GFP constructs were transfected into HEK 293 by nucleoporation using nuclofactor solution V and program A-23 (Lonza, Cologne AG, Germany). According to the manufacturer’s instructions. The transfection efficiency, assessed by flow cytometry, was on average 70%. Forty-eight hours after transfection, cells were trypsinized (0.25% trypsin, Invitrogen), counted using trypan blue exclusion, and plated overnight at a concentration 6 × 105
phosphorylated p38 (phospho-p38) activity by Western blot.

Macrophage cell culture

Head kidney-derived macrophages, considered the fish equivalent of bone marrow-derived macrophages, were prepared as previously described (40, 41). Briefly, carp head kidneys were gently passed through a 100-µm sterile nylon mesh (BD Biosciences, Breda, The Netherlands) and rinsed with homogenization buffer (incomplete NMGFL-15 medium containing 50 U/ml penicillin G, 50 µg/ml streptomycin sulfate, and 20 U/ml heparin [Leo Pharmaceutical, Weesp, The Netherlands]). Cell suspensions were layered on 51% (1.07 g/cm3) Percoll (Amersham Biosciences, Uppsala, Sweden) and centrifuged at 450g for 25 min at 4˚C with the brake disengaged. Cells at the interphase were removed and washed twice in incomplete NMGFL-15 medium. Cell cultures were initiated by seeding 1.75 × 10⁶ cells/well in a 24-well tissue culture plate. The next day, cells were stimulated with (optimized concentrations) 50 µg/ml LTA, 50 µg/ml PGN, 10 µg/ml Pam3CSK4, 10 µg/ml MALP-2, 50 µg/ml P2LPS, 50 µg/ml pIC, 50 µg/ml E. coli LPS for 5 min, or left untreated as control. Cells were lysed for evaluation of gene expression by real-time quantitative PCR (RT-qPCR).

Confocal laser scanning microscopy

Cytospin microscope slides of carp macrophages were made by fixing cells in 100% alcohol and 37% formaldehyde (10:1). HEK 293 cells were grown in 6-well glass bottom culture plate (MatTek, Ashland, MA) fixed in 100% alcohol and 37% formaldehyde (10:1). Sections were embedded in Vectashield containing propidium iodide (PI) (Vector Laboratories, Burlingame, CA) and examined with a Zeiss LSM-510 laser scanning microscope. Green fluorescent signal (rhodamine or green-fluorescent protein) was excited with a 488-nm argon laser and detected using a band-pass filter (505–530 nm) and red-fluorescent signal (PI) was excited with a 543-nm helium-neon laser and detected using a long-pass filter (560 nm).

MACS

Macrophage-enriched fractions of head kidney leukocytes were obtained essentially as previously described (42). Cell suspensions were layered on a discontinuous Percoll gradient (1.020, 1.060, 1.070, and 1.083 g/cm³) and centrifuged 30 min at 800g with the brake disengaged. Cells at 1.070 and 1.083 g/cm³ were collected, pooled, and washed twice with cRPMI (RPMI 1640 adjusted to carp osmolarity 280 mOsm/kg–1). Relative cell populations

FIGURE 1. Schematic representation of carp, human, and mouse TLR2 on the cell membrane. A. All TLR2 molecules have an extracellular, transmembrane, and intracellular domain. LRR motifs are indicated by rectangles and the TIR domain is indicated by an ellipse. Human and mouse TLR2 have 20 LRRs, whereas carp TLR2 has 21 LRRs. The additional LRR is located at the N-terminus (LRR1) and is an irregular LRR belonging to the “bacterial” motif. N-glycosylation sites are indicated by black circle lollipops. Numbered amino acid residues correspond to the position in the human TLR2 molecule. Regions and amino acid residues shown to be important for ligand recognition, protein secretion or interaction with MyD88 for the human TLR2 molecule are shaded in gray. Conserved amino acid residues in carp or mouse, when compared with human TLR2, are depicted in gray and nonconserved amino acid residues are depicted in black. B. LRR and TIR domain percentage sequence identity according to the manufacturer’s instructions and placed into 48-well plate at a concentration 1 × 10⁵ cells/well. The transfection efficiency, assessed by flow cytometry, was on average 25%. After 24 h incubation, the medium was replaced and macrophages were stimulated for 6 or 9 h with (optimized concentrations) 50 µg/ml LTA, 50 µg/ml PGN, 10 µg/ml Pam3CSK4, 10 µg/ml MALP-2, or left untreated as control. Cells were lysed for evaluation of gene expression by real-time quantitative PCR (RT-qPCR).

Transient transfection of carp macrophages

For transient transfection, 2.5 µg carp TLR2WT-GFP or TLR2ΔTIR-GFP constructs were transfected into carp macrophages by nucleoporation using nucleofector Human Macrophage Solution and program Y-001 (Lonza) according to the manufacturer’s instructions and placed into 48-well plate at a concentration 1 × 10⁵ cells/well. The transfection efficiency, assessed by flow cytometry, was on average 25%. After 24 h incubation, the medium was replaced and macrophages were stimulated for 6 or 9 h with (optimized concentrations) 50 µg/ml LTA, 50 µg/ml PGN, 10 µg/ml Pam3CSK4, 10 µg/ml MALP-2, or left untreated as control. Cells were lysed for evaluation of gene expression by real-time quantitative PCR (RT-qPCR).

FIGURE 2. Localization of carp TLR2 constructs in transfected HEK 293 cells. HEK 293 cells were transiently transfected with pTOPO containing full-length carp TLR2 (TLR2WT, 2.5 µg) or TIR-domain truncated TLR2 (TLR2ΔTIR, 2.5 µg) fused to GFP (green) at the 3’-end. PI+ cells are red. Localization of carp TLR2WT and TLR2ΔTIR was determined by confocal laser microscopy (original magnification ×40).
were found to be similar as previously described: 1.070 g/cm³ interphase consisted of macrophages (65%) with granulocytes, small macrophages, and lymphocytes (35%) and 1.083 g/cm³ interphase consisted of neutrophilic granulocytes (85%) with macrophages (15%). The mAb TCL-BE8 (1:50) (43) was used to separate neutrophilic granulocytes from macrophages by MACS. After incubation for 30 min with TCL-BE8 on ice, the leukocyte suspension was washed twice with cRPMI and incubated with PE-conjugated goat anti-mouse (1:50; Dako, Glostrup, Denmark) 30 min on ice. After washing twice, the total cell number was determined with a Bürker counting chamber, and 10⁷ magnetic beads (anti-PE Microbeads, Miltenyi Biotec, GmbH, Gladbach, Germany) was added per 1 × 10⁸ cells. After incubation for 15 min at 4°C, cells were washed twice and resuspended in cRPMI. The magnetic separation was performed on LS-MidiMACS Columns (Miltenyi Biotec) according to the manufacturer’s instructions. The purity of the TCL-BE8⁺ (neutrophilic granulocyte-enriched fraction; >90%) and TCL-BE8⁻ (macrophage-enriched fraction; <10%) fractions was confirmed by flow cytometric analysis using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). TCL-BE8⁺ and TCL-BE8⁻ were washed in cRPMI and resuspended in 1 ml cRPMI⁺⁺ (cRPMI medium supplemented with 1.5% pooled carp serum, 290 μg/ml glutamine [Cambrex], 50 U/ml penicillin G, and 50 μg/ml streptomycin sulfate).

