Expression analysis of the Toll-like receptor and TIR domain adaptor families of zebrafish

Meijer, A.H.; Krens, S.F.G.; Medina Rodriguez, I.A.; He, S.; Bitter, W.; Snaar-Jagalska, B.E.; Spaink, H.P.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Molecular Immunology 40 (2004) 773–783
Molecular Immunology
www.elsevier.com/locate/molimm

Expression analysis of the Toll-like receptor and TIR domain adaptor families of zebrafish☆

Annemarie H. Meijer[a,*], S.F. Gabby Krens[a], Indira A. Medina Rodriguez[a], Shuning He[a], Wilbert Bitter[b], B. Ewa Snaar-Jagalska[a], Herman P. Spaink[a]

[a] *Institute of Biology, Leiden University, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands*
[b] *Department of Medical Microbiology, VU Medical Centre, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands*

Received 25 August 2003; accepted 2 October 2003

Abstract

The zebrafish genomic sequence database was analysed for the presence of genes encoding members of the Toll-like receptors (TLR) and interleukin receptors (IL-R) and associated adaptor proteins containing a TIR domain. The resulting predictions show the presence of one or more counterparts for the human *TLR1*, *TLR2*, *TLR3*, *TLR4*, *TLR5*, *TLR7*, *TLR8*, *TLR9*, *IL-1R* and *IL-18R* genes and one copy of the adaptor genes *MyD88*, *MAL*, *TRIF* and *SARM*. In contrast to data for the pufferfish *Fugu rubripes*, zebrafish has two genes that are highly similar to human *TLR4*. In addition, one fish-specific *TLR* group can be distinguished that is closely related to the *Drosophila melanogaster Toll-9* gene. The sequence of cloned cDNAs for *TLR4*, *TLR2* and *MyD88* show the same intron–exon organisation as in the human counterparts. Expression analysis using reverse transcriptase-PCR (RT-PCR) shows that 17 of the predicted zebrafish *TLR* genes and all the genes encoding adaptor proteins are expressed in the adult stage. A subset of the *TLR* genes are expressed at higher levels in fish infected with the pathogen *Mycobacterium marinum*. The induced genes include the homologues of the human *TLR1* and *TLR2* genes, whose functions are associated with mycobacterial infections, underscoring the suitability of zebrafish as a model for analysis of the vertebrate innate immune system.

Keywords: Innate immunity; Mycobacterium; MyD88; TLR; Zebrafish

1. Introduction

The immune system of teleost fish such as the developmental model species zebrafish (*Danio rerio*), resembles that of higher vertebrates in many respects. Components of both innate and adaptive immune functions have been identified, including highly diversified families of T-cell receptors, immunoglobulins, major histocompatibility class I, II and III proteins, Ikaros and Rag1/Rag2 proteins, cytokines, chemokines and components of the complement system (Yoder et al., 2002). Using the zebrafish as a new immunological model system is particularly attractive for investigations oriented towards developmental and cell biological aspects of immunobiology. The external development and optical clarity of the zebrafish embryos allows examination of the development of lymphoid and myeloid cell types at far earlier points in development than is possible in other vertebrate model systems. Furthermore, the zebrafish is a powerful model because of its suitability for functional genomic approaches, including large-scale mutagenesis screens that could lead to the discovery of novel immune-related genes.

During the recent years, members of the multigene family of Toll-like receptors (TLRs) have been recognised as key players in the recognition of microbes during host defence. The discovery of specific functions of TLRs in microbial recognition, in the induction of anti-microbial genes and in triggering of adaptive immune responses has led to a renewed interest in the field of innate immunity (Medzhitov, 2001; Janeway and Medzhitov, 2002;

Abbreviations: EST, expressed sequence tag; IL-R, interleukin receptor; LPS, lipopolysaccharide; LRR, leucine-rich repeat; MyD88, myeloid differentiation primary response gene 88; PAMP, pathogen-associated molecular pattern; RT-PCR, reverse transcriptase-PCR; SARM, sterile α and HEAT-Armadillo motifs; TIR, Toll/IL-1 receptor homology domain; TLR, Toll-like receptor; TRAM, TRIF-related adaptor protein; TRIF, TIR-domain containing adaptor inducing INF-β; zTLR, zebrafish Toll-like receptor; MAL, MyD88-adaptor-like protein

☆ Comparison of our data with these in the paper by Jault et al., 2003 in this same issue has led to a standardized nomenclature as deposited in GenBank. Our recent unpublished results indicate expression of TLRs and MyD88 during early embryo stages, which was not seen in the study of Jault et al., 2003.

