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The Gene History of Zebrafish tlr4a and tlr4b Is Predictive of Their Divergent Functions

Con Sullivan,‡ Jeremy Charette,* Julian Catchen,§ Christopher R. Lage,* Gregory Giasson,* John H. Postlethwait,¶ Paul J. Millard,† and Carol H. Kim‡

Mammalian immune responses to LPS exposure are typified by the robust induction of NF-κB and IFN-β responses largely mediated by TLR4 signal transduction pathways. In contrast to mammals, Tlr4 signal transduction pathways in nontetrapods are not well understood. Comprehensive syntenic and phylogenetic analyses support our hypothesis that zebrafish tlr4a and tlr4b genes are paralogous rather than orthologous to human TLR4. Furthermore, we provide evidence to support our assertion that the in vivo responsiveness of zebrafish to LPS exposure is not mediated by Tlr4a and Tlr4b paralogs because they fail to respond to LPS stimulation in vitro. Zebrafish Tlr4a and Tlr4b paralogs were also unresponsive to heat-killed Escherichia coli and Legionella pneumophila. Using chimeric molecules in which portions of the zebrafish Tlr4 proteins were fused to portions of the mouse TLR4 protein, we show that the lack of responsiveness to LPS was most likely due to the inability of the extracellular portions of zebrafish Tlr4a and Tlr4b to recognize the molecule, rather than to changes in their capacities to transduce signals through their Toll/IL-1 receptor (TIR) domains. Taken together, these findings strongly support the notion that zebrafish tlr4a and tlr4b paralogs have evolved to provide alternative ligand specificities to the Tlr immune defense system in this species. These data demonstrate that intensive examination of gene histories when describing the Tlr proteins of basally diverging vertebrates is required to obtain fuller appreciation of the evolution of their function. These studies provide the first evidence for the functional evolution of a novel Tlr.

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The landmark discovery of a human homolog to the Droso phila Toll protein (1), which later became known as TLR4 (2), dramatically transformed the immunology paradigm, forcing a reevaluation of our understanding of the immune system to include concepts related to innate immunity. Since this initial breakthrough, additional vertebrate TLR genes have been identified and their fundamental importance to immunity has gathered greater appreciation. The TLR family of pathogen-recognition receptors forms a central pillar in the immune defense of all vertebrates, from fishes to mammals. TLR proteins function as sentinels against infection, participating from the earliest innate immune responses to the eventual transition to adaptive responses (3). Upon recognition of conserved pathogen-associated molecular patterns, TLR proteins trigger signal transduction events resulting in the activation of cytokines, antimicrobial peptides, and other immune response genes.

Mammalian TLR4 function as central proteins in LPS receptor complexes (4–8). LPS is a conserved glycolipid common to Gram-negative bacteria. LPS, facilitated by its association with LPS-binding protein (LBP) (9, 10), interacts with CD14 at its N-terminal binding pocket (11) and then is transferred to TLR4, which is complexed with MD2. MD2 is a glycoprotein responsible for TLR4 localization and responsiveness to LPS (12, 13). The TLR4-MD2 complex homodimerizes and recruits intracellular adaptor proteins to the Toll-IL-1 receptor (TIR) (3) domain of TLR4. Bifurcated signaling processes, which have been termed MyD88-dependent and MyD88-independent, are triggered, resulting in pro- and antiinflammatory immune responses (reviewed in Ref. 14).

There has been an increased effort to identify tlr genes from lower vertebrates, particularly the fishes (15); however, the classification of Tlr proteins in basally diverging vertebrates has tended to rely solely on phylogenies. The failure to investigate the gene histories and functions of tlr genes has led to the false presumption that sequence homology equates to functional conservation. In truth, the way these Tlr proteins are induced and the transduction pathways through which they signal are poorly understood in basally diverging vertebrates. The data presented herein demonstrate that zebrafish are responsive to LPS but through a mechanism that is independent of the prototypical TLR4-MD2 multi-protein LPS receptor complexes seen in mammals. Using a series of

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Abbreviations used in this paper: LBP, LPS-binding protein; TIR, Toll/IL-1 receptor; LRR, leucine-rich repeat; SHRV, snakehead rhadinovirus; dpl, days postinfection; hpe, hours postexposure; ZFL, zebrafish liver cells; mx, myxovirus (influenza virus) resistance gene; qRT-PCR, quantitative RT-PCT; HKEC, heat-killed Escherichia coli; HKLP, heat-killed Legionella pneumophila; TICAM, TIR domain-containing adaptor molecule.
of chimeric mouse-zebrafish and zebrafish-mouse Tlr4 constructs, we demonstrate that the failure of zebrafish Tlr4 proteins to respond to LPS is due to differences in the extracellular domains and not to the transmembrane or intracellular domains. We observe that the transmembrane and intracellular domains of the zebrafish Tlr4a and Tlr4b can mediate a positive NF-κB response when fused to extracellular mouse TLR4 domains, contradicting a recent statement that Tlr4a and Tlr4b TIR domains negatively regulated NF-κB activation (16). Furthermore, we show through analyses of conserved syntenies that zebrafish tlr4a and tlr4b are not orthologous to human TLR4 but rather are paralogs. Our findings confirm the role that these tlr4 paralogs play in zebrafish immunity and support the hypotheses that alternative LPS induction pathways predominate in fishes and that zebrafish Tlr4 proteins offer alternative, LPS-independent ligand specificities.

Materials and Methods

**Nomenclature conventions**

Nomenclature rules for zebrafish, chicken, mouse, and human genes and proteins differ. Gene and protein names are presented according to the respective nomenclature conventions (species, gene, protein: zebrafish, tlr4, Tlr4; human and chicken, TLR4, Tlr4; and mouse, Tlr4, TLR4; see http://zfin.org/zf_info/nomen.html).