Flow cytometric measurement of ROS production

Intracellular ROS levels were evaluated by FACScan flow cytometer using the redox-sensitive dye dihydrorhodamine (DHR) 123 (44, 45) at 0.25 μg/ml (Sigma-Aldrich, D1054). Head kidney-derived macrophages (5 × 10⁵) were resuspended in 100 μl rich-NMGFL-15 medium (incomplete NMGFL-15 medium supplemented with 2.5% heat-inactivated pooled carp serum and 5% FBS) and stimulated with PMA (0.05 μg/ml; Sigma-Aldrich) or left untreated as controls. Concomitantly, cells were also stimulated with LTA or PGN. DHR was added to all samples, and cells incubated for 1 h at 27°C. Cells were resuspended by pipetting, transferred to flow cytometer tubes and PI (0.1 mg/ml; Sigma-Aldrich) was added to each sample to detect and gate out PI⁺ cells. For all cytometric measurements the same settings were used (FS 350 V, gain 2; SS 700 V, gain 10; FL1 600 V; FL2 750 V; FL3 675 V; FL4 660 V). The baseline offset was on and the FS discriminator was set at 50. Per sample, 10⁴ events were measured by flow cytometer.

Nitrite production

Macrophages were stimulated with LTA (50 μg/ml), PGN (50 μg/ml), or left untreated as control, and incubated at 27°C for 18 h. Nitrite production was measured essentially as described previously (46). To 75 μl cell culture supernatant, 100 μl 1% sulfanilamide in 2.5% (v/v) phosphoric acid, and 100 μl 0.1% (w/v) N-naphthyl-ethylenediamine in 2.5% (v/v) phosphoric acid were added in a 96-well flat-bottom plate. The absorbance was read at 540 nm (with 690 nm as a reference) and nitrite concentration (μM) was calculated by comparison with a sodium nitrite standard curve.

Gene expression profiling of LTA- and PGN-stimulated macrophages

Head kidney-derived macrophages (5 × 10⁵) in 100 μl rich-NMGFL-15 were seeded in 96-well flat-bottom culture plates and stimulated with LTA, PGN, Pam₃CSK₄, or left untreated as control, and incubated for the indicated time

![FIGURE 3.](http://www.jimmunol.org/)

**FIGURE 3.** Activation of carp TLR2 by different PAMPs in HEK 293 cells. TLR2WT and TLR2ΔTIR transfected HEK293 cells were stimulated with LTA (50 μg/ml), PGN (50 μg/ml), Pam₃CSK₄ (Pam₃, 10 μg/ml), MALP-2 (10 μg/ml), PgLPS (50 μg/ml), pIC (50 μg/ml), or EcLPS (50 μg/ml) for 5 min or left untreated as control. A, MAPK-p38 phosphorylation was analyzed by immunoblotting for phospho-p38, whereas equal loading was confirmed by immunoblotting for β-tubulin. B, Immunoblot intensity profile (between 0 and 255) of TLR2WT- and TLR2ΔTIR-transfected HEK 293 cells was determined over a horizontal line on each grayscale image. This is one experiment representative of four experiments.
points at 27°C. A number of 3–5 × 10⁶ cells (combination of 6–10 wells) per time point, per treatment, were lysed, and pooled for RNA isolation.

RNA was isolated using the RNaseasy Mini Kit (Qiagen, Leusden, the Netherlands) including the accompanying DNase I treatment on the columns, according to the manufacturer’s protocol. Final elution was performed with 30 μl nuclelease-free water. RNA concentrations were measured by spectrophotometry (Nanodrop, Thermo Scientific, Breda, The Netherlands) and 1 μl was analyzed on a 1% agarose gel to check the RNA integrity. RNA was stored at −80°C until further use. Prior to cDNA synthesis, a second DNase treatment was performed using DNase I, Amplification Grade (Invitrogen). Briefly, 1 μg RNA from each sample was combined with 10X DNase reaction buffer and 1 U DNase I, mixed and incubated at room temperature (RT) for 15 min, followed by inactivation of DNase I by adding 1 μl 25 μM EDTA. Synthesis of cDNA was performed with Invitrogen’s SuperScript III First Strand Synthesis Systems for RT-PCR Systems, according to the manufacturer’s instructions. Briefly, DNase I-treated RNA samples were mixed with five times first strand buffer, 300 ng random primers, 10 μM dNTPs, 0.1 M DTT, 10 U RNase inhibitor, and 200 U SuperScript III Reverse Transcriptase up to a final vol of 20 μl. The mixture was incubated at 37°C for 60 min, followed by an inactivation step at 70°C for 15 min. A nonreverse transcriptase control was included for each sample. cDNA samples were further diluted 50 times in nuclease-free water before use as template in real-time PCR experiments.

RT-qPCR was performed in a 72-well Rotor-Gene 2000 (Corbett Research, Mortlake, Sydney, Australia) with the Brilliant SYBR Green QPCR (Stratagene, Mortlake, Sydney, Australia) as detection chemistry. Primers used for RT-qPCR (Table I) were designed with Primer Express software. IL-1β and TNF-α primers were designed to amplify all known isoforms for each gene. Master mix for each primer set were obtained on Comparative Quantitation Analysis from the Rotor-Gene version 6.0.21 software. Briefly, the E for each primer set was recorded per sample and an averaged E (Eav) was then calculated for each primer set. The relative expression ratio (R) of a target gene was calculated based on the Eav and the C deviation of sample versus control, and expressed in comparison with a reference gene (47, 48).