* Corresponding author. Tel.: +31-71-5274927; fax: +31-71-5274999.
E-mail address: meijer@rulbim.leidenuniv.nl (A.H. Meijer).

0161-5890/$ – see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2003.10.003

Zuany-Amorim et al., 2002). TLRs represent type I transmembrane receptors defined by the presence of leucine-rich repeats (LRRs) in their extracellular domain and by the presence of a Toll/interleukin-1 (IL-1) receptor domain (TIR domain) in the C-terminal, cytosolic part of the protein that initiates signal transduction (Xu et al., 2000). The TIR domain is an evolutionairy ancient protein–protein interaction domain that occurs in a large group of host defence associated proteins from diverse species, including vertebrates, invertebrates and plants (Jebanathirajah et al., 2002). TIR-domain containing proteins in the human genome comprise ten members of the TLR family, eight members of the IL-1/IL-18 receptor group (IL-1R/IL-18R) and five identified or predicted cytosolic proteins that function as adaptor proteins connecting the Toll-like or interleukin receptors with the downstream signalling pathways (O'Neill et al., 2003). The first adaptor protein that was described is encoded by the myeloid differentiation primary response gene MyD88, which has been implicated in signalling by all of the human TLRs with one possible exception (TLR3) (O'Neill et al., 2003; Oshiumi et al., 2003a). In addition to its TIR domain, MyD88 contains a death domain that associates with the death domain of IL-1R-associated kinase (IRAK). Subsequent autophosphorylation of IRAK enables its interaction with tumour-necrosis-factor-associated factor-6 (TRAF-6), ultimately leading through two diverging signalling pathways to the activation of nuclear factor κB (NF-κB) and mitogen-activated protein (MAP) kinase (Barton and Medzhitov, 2003). Recently, two novel adaptors called MAL (MyD88-adaptor-like protein, or TIRAP) and TRIF (TIR-domain containing adaptor inducing INF-β, or TICAM-1) have been described, which are involved in signalling by distinct TLRs (Horng et al., 2001; Fitzgerald et al., 2001; Yamamoto et al., 2002; Oshiumi et al., 2003a). Furthermore, the human genome contains two more potential adaptor proteins, TRAM (TRIF-related adaptor protein) and SARM (sterile α and HEAT-Armadillo motifs), the differential use of which might further explain how distinct pathways are activated by TLRs during host defence (O'Neill et al., 2003).

TLRs were named after the Toll receptor of *Drosophila*, which is required for establishing the dorsal–ventral axis (Belvin and Anderson, 1996). However, in *Drosophila*, the Toll receptor also controls the induction of anti-fungal peptides after infection. The *Drosophila* genome encodes eight other Toll-related receptors thought to carry out both developmental and immune functions (Bilak et al., 2003). The 10 human TLRs, which are essentially conserved in the mouse genome, appear to have evolved specifically to recognize conserved motifs on pathogens that are not found in higher eukaryotes (Aderem and Ulevitch, 2000; Medzhitov, 2001). Such microbial signatures or pathogen-associated molecular patterns (PAMPs) are the targets of innate immunity recognition. PAMPs are detected by TLRs expressed on macrophages and dendritic cells whose arousal both activates natural killer cells and initiates adaptive responses (Dabbagh and Lewis, 2003; Kaisho and Akira, 2003). PAMPs acting as ligands for the different TLRs include lipopolysaccharide (LPS) from the outer membrane of gram-negative bacteria (TLR4), lipoprotein and peptidoglycan from gram-positive bacteria (TLR1, 2 and 6), flagellin from bacterial flagella (TLR5), double stranded RNAs generated during viral infection (TLR3) and unmethylated CpG dinucleotide motifs commonly found in bacterial and viral genomes (TLR9) (Barton and Medzhitov, 2002). Pharmaceutical anti-viral compounds are also recogized by TLRs (TLR7). Triggering of different TLRs produces distinct patterns of gene expression, but it is still unclear what differences in the signalling downstream of TLRs account for such divergence in gene expression. Furthermore, ligand recognition appears to involve protein–protein interactions that are not yet fully understood. For example, the extracellular domain of the TLR4 receptor interacts with the MD-2 protein and associates directly or indirectly with the CD14 receptor that binds complexes consisting of LPS and the LPS-binding protein, LBP (Shimazu et al., 1999).