**DNA constructs**

A full-length zebrafish homolog of human TLR4, designated tlr4a, was identified through BLAST sequence analyses of zebrafish genome and available Tlr4a TIR domain sequence data (17). Based on predicted sequence, we designed primers tlr4a F1 (5'-CTGCGAGTTCTTGATCTGT -H11032) and tlr4a R1 (5'-ACGTTGTTGCATTGTATGCTGA -H11032). Total RNA was extracted from the livers, kidneys, and spleens of adult zebrafish with TRIzol (Invitrogen) and reverse transcribed with random hexamers using the ImProm-II reverse transcription system (Promega), as described previously (18). The tlr4a gene was cloned by PCR from this cDNA pool with Phusion polymerase (New England Biolabs) under the following reaction conditions: 1 cycle at 98°C, 38 cycles at 98°C for 10 s, 58°C for 15 s, 72°C for 90 s; 1 cycle at 72°C for 5 min. A full-length zebrafish tlr4b clone (cDNA clone MGC:85690; IMAGE:6961784) was purchased from American Type Culture Collection. Mouse Tlr4 (mTlr4), human MD2, human CD14, and the NF-κB luciferase reporter construct pBIIx-luc were gifts from R. Medzhitov (Yale University, New Haven, CT). The zebrafish expression vector fm2bl (19) was a gift from P. Gibson (University of Miami, Miami, FL).

For luciferase assays, full-length tlr4a, tlr4b, and mTlr4 were subcloned in fm2bl, resulting in the removal of blue-variant egfp gene. Deletion of the leucine-rich repeat (LRR) of TLR proteins has been shown to result in the creation of constitutively active mutants (1, 8, 20). Due to a truncated N terminus, the Tlr4a extracellular, LRR deletion construct was created by amplification with primers Tlr4aCAF and Tlr4aCAR (supplemental Table S1). Total RNA was extracted from the livers, kidneys, and spleens of adult zebrafish with TRIzol (Invitrogen) and reverse transcribed with random hexamers using the ImProm-II reverse transcription system (Promega), as described previously (18). The tlr4a gene was cloned by PCR from this cDNA pool with Phusion polymerase (New England Biolabs) under the following reaction conditions: 1 cycle at 98°C, 38 cycles at 98°C for 10 s, 58°C for 15 s, 72°C for 90 s; 1 cycle at 72°C for 5 min. A full-length zebrafish tlr4b clone (cDNA clone MGC:85690; IMAGE:6961784) was purchased from American Type Culture Collection. Mouse Tlr4 (mTlr4), human MD2, human CD14, and the NF-κB luciferase reporter construct pBIIx-luc were gifts from R. Medzhitov (Yale University, New Haven, CT). The zebrafish expression vector fm2bl (19) was a gift from P. Gibson (University of Miami, Miami, FL).

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**Paralogy mapping**

To identify paralogons in the human genome, we created a pipeline that modifies the methods of Dehal and Boore (23) using data downloaded from Ensembl (www.ensembl.org/Homo_sapiens/index.html) (24). The pipeline starts with a BLASTN search for each human chromosome 10 (Hsa10) gene against the human genome. The pipeline then scores the top five results, keeping all of the reciprocal best hits. It then looks at the location of each reciprocal best hit and labels the genes as “paralogous duplications” if they are on a different chromosome or more than 1 Mb away on the same chromosome, or tandem duplications if the two sequences are closer than 1 Mb. The distant paralogs constitute paralogous groups. For each of the paralog groups, the pipeline BLASTPs each individual gene in the group against the genome of the urochordate Ciona intestinalis, whose lineage diverged from the vertebrate lineage before the amplification of the vertebrate genome (25). All of the human genes must BLASTP to the same C. intestinalis gene, and the C. intestinalis gene must BLASTP back as one of the top X hits (assuming X human genes in the paralogy group). Nonparalogy gene paralogs that perform as such are considered “verified” and are plotted on a display of human chromosomes (see Fig. 5).

**Zebrafish care and maintenance**

AB strain zebrafish were maintained at 28°C in recirculating systems (flow rate of 150 liters/min) designed by Aquatic Habitats in accordance with procedures and guidelines outlined by the Institutional Animal Care and Use Committee.

**RNA isolation and cDNA synthesis from zebrafish following static immersion in LPS or snakehead rhabdovirus (SHRV)**

Zebrafish aged 20–30 days postfertilization (dpf) were exposed to 10 μg/ml LPS (Escherichia coli 055:B5) (Sigma-Aldrich) for 24 h or 10% 50% tissue culture-infective dose per milliliter of SHRV for 5 h. Mock control exposures were also performed. At the end of the exposure duration, fish were returned to the isolated flow-through system for the remainder of the experiment. Three to five fish were collected at each of the following time points and preserved in TRIZol (Invitrogen) at −80°C until processed: 0, 6, 12, 24, 48, 72, and 96 h postexposure (hpe). Total RNA was extracted from control and control fish by the TRIZol method, with modifications to the manufacturer’s recommendations. First-strand cDNA synthesis was performed with random hexamers using the ImProm-II reverse transcription system (Promega), as described previously (18).
Cell culture and SHRV propagation

Human embryonic kidney 293H cells (Invitrogen) were cultured at 37°C, 6% CO2 in DMEM (high glucose) supplemented with 10% heat-inactivated FBS. Epithelioma papulosum cynepi (EPC) cells were cultured at 28°C, 5% CO2 in MEM supplemented with 10% heat-inactivated FBS. Zebrafish liver (ZFL) cells were cultured at 28°C in LDF media (21). SHRV was propagated in EPC cells, and viral titers were quantified by determining the 50% tissue culture-infective doses per milliliter (22).

PCR and quantitative RT-PCR

Total RNA was extracted from cultured ZFL cells by TRIzol method (Invitrogen) and reverse transcribed into first-strand cDNA with random hexamers using the ImProm-II reverse transcription system (18). Primers were designed to amplify a 976-bp myd88 fragment, a 1971-bp tcam1 fragment, and a 726-bp tirap fragment from this ZFL cDNA pool (supplemental Table S1C). The amplicons were generated using the following PCR conditions: 98°C for 2 min followed by 40 cycles of 98°C for 10 s, 62°C for 15 s, 72°C for 20 s, repeated for 40 cycles. Also, tcam1, tcam2, and mx qRT-PCR were performed under the following conditions, after the initial 3-min polymerase activation step: 95°C for 15 s, 54°C for 15 s, 72°C for 20 s, repeated for 40 cycles. All transcript levels were normalized to β-actin-1 (bactin1). For bactin1, qRT-PCR was performed at 94°C for 30 s, 53°C for 30 s, 72°C for 30 s, repeated for 40 cycles. Representative data are presented as fold induction ± SEM.