Western blot analysis

Cells were resuspended by pipetting and transferred to precooled eppendorf tubes. Cells were washed twice in ice-cold PBS, lysed on ice with lysis solution (0.5% Triton X-100, 20 mM Tris, 100 mM NaCl, 1 mM EDTA, 50 mM NaF [Sigma-Aldrich], 1 mM PMSF [Sigma-Aldrich]), homogenized with a syringe, and incubated on ice for 10 min. Cell lysates were centrifuged at 14,000 rpm for 10 min at 4°C. Supernatant was collected and total protein content was determined by the Bradford method. Samples (20–25 μg) were boiled at 96°C for 10 min with loading buffer containing β-mercaptoethanol and separated by 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Protran, Schleicher and Schuell, Bioscience GmbH, Germany). Membranes were blocked in 3% BSA in TBS (10 mM Tris, 150 mM NaCl, pH 7.5) for 1 h at RT and then incubated with primary Ab overnight at 4°C in 3% BSA in TBS. Abs reactive to both hosts and carp were used: rabbit IgG anti-phospho-p38 (1:1000, Th180/Thy182, BioCat GmBh, Heidelberg, Germany) and rabbit IgG anti-β-tubulin (1:1000, Abcam, Cambridge, U.K.). Membranes were then incubated with goat anti-rabbit HRP-conjugated (1:1000, Dako) in 10% milk powder in TBS for 1 h at RT. Between each incubation step, membranes were washed twice with TBS-Tween/Triton (TBS, 0.05% [v/v] Tween 20, 0.2% [v/v] Triton X-100) and once with TBS, for 10 min at RT. Signal was detected by development with a chemiluminescence kit (Amersham Biosciences) according to the manufacturer’s protocol and visualized by the use of Luminifil chemiluminescent Detection Film (Rochef, Woerden, The Netherlands). The blots were scanned and the intensity profile (between 0 and 255) of each lane was determined over a horizontal line on a grayscale image using the analysis FIVE (Olympus Nederland BV) program.

Protozoan-derived PAMPs

PL-PLC from B. cereus was resuspended in Tris buffer (10 mM Tris-HCl, 144 mM NaCl, 0.05% BSA, pH 7.4) according to the manufacturer’s protocol. The protozoan carp parasite Trypanosoma borrei (49) was incubated for 30 min at 30°C with 30% Tris-buffer or with 1 U PI-PLC (in 30% Tris-buffer) up to a total of 600 μl in incomplete NMFG-15 medium. Samples were centrifuged at 800g for 10 min. Supernatants were collected and filter sterilized (0.22 μm Milllex-GV, Millipore, Ireland) to avoid contamination with parasites. Pellets from PI-PLC-treated were collected and resuspended in 600 μl incomplete NMFG-15 medium. Carp macrophages were stimulated with 25 μl of each fraction.

Statistical analysis

Transformed values (ln) were used for statistical analysis in SPSS software (version 15.0). Homogeneity of variance was analyzed using the Levene’s test. Significant differences (p ≤ 0.05) between a treatment (stimulated

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*The “+” is before the nucleic acid in which the locked nucleic acid bond was placed.*
cells) and the control group (unstimulated cells) were determined by a one-way ANOVA, followed by a Dunnett t test. Significant differences between treatments ($p \leq 0.05$) were determined by one-way ANOVA, followed by Bonferroni test. In case of unequal variances between treatments, the one-way ANOVA was followed by a Games–Howell test.

**Results**

**Carp TLR2 ectodomain contains the critical residues for ligand recognition**

Full-length cDNA of the carp TLR2 gene was cloned (GenBank accession number FJ858800, www.ncbi.nlm.nih.gov/Genbank) encoding for a TLR2 protein of 788 aa. The carp TLR2 protein is composed of an extracellular, transmembrane, and intracellular domain. The ability of carp TLR2 to recognize prototypical TLR2 ligands and to trigger characteristic downstream cascades was evaluated by comparative sequence analysis. The extracellular domain of carp TLR2 has 21 LRRs, whereas human and mouse TLR2 have 20 LRRs (excluding the LRRs at the N-terminal and C-terminal region) (Fig. 1A). This additional but irregular LRR at the N-terminal of carp TLR2 belongs to the “bacterial” type and has also been found in the zebrafish TLR2 sequence (39). The extracellular domain of carp TLR2 region containing S40–I64 compassing the LRR N-terminal and LRR1 motifs, shown to be crucial for the recognition of *S. aureus* PGN (50), contained a number of conserved critical LRR. Furthermore, leucine residues at positions 107, 112, and 115 (at LRR3) that are critical in the recognition of diacylated lipoproteins, lipopeptides, and *S. aureus* PGN in human TLR2 (51), were conserved. The F349 shown to be involved in the recognition of bacterial lipopeptide was also conserved. N-glycosylation sites involved in secretion of the N-terminal ectodomain (52) such as N114 (LLR3) and N442 (LRR16) were conserved (Fig. 1A). Thus, despite a relatively low amino acid sequence identity (<37%) of the carp TLR2 LRR domain to mammalian vertebrate LRR domains (Fig. 1B), the position of the residues critically involved in the recognition of *S. aureus* PGN seems well conserved.

The intracellular (TIR) domain of carp TLR2 showed a much higher sequence identity to the mammalian vertebrate TLR2 sequences than the extracellular LRR domain (Fig. 1B). Proline P681, involved in the interaction with the MyD88 adaptor molecule (53), was conserved in the carp TLR2 sequence. The high sequence identity of the carp TIR domain (>67%) suggests that downstream cascades triggered on TLR2 activation may be conserved in carp.

**LTA, PGN, and Pam3CSK4, but not MALP-2, are ligands of carp TLR2**

HEK293 cells were used to define ligands for carp TLR2 using MAPK-p38 phosphorylation as a measure for responsiveness (15). HEK 293 cells were transfected with carp TLR2 full length (TLR2WT) or with carp TIR-domain truncated TLR2 (TLR2ΔTIR). Both constructs were fused to GFP at the 3’-end that enabled us to investigate the sublocalization of the protein after transfection, by confocal imaging. We could confirm the predominant localization of TLR2WT on the cell surface (TLR2WT; Fig. 2). A more diffuse localization was observed after truncation of the TIR domain (TLR2ΔTIR; Fig. 2).