Although the homologues of many human immune-related genes have been identified in the zebrafish, no members of the zebrafish TLR family have yet been investigated. Furthermore, only very few expressed sequence tags (ESTs) of zebrafish are present in the databases that demonstrate sequence identity with TLRs. However, a prediction of the TLR family in the genome of the pufferfish (*Fugu rubripes*) was recently reported, indicating that this teleost fish contains counterparts of most human TLRs, with the exception of the LPS-specific *TLR4* gene (Oshiumi et al., 2003b). The Sanger Institute (UK) recently released draft assembly sequences of the zebrafish genome. We have used these to characterize the *TLR* gene family and other TIR-domain encoding genes in zebrafish. Our data demonstrate that the zebrafish genome encodes one or two homologues of each of the human TLRs, including two homologues of the *TLR4* gene that seemed absent in *Fugu*. In addition to the counterparts of the human *TLR* genes, a fish-specific cluster of *TLR* genes is identified in zebrafish and *Fugu*. We also report here the presence of multiple interleukin receptors and TIR domain adaptor proteins in the zebrafish genome. The expression of all putative TLRs and adaptor proteins and examples of the interleukin receptors was investigated by RT-PCR in adult fish. Finally, expression levels were compared between healthy fish and fish infected with *Mycobacterium marinum*, a fish pathogen that induces a tuberculosis-like pathology in zebrafish strongly resembling the pathology of human tuberculosis.

2. Materials and methods

2.1. In silico analysis of genome sequences

BLAST searches of the zebrafish genomic sequence were carried out with the tblastn program at the Sanger

Institute Ensembl BLAST server (http://www.ensembl.org/Danio_rerio/blastview). Initially, the first release of the zebrafish whole genome shotgun assembly (version z06) was used for tblastn searches with the full length sequences of all human *TLRs* and *MyD88*. Hits identified in version z06 were subsequently used to search the zebrafish whole genome shotgun assembly sequence version 2 (Zv2) as released in April 2003 by the *Danio rerio* Sequencing Group at the Sanger Institute (ftp://ftp.ensembl.org/pub/assembly/zebrafish/Zr2release/). Additional tblastn searches in version Zv2 were performed using the TIR domain sequences of human *TLR4*, *IL-1R*, *IL-18R*, *MAL*, *TRIF*, *TRAM* and *SARM*. The BLAST server at the National Center for Biotechnology Information (NCBI) was used to check the *F. rubripes*, *Drosophila melanogaster* and human genome sequences for the possible presence of TIR domain encoding sequences other than those that have been reported. Two novel putative *TLR* genes were identified in *Fugu* upon tblastn searching with *TLR4* and *TLR3*. Furthermore, the *Fugu* genes for *IL-18R*, *MyD88*, *MAL*, *TRIF* and *SARM* were identified by searches with the corresponding genes of zebrafish. A tblastn search with *TLR2* revealed an unreported duplication of *TLR2* in the human genome. Tblastn searches of the *Drosophila* sequence with *TLR4*, *MyD88* and *SARM* did not reveal novel TIR domain sequences.

To predict intron–exon boundaries of the zebrafish genes we used the GENSCAN web server at the Massachusetts Institute of Technology (http://genes.mit.edu/burgelab/) or the GENSCAN predictions of the Sanger Ensembl. The predicted sequences were adjusted manually based on comparisons with the homologous human genes. Signal peptide sequences were predicted using the SignalP V2.0.b2 program (http://www.cbs.dtu.dk/services/SignalP-2.0/) with the settings 'eukaryotes' and 'neural networks and hidden Markov models'. Hydrophobicity analysis for predicting transmembrane regions was done by the method of Guy (Guy, 1985) using the program Vector NTI version 8.