Heat-killed E. coli (HKEC) and Legionella pneumophila (HKLP)

Colonies of E. coli (29839; American Type Culture Collection) were isolated on Luria-Bertani agar plates. An individual colony was cultured in Luria-Bertani broth until an OD600 of 2.4 was reached. Cells were pelleted and washed in Dulbecco’s PBS. Cells were pelleted again, resuspended in Dulbecco’s PBS to a concentration of 1010 CFU/ml, and then heat-killed at 70°C for 15 min. HKLP was purchased from InvivoGen and resuspended to 10^9 CFU/ml in endotoxin-free distilled water before use.

NF-κB luciferase reporter assays

293H and ZFL cells were plated in 24-well plates (Corning) so that they were 90–95% confluent on the day of transfection. To assay the responsiveness of Tlr4a and Tlr4b to LPS, HKEC, or HKLP in the presence of mammalian TLR4 pathway accessory proteins MD2 and CD14 (mTLR4-positive control), 293H or ZFL cells were transfected with 133 ng of a TLR4 construct (tlr4a, tlr4b, mTLR4, mTLR2 (for L. pneumophila experiment), or frm empty vector control), 133 ng of MD2, and 133 ng of CD14, along with 400 ng of pBIIx-luc NF-κB-luciferase reporter construct, and 10 ng of pRL-CMV (Promega) Renilla luciferase internal control construct using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol. For coexpression studies, 66.5 ng of tlr4a and 66.5 ng of tlr4b were used. To determine the responsiveness of Tlr4a and Tlr4b to LPS in the absence of MD2 and CD14 (mTLR4-negative control), 293H or ZFL cells were transfected with 133 ng of a TLR4 construct (tlr4a, tlr4b, mTLR4, or frm empty vector control), 266 ng of pcDNA3.1 empty vector, along with 400 ng of pBIIx-luc NF-κB-luciferase reporter construct, and 10 ng of pRL-CMV Renilla luciferase internal control construct using Lipofectamine 2000. As described, when expressed in combination, 66.5 ng of tlr4a and 66.5 ng of tlr4b were transfected. The responsiveness of the chimeric constructs to LPS was measured following transfection of 293H with 133 ng of the chimeric construct (tlr4amTLR4, tlr4b-mTLR4, tlr3a-tlr4a, or tlr3b: tlr4b), 133 ng of MD2, 133 ng of CD14, 400 ng of pBIIx-luc NF-κB-luciferase reporter construct, and 10 ng of pRL-CMV Renilla luciferase internal control construct.

Twenty-four hours posttransfection, cells were exposed to one of the following treatments: LPS (E. coli) 011:B4; InvivoGen at a concentration 10 μg/ml for 6 h, HKEC at a concentration of 10^6 CFU/ml for 6 h, or HKLP at 10^8 CFU/ml for 6 h. Mock exposures were simultaneously performed using respective diluents. Following exposure, cells were lysed and firefly and Renilla luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega).

Deletion of the LRR from TLR4 proteins has been shown to lead to their constitutive activation (1, 8, 20). To determine the capacity of Tlr4a and Tlr4b to constitutively activate an NF-κB-luciferase reporter, 293H and ZFL cells were transfected with 400 ng of frm plasmid encoding an individual Tlr4 construct (Tlr4a, Tlr4aΔLRR, Tlr4b, or Tlr4bΔLRR), 400 ng of pBIIx-luc NF-κB-luciferase reporter construct, and 10 ng of pRL-CMV (Promega) Renilla luciferase internal control construct using Lipofectamine 2000, according to the manufacturer’s protocol. To test for synergism between Tlr4a and Tlr4b and between Tlr4aΔLRR and Tlr4bΔLRR, 293H and ZFL cells were transfected with 200 ng of Tlr4 and 200 ng of Tlr4b or 200 ng of Tlr4aΔLRR and 200 ng of Tlr4bΔLRR, along with 400 ng of pBIIx-luc NF-κB-luciferase reporter construct, and 10 ng of pRL-CMV Renilla luciferase internal control construct (Promega) 2000. For each experiment, cells were lysed 24 h posttransfection, and luciferase activities were measured using the Dual-Luciferase reporter assay system.

An frm expression plasmid containing myd88 was used to assay its effect on NF-κB activation, using the aforementioned NF-κB-luciferase reporter. ZFL cells were transfected with 10 ng, 100 ng, or 400 ng of the frm-myd88 plasmid, along with 400 ng of pBIIx-luc NF-κB-luciferase reporter construct, and 10 ng of pRL-CMV Renilla luciferase internal control construct using Lipofectamine 2000, according to the manufacturer’s protocol. Empty frm plasmid was used as a negative control and was also added when necessary to ensure that the total amount of frm plasmid was 400 ng (0 ng of frm-myd88, 400 ng of frm-empty; 10 ng of frm-myd88, 390 ng of frm-empty; 100 ng of frm-myd88, 300 ng of frm-empty; and 400 ng of frm-empty). Cells were lysed 24 h posttransfection, and luciferase activities were measured using the Dual-Luciferase reporter assay system.

Results

Zebrafish exposed by static immersion to LPS exhibit elevated il1 and tnfa transcript levels but no antiviral response

In mammals, exposure to the LPS present on Gram-negative bacteria triggers inflammatory immune responses through TLR4. TLR4 signaling initiates bifurcated signal transduction cascades leading to the up-regulation of assorted immune-responsive genes. One part of this signaling cascade is MYD88-dependent and relies on MYD88 and TLR4 (TRAP) to initiate an early proinflammatory response typified by the increased expression of il1 and tnfα transcripts (26). The other part of this signaling cascade is MYD88-independent and relies on TIR domain-containing adaptor molecule-1 (TICAM1, also known as TRIF) and TICAM2 (TRAM) to initiate a later NF-κB and an antiviral type I IFN response (27). In zebrafish, myd88 and mal are present in the genome, but tcam2 is not (21), and thus we hypothesize that if zebrafish Tlr4 proteins could recognize LPS, their signaling cascades would involve a proinflammatory component but not an antiviral response.