MAPK-p38 phosphorylation in transfected HEK 293 cells was used to study carp TLR2 activation by different TLR ligands, using

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**FIGURE 4.** Kinetics of immune gene expression in carp macrophages after stimulation with different PAMPs. RT-qPCR analysis of gene expression in carp macrophages after stimulation for 6, 9, 12, and 18 h with LTA (50 μg/ml), PGN (50 μg/ml), Pam3CSK4 (Pam3, 10 μg/ml), MALP-2 (10 μg/ml), or left untreated as control. mRNA levels of IL-1β, iNOS, IL-11, TNFα, IL-12p35, and IL-12p40 relative to the housekeeping 40S ribosomal protein gene level are expressed as fold change relative to unstimulated cells at 0 h. Bars show averages ± SD of $n = 3$ fish. *Significant ($p \leq 0.05$) difference compared with unstimulated cells (control group). Note the differences in scale of the y-axes.
an Ab specific for phospho-p38. Stimulation of TLR2ΔTIR transfected HEK 293 cells with known TLR2 ligands did not increase p38 phosphorylation (TLR2ΔTIR; Fig. 3A, 3B). This negative control thereby showed the unresponsiveness of the parental HEK 293 cells to TLR2 agonists, confirming that HEK 293 cells themselves do not bear receptors for TLR2 ligands. In contrast, stimulation with LTA, PGN, and the synthetic agonist Pam3CSK4 [ligand for TLR2/TLR1 (54)] of TLR2WT transfected HEK 293 resulted in a clear increase of MAPK p38 activity. Neither MALP-2 [ligand for TLR2/TLR6 (12)], nor PgLPS, EcLPS, nor polyinosinic polycytidylic acid were able to modulate MAPK-p38 phosphorylation at any concentration of n = 4 fish. *Significant (p ≤ 0.05) difference compared with TLR2ΔTIR transfected carp cells. Note the differences in scale of the y-axes.

**FIGURE 5.** Overexpression of TLR2 in carp macrophages. TLR2WT and TLR2ΔTIR transfected carp macrophages were stimulated with LTA (50 μg/ml), PGN (50 μg/ml), Pam3CSK4 (Pam3, 10 μg/ml), MALP-2 (10 μg/ml) for 6 and 9 h, or left untreated as control. mRNA levels of IL-1β, iNOS, and TNF-α relative to the house keeping 40S ribosomal protein gene level are expressed as fold change relative to unstimulated cells. mRNA levels of IL-11, IL-12p35, and IL-12p40 after stimulation were also determined but no changes were observed (data not shown). Bars show averages ± SD of n = 4 fish.

**Activation of carp macrophages by TLR2 ligands**

Basal TLR2 gene expression was measured in different cell types and shown to be most ubiquitous in myeloid-derived cells and highest in macrophages (data not shown). In mammalian vertebrates, TLR activation results in downstream gene expression of immune modulators, including several cytokines, chemokines, and radicals such as NO. This immune profile is often used as marker for TLR(2) activation. We tested the ability of LTA, PGN, Pam3CSK4, and MALP-2 to induce an immune gene expression profile in carp macrophages (Table 1). Pam3CSK4 modulated immune gene expression to a minor extent, whereas MALP-2 clearly did not modulate immune gene expression at any concentration (1–10 μg/ml) or time point (6–18 h) analyzed. In contrast, both LTA and PGN modulated immune gene expression in carp macrophages, albeit at high concentrations of 50 μg/ml. Both the magnitude and the kinetics of the response were different between the two stimulants, with PGN consistently inducing a higher response than LTA. The kinetics of downstream activation of carp IL-1β, iNOS, IL-11, IL-12–p35 (p = 0.065), and IL-12–p40 indicated highest gene expression at 6 h after PGN-stimulation, whereas LTA stimulation induced highest gene expression at 9 h (Fig. 4). Our results show that the TLR2 ligands LTA, PGN, and Pam3CSK4 (to a minor extent) but not MALP-2, stimulated downstream signaling pathways with ligand-specific kinetics profiles in carp macrophages.

**Higher activation of carp macrophages by LTA, PGN, and Pam3CSK4, but not MALP-2 after overexpression of TLR2**

To verify whether macrophage activation by LTA and PGN would be TLR2-mediated, we overexpressed TLR2 in carp macrophages. The expression of both TLR2WT and TLR2ΔTIR constructs after transfection of carp macrophages were confirmed by Western blot (data not shown). Stimulation of macrophages overexpressing TLR2ΔTIR was used as the negative control (TLR2ΔTIR; Fig. 5).
Carp macrophages overexpressing TLR2WT could be further activated with PGN and LTA and to a minor extent with PamC_3K_4, but not with MALP-2 (TLR2WT; Fig. 5). Again, gene expression (IL-1β, iNOS) was highest at 6 h, after PGN-stimulation, and highest at 9 h after LTA stimulation.

**LTA, PGN, and protozoan GPI-anchors induce expression and increase mRNA stability of the TLR2 gene in carp macrophages**

TLR2 ligands LTA and PGN could activate carp macrophages via TLR2 as shown by increased MAPK-p38 phosphorylation in HEK 293 cells and enhanced immune gene expression in TLR2WT-transfected cells. We subsequently tested whether LTA and PGN could modulate the expression of the carp TLR2 gene itself (Table 1). TLR2 gene expression in carp macrophages was highest at 6 h and then declined (Fig. 6A). Stimulation with either LTA or PGN induced a low but significant 1.5–2-fold upregulation of TLR2 gene expression.

To verify the low but consistent fold upregulation of TLR2, two strategies were taken. First, additional sources of carp macrophages were examined for TLR2 gene expression. Both macrophage-enriched Percoll fractions of freshly isolated head kidney leukocytes and macrophage-enriched MACS-sorted leukocytes (TCL-BE8^-) showed a consistent but maximum 2-fold upregulation of expression of the carp TLR2 gene (data not shown). Second, GPI anchors from protozoan parasites of carp, T. borrelii (Kinetoplastida), were examined as TLR2 ligands. In mammalian vertebrates, TLR2 recognizes GPI-anchors from protozoan parasites (8). Macrophages stimulated with supernatant from PI-PLC-treated parasites as source of GPI-anchors showed a dose-dependent upregulation of TLR2 gene expression (Fig. 6B). Macrophages stimulated with nontreated parasites or the resultant pellet did not show upregulation of TLR2 gene expression (negative controls).