2.2. Sequence alignments and phylogenetic analysis

The alignment of zebrafish TIR domain sequences shown in Fig. 2 was made using the program Vector NTI version 8.0. The dendrogram of zebrafish sequences and the phylogenetic tree were constructed by the neighbor-joining method (Saitou and Nei, 1987) using ClustalW (http://hypernig.nig.ac.jp) at the web server of the DNA Data Bank of Japan (DDBJ). Clustal W analysis was done with default settings, without Kimura's correction. Bootstrap sampling was reiterated 10,000 times. For the matrix table 'blosum' was used. The gap extension penalty was set at 0.2 and the gap distance was set at 8. Trees were printed using the program Treeview (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Genebank or swiss protein accessions, contig (ct) or scaffold (s) numbers of *Caenorhabditis elegans*, *Drosophila*, *Fugu* and human sequences used for the phylogenetic tree are: cToll-1, AC006694.1; cSARM, Z49936.1; dToll, AE003758.4; d18w, AE003793.3; dToll-3, AE003672.3; dToll-4, AE003623.2; dToll-5, AAE003640.3; dToll-6, AE003531.3; dToll-7, AE003794.3; dToll-8, AE003531.3; dToll-9, AAF51581; dMyD88, AAL56570; dSARM, ct3L_2023; fTLR1, s000614; fTLR2, s006262; fTLR3, s002098; fTLR5, s000195; fTLR7, s000095; fTLR8, s000095; fTLR9, s000035; fTLR18, s027305; fTLR21, s002667; fTLR22a, s000080; fTLR22b, s003381; fIL-18R, s000368; fMyD88, s011488; fMAL, s002141; fTRIF, s000989; fSARM, s003257; hTLR1, XP113437; hTLR2, NP 003255; hTLR3, NP 003256; hTLR4, O00206; hTLR5, XP029434; hTLR6, NP 006059; hTLR7, Q9NYK1; hTLR8, Q9NR97; hTLR9, Q9NR96; hTLR10, Q9BXR5; hIL-1R, P14778; hIL-18R, Q13478; hMyD88, NM 002468; hMAL, NM 148910; hTRIF, AB093555; hTRAM, AY232653; hSARM, NM 015077.

2.3. RT-PCR and gene cloning

Total RNAs were isolated from zebrafish homogenized in liquid nitrogen and extracted using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. Traces of genomic DNA were removed by incubation with RNase-free DNaseI (Roche), followed by phenol/chloroform extraction and ethanol precipitation. RT-PCR analysis was performed using the SuperScript II one-step RT-PCR system with Platinum Taq (Invitrogen). Reactions were performed on 100 ng of total RNA using 25 pmol of the forward and reverse primers indicated in Fig. 1. For another putative *TLR* gene present on the same contig as *zTLR20a* and *zTLR20b*, the reverse primer, 5′-CAACTTTACAATAAACAGAAGGAAAGT was used in combination with the *zTLR20a/b* forward primer. Furthermore, the reverse primer 5′-CTGCCCAATAAACAGAAGGAAAGT was used in combination with the *zTLR20a/b* forward primer to detect expression of a putative *zTLR20* homologue identified on contig ctg10265.3. Reverse transcription was performed at 50 °C for 30 min. PCR conditions were 2 min of initial denaturation at 94 °C, 30–40 cycles of denaturation at 94 °C for 20 s, annealing at 52–58 °C for 30 s and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. For detection of *zTLR19*, one-tenth of the RT-PCR reaction was used as template for a second round of 40 cycles amplification. Amplification of β-actin (AF057040; primers 5′-CGAGCAGGAGATGGGAACC and 5′-CAACGGAAACGCTCATTGC) was used as a control for constitutive expression. The PCR products were separated by electrophoresis in an 1.5% gel of Ultra-Pure Agarose 1000 (Invitrogen) and stained with ethidium bromide. To confirm the identity of the amplified sequences, the PCR products were cloned in pCRII-TOPO vector (Invitrogen) and sequenced using the sequencing service of ServiceXS (Leiden, The Netherlands). Sequences were deposited in the GenBank database under the accession numbers: *zTLR1*, AY389444; *zTLR2*,