To test our hypothesis, zebrafish aged 20–30 dpf were exposed by static immersion to 10 μg/ml LPS for 24 h. Whole body samples were collected at specific time points following this initial exposure and stored in TRIzol. Previous studies have shown that zebrafish exposed to bacterial and viral pathogens by static immersion exhibit potent proinflammatory immune responses (18, 28). Robust increases in il1 and il10 transcript levels have been measured as part of the antibacterial response (28). Similarly, remarkable elevations in ifnα and ifnβ transcript levels are measured as part of the antiviral response (18). Upon exposure to LPS, our studies showed that zebrafish exhibited elevated levels of il1 transcripts, as measured by qRT-PCR, relative to controls (Fig. 1A). Changes were noted as early as 6 h, with an observed 11.4-fold increase in il1 expression relative to controls. Expression of il1 increased in the LPS-exposed groups and remained elevated through 96 h. Similarly, ifnα expression (Fig. 1B) was up-regulated most during the initial 24 hpe, peaking at 6 h when a
of protein responsive to Ifn stimulation (29), were minimally induced with peak expression of 178-fold over control observed at 24 hpe. The data contrasted, SHRV exposure induced very strong expression of 29.8-fold induction was observed. By 48 hpe, expression, with an initial peak of 1.9-fold over control at 6 hpe and a second peak of 2.5-fold over control at 72 hpe. In stark contrast, SHRV exposure induced very strong expression of mx, with peak expression of 178-fold over control observed at 24 hpe. Levels of mx remained strongly elevated relative to the controls through 96 hpe, when a 31.1-fold induction was recorded. The data indicate that, in contrast to mammals (30, 31), zebrafish do not exhibit a potent antiviral response to LPS stimulation, as is observed upon SHRV exposure.

Identification of zebrafish tlr4 homologs

Sequence encoding the TIR domain of zebrafish tlr4a, originally described by Meijer et al. (17), was used to search genomic databases in an effort to identify a putative full-length clone based upon an in silico prediction of the entire coding sequence. We amplified a 2297-bp clone of tlr4a containing a 2253 nt open reading frame by PCR and deposited the sequence in GenBank (accession no. EU551724 (www.ncbi.nlm.nih.gov/nuccore/190341704)). A full-length zebrafish tlr4b clone had previously been amplified (accession no. NM_212813 (www.ncbi.nlm.nih.gov/nuccore/47086406)), but its role has not been directly investigated. The zebrafish tlr4a gene encodes a 750-aa protein, with 38% identity and 57% similarity to 839-aa human TLR4 protein and 36% identity and 56% similarity to 835-aa mouse TLR4 protein. An in-frame stop codon was observed just upstream of the tlr4a start codon and accounts for the truncated N terminus. The zebrafish tlr4b gene encodes a slightly larger 819-aa protein, with 38% identity and 55% similarity to human TLR4 and 35% identity and 54% similarity to mouse TLR4. Zebrafish Tlr4a and Tlr4b share 67% identity and 78% similarity with each other. A ClustalW-based AlignX amino acid alignment of human TLR4, mouse TLR4, zebrafish Tlr4a, and zebrafish Tlr4b reveals sequence differences in the extracellular protein domains that may have functional consequences (Fig. 2). For example, in humans, the region between Glu24 and Lys47 has been shown to be essential for MD2 binding (32, 33). Close examination of our amino acid alignment reveals strong conservation of this region between mouse TLR4 and human TLR4. In contrast, zebrafish Tlr4a does not possess a homologous MD2 binding region, and zebrafish Tlr4b exhibits only 35% identity in this area. This difference, combined with the fact that an MD2 homolog is not observed in the zebrafish (34), strongly indicates that this structural difference may have significant effects on the mechanisms of signaling that zebrafish Tlr4a and Tlr4b use. Human TLR4 requires MD2 and the glycosylations that MD2 facilitates at Asn526 or Asn575 of TLR4 to be expressed on the cell surface and be LPS-responsive. Analysis of homologous residues in the zebrafish reveals that glycosylation sites with the canonical sequence Asp-Xxx-Ser/Thr (where Xxx represents any amino acid) are not present in either Tlr4a or Tlr4b. The significance of these differences is in question, however, due to the lack of MD2 in the zebrafish genome and the potential for compensatory adaptations through evolution. Indeed, when we stably expressed zebrafish Tlr4a-GFP and Tlr4b-GFP in ZFL cells, fluorescence appeared on the cell surface (supplemental Fig. S1), suggesting that Tlr4a and Tlr4b are transported to the cell surface through an MD2-independent mechanism.

In contrast to the extracellular domains, the intracellular portions of Tlr4a and Tlr4b, particularly the TIR domains, are more similar to mammalian TLR4 proteins. Alignment of the intracellular portions of the proteins reveal 50% identity and 66% similarity of residues for both zebrafish Tlr4a and Tlr4b when compared with human TLR4. In this intracellular region of the polypeptide, zebrafish Tlr4a and Tlr4b share 91% identity and 96% similarity with each other. This sequence homology is significant because it supports the notion that Tlr4a and Tlr4b differ from mammalian TLR4 proteins at the point of ligand recognition rather than at the level of signal transduction through adaptor proteins like Myd88.
Phylogenetic analyses of the extracellular and transmembrane portions from a broad representation of the TLR proteins predict that zebrafish Tlr4a and Tlr4b form a monophyletic group with mouse, human, and chicken TLR4s well separated from other TLRs (Fig. 3). These findings are supported by other analyses (15) and bolster the notion that tlr4a and tlr4b share a common evolutionary history with other TLR4 genes.