Stimulation with LTA more than stimulation with PGN stabilized carp TLR2 mRNA (increased mRNA half-life: Fig. 6C). Although IL-1β and iNOS mRNA (control genes) were also stabilized by LTA, this was not the case for stimulation by PGN. Using an inhibitor of MAPK-p38, we also studied the posttranscriptional effect of MAPK-p38 phosphorylation on the mRNA stability of the carp TLR2 gene, again including IL-1β and iNOS as control genes. Inhibition of MAPK-p38 destabilized all three gene transcripts. Our results suggest that the TLR2 ligands LTA and PGN, but also the signaling molecule MAPK-p38, lead to increased mRNA stability of the carp TLR2 gene. Increased mRNA stability may lead to an increase in the amount of TLR2 protein induced after ligand binding.

**FIGURE 6.** TLR2 gene expression and mRNA stability after stimulation with TLR2 ligands. **A**, Kinetics of TLR2 gene expression in carp macrophages after stimulation for 6, 9, 12, and 18 h with LTA (50 μg/ml), PGN (50 μg/ml), or left untreated as control. RT-qPCR analysis of gene expression in carp macrophages. mRNA levels of TLR2 relative to the house keeping 40S ribosomal protein gene level are expressed as fold change relative to unstimulated cells at 0 h. Bars show averages ± SD of n = 4 fish. *Significant (p ≤ 0.05) difference compared with unstimulated cells (control group). **B**, TLR2 gene expression in carp macrophages after stimulation for 6 h with supernatants from PI-PLC–treated T. borrelii parasites. mRNA levels of TLR2 relative to the house keeping 40S ribosomal protein gene level are expressed as fold change relative to unstimulated cells at 0 h. Bars show averages ± SD of n = 3 fish. *Significant (p ≤ 0.05) difference compared with unstimulated cells (control group). **C**, TLR2 mRNA stability. TLR2 gene transcription in carp macrophages after stimulation for 6 h with LTA (50 μg/ml) or PGN (50 μg/ml) or pre-incubated for 30 min with MAPK-p38 inhibitor (SB203530, 25 μM), followed by stimulation for 6 h with LTA (50 μg/ml). At 6 h, cells were treated with RNA synthesis inhibitor (actinomycin D, 5 μg/ml) and collected at the time points indicated. mRNA levels of TLR2, IL-1β, and iNOS, corrected for differences in mRNA levels of the house keeping 40S ribosomal protein gene, are expressed as % remaining mRNA (ratio mRNA at indicated time point relative to mRNA of the same gene prior to the addition of actinomycin D). Graphs do not reflect differences in gene transcription between treatments before the addition of actinomycin D. This is one experiment representative of three experiments. Exponential functions fitting the TLR2 mRNA levels for each treatment were used to estimate t_1/2 in hours. Estimated t_1/2 of TLR2 gene transcripts were t_1/2 = 1.18 h for unstimulated cells; t_1/2 = 4.30 h (LTA); t_1/2 = 1.62 h (PGN), and t_1/2 = 0.69 h for MAPK-p38/iNOS/LTA–stimulated macrophages.
PGN induces MAPK-p38 activation and radical production in carp macrophages

Stimulation of carp macrophages with LTA and PGN resulted in higher phosphorylation of MAPK-p38 in carp macrophages within 5 min (Fig. 7A). The signal was stronger in PGN- than in LTA-stimulated carp macrophages.

The MAPK-p38 pathway is known to be redox-sensitive in mammalian vertebrates (55). PGN, but not LTA, induced the production of nitrogen radicals (NO) in carp macrophages (Fig. 7B). Similarly, oxygen radical (ROS) production by carp macrophages could be induced with PGN (141%) (Fig. 7D), but not with LTA (102%). The ROS production was measured with the DHR 123 probe, which is reduced to the green fluorescent rhodamine 123 (Fig. 7C) under influence of ROS formation (e.g., H₂O₂, HO⁻) (45).

Treatment of carp macrophages with SOD (which dismutates O₂⁻ into H₂O₂ and O₂) further increased the ROS production (Fig. 7D) indicating that TLR2-mediated H₂O₂ production can be detected by this method.

ROS production modulates TLR2 gene expression

PGN induced MAPK-p38 activation, TLR2 gene expression, NO, and also ROS production in carp macrophages. To examine the influence of MAPK-p38 activation and ROS production on carp TLR2 gene expression, we used inhibitors of signal transduction and inhibitors of radical production and measured TLR2 gene expression. Cell viability (assessed by trypan blue exclusion), TLR2 gene expression, and ROS production were not affected by the presence of these inhibitors (data not shown).

Signal transduction pathways associated with TLR2 gene regulation were studied by preincubation of carp macrophages with inhibitors of NF-κB (PDTC) and MAPK-p38 (SB 208530). TLR2 gene expression, induced by PGN or by LTA (not shown), could be inhibited by the presence of either inhibitor, although the strongest by MAPK-p38 inhibition (Fig. 8A). Radical production, induced by PGN (LTA did not induce radical production, see Fig. 7D), was inhibited only by inhibition of the MAPK-p38 pathway (Fig. 8B). This suggests that MAPK-p38 activation is involved in regulating both TLR2 gene expression and radical production.

Enzymatic pathways associated with radical production were studied by preincubation of carp macrophages with inhibitors of the mitochondrial electron transfer chain subunit I (rotenone), NADPH oxidase (DPI), and xanthine oxidase (allopurinol). TLR2 gene expression, induced by PGN or LTA (not shown), could be inhibited by the presence of any of the three inhibitors, although to a lesser extent by the inhibitor of xanthine oxidase (Fig. 8C). Radical production, induced by PGN, was significantly inhibited only in the presence of the NADPH oxidase inhibitor (Fig. 8D). This indicates that oxygen radicals, including the NADPH oxidase-derived intracellular ROS (e.g., H₂O₂), are involved in the regulation of TLR2 gene expression in carp macrophages.