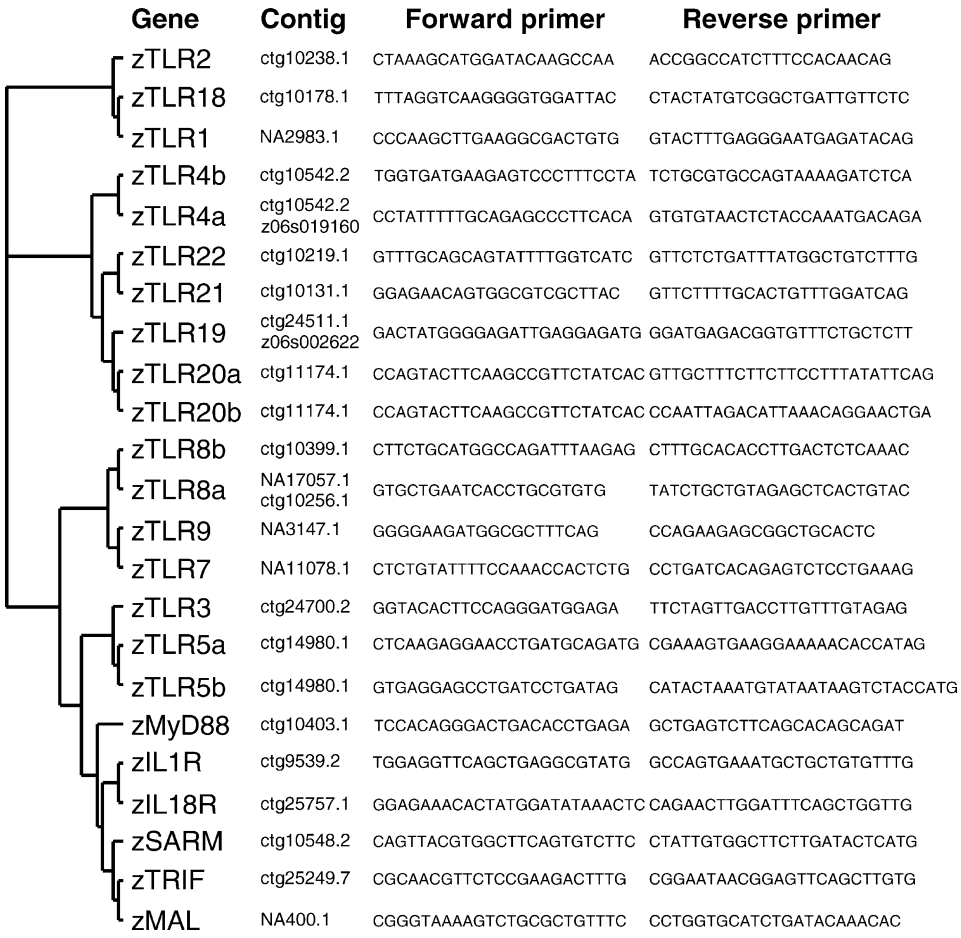

Fig. 1. TIR domain containing proteins identified in the zebrafish genome. The structure of the TLR, IL-1R/IL-18R and adaptor protein families is shown by a dendrogram based on the alignment of the TIR domains as shown in Fig. 2. Names of the corresponding contigs in the Zv2 release of the Sanger Institute are shown. Contig names of the earlier z06 release are mentioned when these provided additional useful information. Primer sequences used for RT-PCR analysis are presented in the 5′ to 3′ direction.

AY389445; *zTLR3*, AY389446; *zTLR4a*, AY389447; *zTLR4b*, AY389448; *zTLR5a*, AY389449; *zTLR5b*, AY389450; *zTLR7*, AY389451; *zTLR8a*, AY389452; *zTLR8b*, AY389453; *zTLR9*, AY389454; *zTLR18*, AY389455; *zTLR19*, AY389456, *zTLR20a*, AY389457; *zTLR20f*, AY389458; *zTLR21*, AY389459; *zTLR22*, AY389460; *zIL-1R*, AY389460; *zIL-18R*, AY389462; *zMyD88*, AY389463; *zMAL*, AY389464; *zTRIF*, AY389465; *zSARM*, AY389466.