Genomics of the origin of tlr4a and tlr4b

To identify the location of tlr4a and tlr4b in the zebrafish genome, we designed mapping primers and located the genes on the LN54 radiation hybrid panel (22). Results placed tlr4a and tlr4b at a distance of 4.71 and 2.63 cR, respectively, from Z9049, showing that these two genes lie very close together on linkage group 13. The zv6 assembly of the zebrafish genome agrees with our mapping data and places tlr4a and tlr4b as nearest neighbors separated by ~7.2 kb and transcribed in the same direction from the same DNA strand.

The hypothesis that tlr4a and tlr4b are co-orthologs of the human TLR4 gene predicts that they should share conserved syntenies. To test this proposition, we examined the orthologies of loci surrounding the zebrafish tlr4 genes using the best reciprocal BLAST hit as the criterion for orthology (36). Results showed that of the 12 annotated sequences surrounding the tlr4 genes (Fig. 4B), seven were orthologous to genes in three small regions on the long arm of human chromosome 10 (Hsa10q, Fig. 4A). Others were orthologous by this criterion to human genes in Hsa2, Hsa4, but none were from Hsa9, the location of TLR4. This situation is not consistent with the hypothesis of orthology.
as predicted if tlr4a and tlr4b occupy a chromosome segment conserved with the region surrounding TLR4 in the human genome.

To investigate whether this situation is similar among teleost fish, we examined the genome of the green-spotted pufferfish, *Tetraodon nigroviridis* (Tni). Results showed that a series of six genes in pufferfish were orthologous to genes to the right and left of the zebrafish tlr4 genes, but there was no tlr4 sequence in this portion of the pufferfish genome (Fig. 4C), and in fact, the pufferfish genome database has no ortholog of TLR4. The closest sequence to human or zebrafish Tlr4 genes in *T. nigroviridis* is GSTENT00026075001, which is orthologous to human TLR7 on HsaXp22. Furthermore, neighbors of GSTENT00026075001 are also orthologous to HsaXp22 genes, confirming orthology by conserved synteny. Two possible hypotheses can explain these results. First, the zebrafish tlr4 genes may have been translocated into their current location in the middle of Hsa10q orthologs after the divergence of the zebrafish and pufferfish lineages and, in addition, the teleost tlr4 gene was deleted from the pufferfish lineage, a hypothesis requiring two steps. Second, and alternatively, the teleost tlr4 gene was in a location orthologous to the zebrafish location in the last common ancestor of zebrafish and pufferfish and a single event deleted it from the pufferfish lineage. Because the latter hypothesis requires a single step, it is preferred by parsimony. The loss of the tlr4 genes from the pufferfish genome and their retention in the zebrafish genome leads us to speculate that there may be functional redundancies in the pufferfish that make tlr4 genes dispensable. As a corollary, their presence in the zebrafish genome is suggestive of a potential loss of this functional redundancy in the zebrafish through the sub- or neofunctionalization of the Tlr4 or other Tlr proteins.
paralogy mainly with Hsa8p, Hsa5q, Hsa4, and Hsa2p. Significantly, the genes surrounding tlr4 genes in zebrafish that are not orthologous to genes on Hsa10q are highly similar to genes on Hsa2p or Hsa4q, suggesting that they may be paralogs, rather than orthologs of the human genes. This raises the hypothesis that the zebrafish tlr4 genes are paralogs, rather than orthologs, of the human TLR4 gene.

To explore this hypothesis, we determined the closest human paralogs of the human orthologs of the genes surrounding zebrafish tlr4 genes. The closest human paralog to PDZD7 is DFNB31, which is located ~3 Mb from the human TLR4 gene (Fig. 5B–D). Likewise, the closest human paralog of STOX1, whose apparent ortholog is the nearest neighbor of zebrafish tlr4a, is ~2 Mb from the human TLR3 gene (Fig. 5D–F). EIF4E, the human ortholog of the zebrafish sequence zgc:110154, is found on Hsa4q along with TLR2 and TLR3 (Fig. 5F). Human paralogs of the other neighbors of tlr4 genes are almost exclusively on Hsa2p (CXXC6/MGC22014, MAT1A/MAT2A), Hsa5 (SFXN3/SFXN1, MAT1A/MAT2B, EIF4E/EIF4E1B), and Hsa8 (CXXC6/KIAA1967). This evidence from the analysis of conserved syntenies suggests that the zebrafish tlr4 genes are paralogs, not orthologs, of the human TLR4 gene despite their position in the phylogenetic analysis.

Zebrafish Tlr4a and Tlr4b are unresponsive to LPS stimulation

Due to the significant sequence divergence observed between zebrafish Tlr4 proteins and mammalian TLR4 proteins, particularly in the extracellular domain, we sought to determine whether zebrafish Tlr4a and Tlr4b, alone or in combination as a potentially heterodimeric complex, were capable of activating an NF-κB-luciferase reporter, even though it was not anticipated that MD2 could interact with these proteins. In 293H cells and in the presence of CD14 and MD2, zebrafish Tlr4a alone, Tlr4b alone, and Tlr4a combined with Tlr4b exhibited no induction (1.0-fold) of the NF-κB-luciferase reporter (Fig. 6A) when compared with uninduced controls. In contrast, mTLR4 overexpression, when combined with CD14 and MD2, exhibited 2.2 ± 0.05-fold activation.
Forty-eight hours posttransfection, cells were lysed and luciferase activities were measured. The data are presented as normalized mean fold induction of NF-κB-luciferase reporter activity ± SEM. B, RT-PCR reveals expression of myd88, ticam1 (trif), and tirap (mal) in ZFL cells. Total RNA was extracted from ZFL cells and reverse transcribed to cDNA (+). A mock reverse transcription, in which the reverse transcriptase was excluded, was also performed (−). PCR was performed using gene-specific primers for myd88, ticam1 (trif), and tirap (mal) (see supplemental Table S1C). C, Full-length zebrafish myd88 was subcloned in frm2bl so that the egfp was replaced. ZFL cells were transfected with 10, 100, and 400 ng of myd88 plasmid, along with empty frm (no egfp) so that the total frm plasmid transfected was 400 ng. Additionally, 400 ng of pBIIx-luc and 10 ng of pRL-CMV were cotransfected. Forty-eight hours posttransfection, cells were lysed and luciferase activities were measured. The data are presented as normalized mean fold induction of NF-κB-luciferase reporter activity ± SEM over empty vector control. D, As in A, zebrafish Tlr4a and Tlr4b, alone and in combination, were overexpressed in ZFL cells, in the presence or absence of human CD14 and human MD2. mTLR4 was overexpressed as a positive control. pBIIx-luc and pRL-CMV were also cotransfected. Following transfection, cells were exposed to ultrapure LPS (10 µg/ml) for 6 h and then lysed. Firefly and Renilla relative luminescence units were recorded. The data are presented as the normalized mean fold induction of NF-κB-luciferase reporter activity ± SEM.