**FIGURE 7.** Effect of TLR2 ligands on MAPK-p38 activation and ROS production in carp macrophages. A, MAPK-p38 activation in carp macrophages analyzed by immunoblotting for phospho-p38. Equal loading was confirmed by immunoblotting for β-tubulin. Macrophages were stimulated with LTA (50 μg/ml) or PGN (50 μg/ml) for the time points indicated. B, NO production in carp macrophages. Macrophages were incubated with LTA (50 μg/ml) or PGN (50 μg/ml) for 18 h. Bars show averages ± SD of n = 4 fish. *Significant (p ≤ 0.05) difference compared with unstimulated cells (control group). C, Radical production in carp macrophages analyzed by fluorescence microscopy. Macrophages were incubated for 1 h with DHR (0.25 μg/ml; green) or left untreated as control. PI⁺ cells are red (original magnification ×40). D, Radical production in carp macrophages by means of DHR fluorescence intensity. Macrophages were incubated with DHR (0.25 μg/ml) and PMA (0.05 μg/ml), followed by LTA (50 μg/ml) or PGN (50 μg/ml) in the presence or absence of SOD (20 U/ml) for 1 h. Gray shaded histograms represent a control sample that corresponds to PMA-stimulated cells. This is one experiment representative of four experiments.
H$_2$O$_2$ is an essential signaling molecule for TLR2 gene expression and MAPK-p38 activation

ROS, such as H$_2$O$_2$, are able to diffuse across membranes and therefore can act as signaling molecules (56). TLR2 gene expression in carp macrophages was significantly modulated by H$_2$O$_2$ in a concentration-dependent manner (Fig. 9A). Consistent with these findings, PGN-induced TLR2 gene expression in carp macrophages could be inhibited by a H$_2$O$_2$ scavenger (catalase) (Fig. 9B).

To investigate whether H$_2$O$_2$ influences TLR2 gene expression via MAPK-p38 activation, carp macrophages were stimulated with increasing concentrations of H$_2$O$_2$. Indeed, MAPK-p38 activation was considerably upregulated by H$_2$O$_2$ in a concentration-dependent manner, within 5 min after addition of H$_2$O$_2$ (Fig. 9C). MAPK-p38 activation was significantly upregulated by H$_2$O$_2$ at concentrations >1 mM H$_2$O$_2$. The concentration of >1 mM H$_2$O$_2$ required to activate MAPK-p38 (57) implies that TLR2 gene expression is highly sensitive to modulation by H$_2$O$_2$ (0.1 mM) (Fig. 8A).

Our data indicate that H$_2$O$_2$ is able to induce TLR2 gene expression and activate the MAPK-p38 signaling cascade in carp macrophages. Furthermore, catalase (H$_2$O$_2$ scavenger) but not SOD (dismutates O$_2^\cdot$ into H$_2$O$_2$) inhibited PGN-induced MAPK-p38 phosphorylation (data not shown). These observations suggest that NADPH oxidase-derived radicals such as H$_2$O$_2$ in particular, are essential for a complete MAPK-p38 phosphorylation and maximal expression of the TLR2 gene.

**Discussion**

Previous studies have identified TLR2 orthologs in teleost fish based on sequence homology, but the role of the TLR2 receptor in the recognition of ligands from Gram-positive bacteria has not been studied. Transfection of human cells (HEK 293) with the carp TLR2 receptor showed activation of MAPK-p38 by LTA and PGN from S. aureus, which are prototypical TLR2 ligands from Gram-positive bacteria. The synthetic triacylated lipopeptide Pam$_3$CSK$_4$ but not the diacylated lipopeptide MALP-2 also activated TLR2 transfected human cells. Overexpression of TLR2 in carp macrophages confirmed the response to LTA and PGN, low-responsiveness to Pam$_3$CSK$_4$ and nonresponsiveness to MALP-2. Activation of carp macrophages by LTA and PGN from S. aureus resulted in increased TLR2 gene expression and enhanced TLR2 mRNA stability. ROS production and MAPK-p38 activation cooperatively determined the level of TLR2 gene expression, indicating that the H$_2$O$_2$–MAPK-p38–dependent axis is crucial for regulation of TLR2 gene expression in fish cells.

Similar to mammalian vertebrate TLR2, carp TLR2 is primarily expressed in myeloid cell types as a type I transmembrane protein composed of an extracellular, a transmembrane and an intracellular C-terminal domain. The extracellular domains of mammalian vertebrate TLR2 recognize LTA (6, 7), PGN (58–60), and lipopeptides (5). Mammalian vertebrate TLR2 contains 20 LRRs (39), whereas the extracellular domain of carp TLR2 is composed of 21 LRR motifs, one of which has a “bacterial” motif and may not be important for ligand recognition. Although, in general, the extracellular LRR domain appears to have evolved more rapidly than the intracellular TIR domain (36), comparative sequence analysis revealed a conservation of the position of the critical PGN recognition leucine residues (50, 51, 61) in the carp TLR2 extracellular domain. Transfection of human (HEK 293) cells with carp TLR2 confirmed the ability of prototypical TLR2 ligands (LTA, PGN, and Pam$_3$CSK$_4$) to trigger MAPK-p38 activation. In contrast, carp TLR2 did not recognize the TLR2 ligand MALP-2 in transfected HEK 293 cells.

TLR activation in humans is often measured via quantification of NF-κB activation or downstream expression of cytokines such as IL-1β and TNF-α. We characterized activation of fish macrophages by determining the expression profile of several cytokine genes, ROS and NO production and MAPK-p38 phosphorylation (62). We identified

**FIGURE 8.** Effect of MAPK-p38 activation and ROS production on TLR2 gene expression. Macrophages were preincubated for 30 min with inhibitors of NF-κB (PDTC, 5 μM), MAPK-p38 (SB203580, 25 μM), xanthine oxidase (allopurinol, 200 μM), NADPH oxidase (DPI, 20 μM), or mitochondrial electron transfer chain subunit I (rotenone, 10 μM). A and C. Macrophages were further stimulated for 6 h with PGN (50 μg/ml). TLR2 gene expression was measured by means of RT-qPCR. 40S ribosomal protein gene was used as a housekeeping gene and results are expressed as mRNA fold change in stimulated cells relative to untreated cells (fold change = 1). Averages ± SD of n = 4 fish are given. *Significant (p ≤ 0.05) difference compared with PGN-stimulated cells. B and D. Macrophages were further incubated for 1 h with DHR (0.25 μg/ml) and PMA (0.05 μg/ml), followed by PGN (50 μg/ml). Radical production was measured by means of DHR fluorescence intensity. Results are expressed as mean fluorescence intensity relative to PMA-stimulated cells (mean fluorescence intensity = 15). Averages ± SD of n = 4 fish are given. *Significant (p ≤ 0.05) difference compared with PGN-stimulated cells.
The unresponsiveness of fish cells to MALP-2, recognized by TLR2-TLR6 heterodimers in mammalian vertebrates (12), is consistent with the unresponsiveness of TLR2-transfected HEK 293 cells to MALP-2. Apparently, overexpression of TLR2 homodimers in HEK 293 cells does not overcome the requirement for TLR2-TLR6 heterodimerization. In mammalian vertebrates, it is clear that distinct TLR2-containing receptor complexes allow for the accommodation of structurally diverse TLR2 ligands (62) and the ability of TLR2 to detect a relatively wide array of PAMPs has been attributed to a functional interaction with a number of other receptors (64, 65), including TLR1 and TLR6. TLR1, 6 and 10 are thought to have diverged from a common ancestral gene (66). The presence of a putative TLR1 homolog in fish (17, 67) is presently under investigation, which would allow for future studies on TLR2-TLR6 heterodimerization and detailed studies into recognition of triacylated lipopeptides. TLR6 does not seem to have an ortholog in teleost fish (17, 21). The apparent absence of a functional TLR6 homolog in carp could contribute to the unresponsiveness to MALP-2 observed in carp macrophages.