In the absence of CD14 and MD2, similar results were observed. Tlr4a exhibited 1.1 ± 0.25-fold activation; Tlr4b was induced 0.88 ± 0.27-fold; and the combined Tlr4a plus Tlr4b were activated 1.0 ± 0.21-fold. Without MD2 and CD14 coexpression, mTLR4 showed 0.72 ± 0.26-fold activation.

We have previously shown that ZFL cells were useful for measuring NF-κB activities in response to the overexpression of constitutively-activated tir3, traf6, irak4 (20), and ticam1 (trif) (21). We also demonstrate that ZFL cells express full-length myd88, tirap (mal), and ticam1 (Fig. 6B). Furthermore, ectopic expression of Myd88 leads to dose-dependent activation of an NF-κB-luciferase reporter (Fig. 6C). Despite the presence of these intact signaling cascades in ZFL cells, none of the Tir constructs tested exhibited significant activation of the NF-κB-luciferase reporter when cells were stimulated with LPS (Fig. 6D). When overexpressed in combination with CD14 and MD2, Tlr4a exhibited 0.73 ± 0.25-fold activation when compared with uninduced controls; Tlr4b was induced 0.79 ± 0.16-fold; Tlr4a and Tlr4b together was activated 0.77 ± 0.23-fold; and mTLR4 was induced 1.3 ± 0.29-fold. Likewise, in the absence of CD14 and MD2, Tlr4a showed 0.83 ± 0.17-fold activation; Tlr4b exhibits 1.1 ± 0.08-fold induction; Tlr4a and Tlr4b combined is activated 0.92 ± 0.06-fold; and mTLR4 is induced 0.87 ± 0.19-fold. Based on these data, we conclude that in ZFL cells, ectopic expression of zebrafish or mammalian TLR4 proteins followed by stimulation with LPS is not enough to activate a robust NF-κB response.

The failure of zebrafish Tlr4a and Tlr4b to respond to LPS is due to differences in their extracellular domains

We wanted to determine whether the lack of responsiveness by zebrafish Tlr4a and Tlr4b to LPS stimulation was due to differences in the extracellular domain or the transmembrane and intracellular domains, or both. Previous experiments have shown that invertebrate Toll proteins (37) and vertebrate Tlr proteins (1, 8, 20)
can be made constitutively active by the removal of the LRR regions. We sought to determine whether the removal of the LRRs from the zebrafish Tlr4a and Tlr4b proteins would result in their constitutive activation (Fig. 7A). In 293H cells, overexpression of the Tlr4aΔLRR led to a 9.5 ± 0.1-fold activation of the NF-κB-luciferase reporter (Fig. 7B). Similarly, a 3.8 ± 1.3-fold activation was observed in ZFL cells (Fig. 7C). Overexpression of the full-length Tlr4a failed to activate the NF-κB-luciferase reporter (1.1 ± 0.1-fold in 293H cells; 0.9 ± 0.2-fold in ZFL cells) (Fig. 7B and C). In stark contrast, overexpression of the Tlr4bΔLRR in 293H and ZFL cells failed to activate NF-κB-luciferase reporter expression (1.1 ± 0.02-fold in 293H cells; 0.9 ± 0.2-fold in ZFL cells) (Fig. 7B and C). Full-length Tlr4b similarly failed to activate the NF-κB-luciferase reporter (1.4 ± 0.1-fold in 293H cells; 1.3 ± 0.4-fold in ZFL cells). We tested for potential synergism between the Tlr4a and Tlr4b proteins by coexpression of the full-length or ΔLRR constructs in 293H cells, along with CD14, MD2, pBIIx-luc, and pRL-CMV. Cells were exposed to LPS (10 μg/ml) for 6 h and then lysed. Firefly and Renilla relative luminescence units were recorded. The data are presented as the normalized mean fold induction of NF-κB-luciferase reporter activity ± SEM.

FIGURE 7. The lack of LPS responsiveness is due to the extracellular domains of Tlr4a and Tlr4b. A, Schematic of native and chimeric Tlr4 constructs created by PCR sewing of DNA sequence encoding the mTLR4 extracellular domain to the transmembrane and intracellular domains of zebrafish Tlr4a and Tlr4b or the zebrafish Tlr4a and Tlr4b extracellular domains to the transmembrane and intracellular domains of mTLR4. The extracellular domain, including the LRR region, the transmembrane (TM) domain, and the intracellular domains, including the TIR domain, for the native and chimeric proteins are labeled accordingly. Numbers below each of the native proteins indicate the C terminus of the protein (left), the N-terminal portion of the transmembrane domain (middle), and the N terminus of the protein (right). B and C, Plasmids encoding Tlr4aΔLRR and Tlr4bΔLRR proteins were overexpressed by 200 ng of each, 400 ng total. D, Chimeric Tlr4 constructs were overexpressed in 293H cells, along with CD14, MD2, pBIIx-luc, and pRL-CMV. Cells were exposed to LPS (10 μg/ml) for 6 h and then lysed. Firefly and Renilla relative luminescence units were recorded. The data are presented as the normalized mean fold induction of NF-κB-luciferase reporter activity ± SEM.
293H or ZFL cells (Fig. 7, B and C). In light of 91% identity and 96% similarity between Tlr4a and Tlr4b across their TIR domains, these data were both surprising and concerning, particularly in light of recent findings by Sepulcre et al. (16). Our findings indicate that the overexpression of the Tlr4b TIR domain alone may not give an accurate representation of Tlr4b function.

To further investigate the roles of the LRR, transmembrane, and TIR domains and address this question about the lack of LPS responsiveness, we created chimeric Tlr4 proteins consisting of portions of the zebrafish Tlr4a or Tlr4b protein fused to a portion of the mTLR4 protein (Fig. 7A) through PCR sewing of genes: zebrafish Tlr4a or Tlr4b extracellular domains fused to mTLR4 transmembrane and intracellular domains (Tlr4a-mTLR4 or Tlr4b-mTLR4 respectively) and mTLR4 extracellular domain fused to zebrafish Tlr4a or Tlr4b transmembrane and intracellular domains (mTLR4-Tlr4a or mTLR4-Tlr4b, respectively). Each of these constructs was overexpressed in 293H cells, stimulation of Tlr4a-mTLR4 and Tlr4b-mTLR4 chimeric proteins with LPS failed to activate NF-κB-luciferase reporter activity (1.0 ± 0.01-fold and 0.98 ± 0.05-fold, respectively) (Fig. 7D). As a result, it is apparent that the extracellular portions of the zebrafish Tlr4a and Tlr4b fail to recognize LPS. In contrast, the expression construct encoding both mTLR4-Tlr4a and mTLR4-Tlr4b chimeric proteins exhibited robust activation upon stimulation with LPS (6.5 ± 0.27-fold and 9.5 ± 0.38-fold). These data support the notion that the transmembrane and intracellular domains of zebrafish Tlr4a and Tlr4b retain a capacity to interact with components of the intracellular downstream TLR signal transduction pathway (in human 293H cells, MyD88 and Mal and/or TICAM2 and TICAM1) and positively regulate NF-κB. These data directly contradict findings by Sepulcre et al. (16), who determined through overexpression of the TIR domain alone, rather than the entire protein, that Tlr4a and Tlr4b were negative regulators of NF-κB. Taken together, these data indicate that there may be ligands other than LPS that are recognized by Tlr4a and Tlr4b. It is also possible that Tlr4a and Tlr4b may function as coreceptors for each other or for other zebrafish Tlr proteins in a manner similar to the role mammalian TLR1 and TLR6 play in supporting the alternative ligand specificities of TLR2.

**Zebrafish Tlr4a and Tlr4b are unresponsive to stimulation by HKEC and HKLP**

The failure of Tlr4a and Tlr4b to respond to LPS leads to speculation regarding their ligand specificities. To begin to address this question, we attempted to stimulate Tlr4a and Tlr4b with HKEC and HKLP. Both HKEC and HKLP can present an assortment of ligands, in addition to LPS, to the receptors (Fig. 8). For HKEC stimulation, empty vector, Tlr4a, Tlr4b, Tlr4a and Tlr4b in combination, and mTLR4 were overexpressed in 293H cells along with CD14, MD2, pBluescript and pRS-CMV. For HKEC exposure, empty vector was transfected as a negative control, and plasmid encoding mTLR4 was transfected as a positive control. For HKLP exposure, empty vector and plasmid encoding mTLR4 were transfected as negative controls, and plasmid encoding mTLR2 was transfected as a positive control. Following transfection, cells were exposed to (A) HKEC (10⁶ CFU/ml) or (B) HKLP (10⁶ CFU/ml) for 6 h and then lysed. Firefly and Renilla luciferase luminescence units were recorded. The data are presented as the normalized mean fold induction of NF-κB luciferase reporter activity ± SEM.

![Figure 8](image-url)

*FIGURE 8.* Zebrafish Tlr4a and Tlr4b are unresponsive to stimulation by HKEC or HKLP. Constructs encoding zebrafish Tlr4a and Tlr4b, alone and in combination, were overexpressed in 293H cells along with CD14, MD2, pBluescript-luc, and pRS-CMV. For HKEC exposure, empty vector was transfected as a negative control, and plasmid encoding mTLR4 was transfected as a positive control. For HKLP exposure, empty vector and plasmid encoding mTLR4 were transfected as negative controls, and plasmid encoding mTLR2 was transfected as a positive control. Following transfection, cells were exposed to (A) HKEC (10⁶ CFU/ml) or (B) HKLP (10⁶ CFU/ml) for 6 h and then lysed. Firefly and Renilla luciferase luminescence units were recorded. The data are presented as the normalized mean fold induction of NF-κB luciferase reporter activity ± SEM.

**Discussion**

Zebrafish Tlr pathways consist of evolutionarily conserved and evolutionarily divergent parts. We and others have previously shown that many, but not all, of the components present in the mammalian TLR signal transduction pathways are present in the zebrafish (17, 20, 21, 29, 43–45). For example, it appears that Myd88 (45) and Ticam1 (21) play essential roles in mediating...
antibacterial and antiviral immunity, as they do in mammals. At this point, however, it has not been determined how the Tlr proteins identified in zebrafish respond to their environments. The temptation to presume that phylogenetic similarities equate to functional conservation needs to be tempered in light of the data we present herein. The data show that while zebrafish do respond to LPS stimulation, this activation is not dependent on mechanisms homologous to the prototypical LPS receptor complexes observed in mammals, which require CD14 and involve TLR4 and MD2 as its core components. Our findings have been bolstered by Sepulcre et al. (16), who demonstrated, using a novel in vivo assay, that the knockdown of Tlr4a and Tlr4b expression, through a morpholino-mediated translation blocker and a morpholino-meditated splice blocker, did not disrupt the immune response by zebrafish to LPS exposure. Future studies will be directed toward identifying the role the zebrafish Tlr4 proteins play in the immune system. The failure of Tlr4a and Tlr4b to respond to LPS, HKCLP, and HKLP allows for broad speculation regarding their roles. It may be possible that they respond to heretofore unidentified pattern-associated molecular patterns of bacterial, fungal, protistan, helminthic, and/or viral origins. It is also possible that Tlr4a and Tlr4b contribute to the ligand-recognizing diversity of other zebrafish Tlr proteins by functioning as coreceptors. This particular possibility is reminiscent of what is observed with mammalian TLR1 and TLR6, which heterodimerize with TLR2 to broaden the diversity and specificity of TLR2 signaling to include responses to diacyl (TLR2:TLR6) and triacyl (TLR2:TLR1) lipoproteins (46, 47). In zebrafish, Tlr4a and Tlr4b may lack the capacity to function as “stand-alone” receptors and thus may operate in this manner. It is also possible that the zebrafish Tlr4s may behave as “decoy” receptors, modulating the activation of other TLR pathways, although the capacity for the mTLR4-Tlr4a and mTLR4-Tlr4b chimeric proteins to be stimulated by LPS (Fig. 7) more than likely precludes this notion (48).