LTA consistently stimulated downstream cytokine gene expression with a delayed kinetic profile when compared with PGN, an observation also made for mammalian vertebrates (62). PGN always more clearly than LTA induced NO, ROS production, and MAPK-p38 phosphorylation. Differences in kinetics were previously suggested to be the result of distinct TLR2 coreceptors usage and signaling after cellular trafficking of these complexes (62). Although yet to be characterized, the distinct cellular responses could possibly be attributed to the use of distinct TLR2-containing receptor complexes on fish macrophages. Different recruitment and/or kinetics of TLR 2-specific adaptor proteins (68) on activation of TLR2-containing complexes may lead to ligand-specific responses and ultimately have profound effects on cellular response to LTA and PGN.

Fish cells did respond to both LTA and PGN, albeit at high concentrations (35, 63, 69), which fact could not simply be ascribed to the ligand preparations because we used highly purified TLR2 ligands throughout our study. LTA from <i>S. aureus</i> was obtained by butanol extraction that preserves its molecular structure (70, 71). PGN was a soluble polymeric high molecular-weight preparation purified from supernatants of <i>S. aureus</i> grown in the presence of penicillin, devoid of teichoic acids or other proteins (72). As stated previously, the ability of TLR2 to detect a relatively wide array of PAMPs has been attributed to a functional interaction with a number of other receptors (64, 65). These not only include TLR1 and TLR6, but also the lipid scavenger receptor CD36 and the CD14 protein. A CD36 gene sequence is present in zebrafish but no functional studies have been reported on the formation of a functional receptor complex of fish TLR2 with the CD36 lipid scavenger receptor. LTA and PGN also can interact with CD14 (73–77). A recent report (78), in fact, suggests that the main function of CD36 is to bind and transfer diacylglycerol ligands (e.g., lipomannan, LTA) onto TLR2/TLR6, in a CD14-dependent manner. So far, CD14 has not been detected in any fish genome or expressed in fish macrophages under influence of hydrogen peroxide. A, TLR2 gene expression in carp macrophages after stimulation for 6 h with H2O2 (0.1, 0.25, 0.5, and 1 mM). Averages and SD of n = 4 fish are given. mRNA levels of TLR2 are relative to the housekeeping 40S ribosomal protein gene level and expressed as fold change in stimulated cells relative to unstimulated cells (fold change = 1). *Significant (p ≤ 0.05) difference compared with unstimulated cells (control group). B, TLR2 gene expression in carp macrophages after preincubation with catalase (1 or 2 U/ml) for 30 min, followed by stimulation with PGN (50 μg/ml). Averages ± SD of n = 4 fish are given. *Significant (p ≤ 0.05) difference compared with PGN-stimulated cells. C, MAPK-p38 activation in carp macrophages incubated for 5 min with increasing concentrations of H2O2 (1, 2.5, and 5 mM). MAPK-p38 phosphorylation was analyzed by immunoblotting for phospho-p38, whereas equal loading was confirmed by immunoblotting for β-tubulin.

FIGURE 9. TLR2 gene expression and MAPK-p38 activation in carp macrophages under influence of hydrogen peroxide. A, TLR2 gene expression in carp macrophages after stimulation for 6 h with H2O2 (0.1, 0.25, 0.5, and 1 mM). Averages and SD of n = 4 fish are given. mRNA levels of TLR2 are relative to the housekeeping 40S ribosomal protein gene level and expressed as fold change in stimulated cells relative to unstimulated cells (fold change = 1). *Significant (p ≤ 0.05) difference compared with unstimulated cells (control group). B, TLR2 gene expression in carp macrophages after preincubation with catalase (1 or 2 U/ml) for 30 min, followed by stimulation with PGN (50 μg/ml). Averages ± SD of n = 4 fish are given. *Significant (p ≤ 0.05) difference compared with PGN-stimulated cells. C, MAPK-p38 activation in carp macrophages incubated for 5 min with increasing concentrations of H2O2 (1, 2.5, and 5 mM). MAPK-p38 phosphorylation was analyzed by immunoblotting for phospho-p38, whereas equal loading was confirmed by immunoblotting for β-tubulin.

similarities but also clear differences between the mammalian vertebrate and carp TLR2-mediated response. We observed in fish macrophages 1) low responsiveness to Pam3CSK4, 2) nonresponsiveness to MALP-2, 3) delayed gene expression kinetics and distinct cellular responses (radical production and MAPK pathway activation) to LTA as compared with PGN, and 4) a requirement for at least five times higher doses of LTA and PGN than in humans.

The low responsiveness of fish cells to Pam3CSK4 is in contrast with the observation that carp TLR2-transfected HEK 293 cells clearly do bind Pam3CSK4. In mammalian vertebrates, Pam3CSK4 is recognized by a TLR2-TLR1 heterodimer (54). Apparently, overexpression of TLR2 homodimers in HEK 293 cells overcomes the requirement for TLR2-TLR1 heterodimerization and is sufficient for the recognition of Pam3CSK4. Overexpression of TLR2 homodimers in carp macrophages indeed improved recognition of Pam3CSK4 as shown by increased iNOS gene expression. The low responsiveness to Pam3CSK4 observed in carp is in line with previous reports in gilthead seabream (63) and rainbow trout (35). In both studies a minor modulation of cytokine gene expression with Pam3CSK4 was observed; however, no additional prototypical TLR2 ligands were used.