Another caveat that may need explanation to ascertain the true function of Tlr4a and Tlr4b relates to the role adaptor proteins play in their activation. In the zebrafish genome, orthologs of CD14 and MD2 are not observed (34). A homolog of MD1 (also known as LY86) is present, but MD1 protein is typically associated with the non-TLR CD180 (also known as RP105) receptor. MD1 facilitates the role CD180 plays as a negative regulator of TLR4 signaling. Phylogenetic analyses reveal that despite the absence of a TIR domain, CD180 and TLR4 are more closely related to each other than any other TLR is to TLR4 (34, 49). The relative phylogenetic closeness of CD180 to TLR4 makes it possible that perhaps in the zebrafish, CD180 and Tlr4a or Tlr4b share Md1 to function. Future experiments aimed at establishing a definitive role for CD180 and the Tlr4s in the zebrafish immune system will be designed to address this specific issue. Additionally, a zebrafish gene showing identity with mammalian LBP also has strong similarity to the mammalian BPI (34), an LPS binding protein with antimicrobial activity (50). The unclear origins of this gene and the lack of functional data confound the role of this LBP/BPI protein homolog in both LPS recognition (associated with mammalian LBP function) and antimicrobial activity (associated with mammalian BPI function). With this in mind, it has become apparent that strong consideration for additional adaptor molecules in any zebrafish Tlr signaling complex must be considered to correctly and comprehensively characterize a particular Tlr function. In this regard, ectopic expression of tlr4a and tlr4b in ZFL cells, which we have used in previous Tlr pathway functional studies (20, 21, 44), may not allow for the full exploration of Tlr4a and Tlr4b function. It may be that heretofore unrecognized but essential adaptor proteins not present in these cells may be required for Tlr4a and Tlr4b to achieve functional competence. The derivation of alternative zebrafish cell lines, perhaps expressing Tlr4a and/or Tlr4b, and the utilization of gene knockdown strategies including morpholinos and/or RNA interference may be required to address these functional questions in vitro. Additionally, novel in vivo approaches, like the one utilized by Sepulcre et al. (16), may prove useful in the ultimate characterization of these receptors.

Results from the analysis of conserved syntenies are as expected by the hypothesis that the zebrafish tlr4 genes are paralogous, not orthologous, to human TLR4 (Fig. 5). This contrasts with the data from phylogenetic analyses, which show tlr4a and tlr4b falling clearly in the clade with mammalian and bird Tlr4 genes (Fig. 3). The following gene history model can account for these results (Fig. 9). It is likely that two rounds of whole genome duplication (R1 and R2) occurred at the base of the vertebrate radiation (23, 51). These events produced paralogs that appear today in the human genome, generally four copies of each paralogon, but occasionally five if a translocation subsequently divided one of the original four paralogs, as apparently happened with the Hsa2p, Hsa4, Hsa5q, Hsa8p, and Hsa10 paralogon shown in Fig. 5A. Paralogous genes derived from these genome duplications are called ohnologs (52, 53). After the R1 and R2 duplication events, genes were gradually lost, and sometimes different lineages lost different ohnologs (reviewed in Ref. 54). Our historical model suggests that the last common ancestor of zebrafish and humans had a precursor of vertebrate TLR4 genes present on at least two of the paralogous chromosomes from the R1 and R2 genome duplication events. The model imagines that the Hsa10q member of the paralogon lost the TLR4 ohnolog in the human lineage, and a different member of the paralogon lost its TLR4 ohnolog in the zebrafish lineage. Thus, the gene in today’s human genome that is most similar to tlr4a and tlr4b would be TLR4, as the phylogenetic analysis shows, although these two genes would not be orthologs (genes in two different species descended from a single gene in the last common ancestor of two species). Instead, the tlr4 genes of zebrafish and the TLR4 gene of humans are descended from a single gene in a distant common ancestor just after the R2 genome duplication event. A translocation is required to explain why TLR4 is on Hsa9, which otherwise has few genes paralogous to those in humans.
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References


Supplementary Figure 1. Tlr4a and Tlr4b proteins are expressed on the surface of ZFL cells.

ZFL cells were stably-transfected under G418 selective pressure to express Tlr4a-GFP or Tlr4b-GFP chimeric proteins. Cells were fixed for 15 min in 4% paraformaldehyde in PBS, rinsed with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Cells were subsequently counterstained with DAPI, rinsed with deionized water, air-dried, and mounted in Cytoseal 60. Fluorescence images were acquired with an IX-81 inverted epifluorescence microscope (Olympus) with a 60× oil immersion objective lens, GFP filter cube (Chroma Technologies), Hamamatsu Orca ER cooled CCD camera, and IP-Lab imaging software.

Tlr4a-GFP- (A) and Tlr4b-GFP-expressing cells (B) exhibited GFP fluorescence associated with the cell surface and within cytoplasmic inclusions that was not observed within or on the surface of non-transfected ZFL cells (C). Continuous fluorescence around the margins of the cells indicated Tlr4a-GFP (A) and Tlr4b-GFP (B) localization in the plasma membrane (white arrows). The distribution of punctate cytoplasmic fluorescence, particularly in the perinuclear region, is consistent with additional localization of Tlr4a-GFP or Tlr4b-GFP within the endoplasmic reticulum. Non-transfected ZFL cells (C) exhibited were used as negative controls and exhibited negligible autofluorescence. Nuclei (blue) were stained with DAPI.
Supplementary Figure 1

A

B

C