The unresponsiveness of fish cells to MALP-2, recognized by TLR2-TLR6 heterodimers in mammalian vertebrates (12), is consistent with the unresponsiveness of TLR2-transfected HEK 293 cells to MALP-2. Apparently, overexpression of TLR2 homodimers in HEK 293 cells does not overcome the requirement for TLR2-TLR6 heterodimerization. In mammalian vertebrates, it is clear that distinct TLR2-containing receptor complexes allow for the accommodation of structurally diverse TLR2 ligands (62) and the ability of TLR2 to detect a relatively wide array of PAMPs has been attributed to a functional interaction with a number of other receptors (64, 65), including TLR1 and TLR6. TLR1, 6 and 10 are thought to have diverged from a common ancestral gene (66). The presence of a putative TLR1 homolog in fish (17, 67) is presently under investigation, which would allow for future studies on TLR2-TLR6 heterodimerization and detailed studies into recognition of triacylated lipopeptides. TLR6 does not seem to have an ortholog in teleost fish (17, 21). The apparent absence of a functional TLR6 homolog in carp could contribute to the unresponsiveness to MALP-2 observed in carp macrophages.
Sequence Tag database, suggesting the absence of this coreceptor in fish. If true, the absence of CD14 could not only contribute to the well-known hyporesponsiveness of fish cells to Gram-negative (26, 79) bacteria but also to the hyporesponsiveness of fish cells to ligands from Gram-positive bacteria reported in this study.

Of course, carp macrophages undoubtedly express innate receptors additional to TLR2 and it is impossible to unambiguously ascribe activation of carp macrophages by TLR2 ligands to the activation of TLR2 receptors only. In mammalian vertebrates, several families of PRRs have been shown to detect PGN from Gram-positive bacteria including TLR2 (60), nucleotide-binding oligomerization domain 2 (Nod2) and PGN recognition proteins (PGRs) (80). In zebrasfish it was shown that suppression of PGRP6 decreased significantly the expression of TLR2 mRNA suggesting that TLR2 and PGRP may cooperatively recognize PGN (81). Without access to knock-out phenotypes it remains difficult to exclude the involvement of other receptors on carp macrophages, such as Nod2. However, stimulation of carp macrophages with muramyl dipeptide; the minimum PGN fragment recognized by Nod2 (82), did not induce ROS and NO to the same extent as PGN (unpublished observation). This suggests that Nod2 did not play the major role in the stimulation by PGN we observed in carp macrophages. Furthermore, overexpression of TLR2 in carp macrophages led to a more pronounced induction of downstream cytokine gene expression in response to LTA, PGN and, to a minor extent, Pam3CSK4. These results corroborate the ability of carp TLR2 to bind LTA and PGN from S. aureus and to trigger TLR2-dependent downstream activation pathways in response to these ligands in carp macrophages. In conclusion, we provide evidence that fish macrophages can be activated by ligands from Gram-positive bacteria that are prototypically activators of mammalian vertebrate TLR2, but require relatively high concentrations.

We investigated whether TLR2 ligands could modulate the expression of the carp TLR2 gene itself. Clearly, carp TLR2 gene expression was regulated in a consistent manner throughout our studies, although to a maximum of 2-fold upregulation. In mammalian vertebrates, expression patterns of the TLR2 gene itself are divergent. In human monocytes, TLR2 mRNA is upregulated after adherence to tissue culture plates but cannot be further induced (83). In murine monocytes, TLR2 mRNA is low or undetectable in vitro but can be strongly induced (84). In cell lines, 2- to 3-fold increase of TLR2 gene expression has been observed in murine (RAW 264.7) and human (HL-60) macrophages (85). In our study, GPI anchors from protozoan parasites of carp showed a dose-dependent upregulation of TLR2 gene expression. It is known that the carp protozoan T. borelli parasite induces radical production in carp macrophages (86). We are currently defining the involvement of TLR2 in the recognition of GPI anchors from protozoan parasites. Further studies will elucidate the contribution of TLR2 to the immune response induced by protozoan parasites in carp.

To further investigate the regulation of TLR2 gene expression, we studied whether TLR2 ligands could induce posttranscriptional stabilization of carp TLR2 mRNA. Indeed, LTA and PGN specifically increased TLR2 mRNA stability that could contribute to the consistent 2-fold inducibility of TLR2 gene expression. Changes in mRNA stability for TLR2 in mammalian vertebrates are yet to be reported. Stimulation of carp macrophages with PGN clearly induced MAPK-p38 phosphorylation and increased NO and ROS production. MAPK-p38 activation was dependent on NADPH oxidase-derived radicals, in particular H2O2, suggesting that the MAPK-p38 activation pathway in fish is redox sensitive. Complementary, PGN-induced radicals were NADPH oxidase as occurs in humans (87). Moreover, PGN-induced radicals could be further increased by the presence of SOD, suggesting that H2O2 production occurs after TLR2 engagement. We found that PGN-induced radicals derived from NADPH oxidase were necessary for maximal expression of carp TLR2 gene expression. Furthermore, catalase significantly inhibited TLR2 gene expression, whereas H2O2 significantly induced TLR2 gene expression. In our study, MAPK-p38 was required for an effective PGN-induced TLR2 expression and posttranscriptional stability, suggesting a clear role for MAPK-p38 in the regulation of TLR2 gene expression in carp. Collectively, these results suggest that H2O2 radicals via MAPK-p38 activation play an indispensable role in the regulation of TLR2 gene expression itself in carp macrophages. In mice, ROS-dependent activation of the TRAF6-ASK1-p38 pathway is selectively required for TLR4 innate immunity (88). In humans, ASK1-MAPK-p38-p47 phox activation is essential for inflammatory responses during tuberculosis via TLR2-ROS signaling (89). These observations suggest an important role for ROS as second messengers in TLR-mediated signaling pathways. We show an important role for ROS, in particular H2O2, on TLR2 gene expression in carp macrophages. We demonstrated for the first time, in carp, that bidirectional communication between ROS and activated MAPK-p38, besides shaping the TLR-mediated response, has a direct effect on the level of TLR2 gene expression.

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Disclosures

The authors have no financial conflicts of interest.

References


Irnazarow, I. 1995. Genetic variability of Polish and Hungarian carp lines (Cyprinus carpio) TNF-


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