To clone the full length sequences of *zTLR2*, *zTLR4* and *zMyD88* reverse transcription was performed on 100 ng of total RNA of Tuebingen zebrafish using Superscript III (Invitrogen) at 55 °C for 60 min under the reaction conditions of the manufacturer's instructions. Gene-specific primers for reverse transcription were 5′-GGGTTAGGGATTAGGTAGGTTAGGGC for *zTLR2*, 5′-TCTGCGTGCCAGTAAAAGATCTCA and 5′-CGACTCAGGAAAGCAATTGTTGCTACA for two parts of *zTLR4*, and 5′-GCTGAGTCTTCAGCACAGCAGAT for *zMyD88*. One-tenth of the reverse transcription reactions were used for PCR amplification using 1 μl of the proofreading enzyme PfuUltra HF (Stratagene), 10–25 pmol of primers and 2.5 mM of dNTPs in PfuUltra HF reaction buffer. Amplification was by an initial denaturation step at 95 °C for 2 min, 30–35 cycles of denaturation at 95 °C for 30 s, annealing at 55 to 60 °C for 30 s and extension at 72 °C for 1 min per kb of amplification product, followed by a final extension step at 72 °C for 10 min. *zTLR2* was amplified using the primer 5′-ATGAGACTCGTAGGAACAATGACTGCC and the primer used for reverse transcription (30 cycles), followed by a second round of PCR with the same forward primer and a nested reverse primer 5′-AGCCGCTTTACAGAGAGACGAGTGT (35 cycles). Two overlapping parts of *zTLR4b*, together comprising the full length coding sequence, were amplified each in two successive rounds of 30 and 35 cycles using the forward primer 5′-ATGATCATGTCAAATGGGGAACGGA with the reverse transcription primer 5′-TCTGCGTGCCAGTAAAAGATCTCA, and the forward primer 5′-TGGTGATGAAGAGTCCCTTTCCTA with the reverse transcription primer 5′-CGACTCAGGAAAGCAATTGTTGCTACA. *zMyD88* was amplified using the primer 5′-GGTAACGCGGAGATATACAACAAC with the reverse transcription primer

(30 cycles), followed by amplification with the nested primers 5′-CGGGATCCTCACCATGGCATCAAAGTTAAGTCTAGAC and 5′-CGCGCTCGAGTTAGGGCAGTGAGAGTGCTTTG (35 cycles). PCR products of *zTLR2* and *zTLR4* were cloned in pCR-BLUNTII-TOPO vector (Invitrogen). The *zMyD88* product was cloned in the vector pCS2+ (http://sitemaker.umich.edu/dlturner.vectors) at the *Bam*HI and *Xho*I restriction sites. The sequences were determined by the sequence services of Baseclear (Leiden, The Netherlands) or ServiceXS and deposited in GenBank under the accession numbers: *zTLR2*, AY388399; *zTLR4*, AY388400; *zMyD88*, AY388401.

2.4. Zebrafish infection experiments

Young adult male zebrafish were used for the infection experiments. These zebrafish were selected from a laboratory-breeding colony and acclimated to their new environment for 1 week in a quarantine area. These fish were kept at 28 °C on a 12:12 h light/dark rhythm throughout the experiment. Groups of 10 infected zebrafish, infected with the same dose and strain of mycobacteria, were kept in small fish tanks (10 l) with their own separate filtering system (Eheim Ecco). *M. marinum* strain M (Ramakrishnan and Falkow, 1994) and E11 (Puttinaowarat et al., 2000) were grown in ADC supplemented Middlebrook 7H9 medium to late exponential-phase cultures. This medium was also supplemented with 1 mg/ml D-arabinose in order to obtain dispersed growth cultures. Zebrafish were inoculated intraperitoneally with 1 μl bacterial cell suspension or control fluid (PBS) using a pneumatic picopump (PV820, World Precision Instruments) with pulled microcapillar pipettes. Animals were injected with approximately 1×10^3 bacteria, as was determined by optical density (OD 600 nm) and microscopic analysis. After 8 weeks, the zebrafish were sacrificed and used for RNA isolation or histological examination. The infected zebrafish used for RNA isolation showed lethargy, one of the overt signs of fish tuberculosis. All the mycobacteria-infected zebrafish showed, in varying degrees, pathology corresponding to fish tuberculosis upon histological examination, whereas none of the control fish showed these characteristics (van der Sar and Bitter, in preparation).

3. Results

3.1. Prediction of TIR domain encoding genes in the zebrafish genome

Blast analysis of the zebrafish genome database (release Zv2) of the Sanger Institute identified 21 reading frames in 17 contigs that encode proteins with homology to the TIR domain of the human *TLR* genes. Genscan predictions for these frames were analysed for the presence of a predicted N-terminal signal peptide, LRRs and a predicted single transmembrane region at the characteristic distance of approximately 30 amino acids upstream of the TIR domain (Table 1). According to these criteria, nine of these reading frames represent a full length TLR. We were able to manually annotate the presence of LRR regions and a transmembrane domain linked to the TIR domain for another six predicted TLR encoding sequences. In two cases, the older released version of the zebrafish genome (version 06) contained additional information that was helpful to determine reading frames for TLR homologues. In the remaining four cases, the quality of the genome dataset was insufficient to predict the presence of a TLR sequence using detailed homology analysis of all reading frames encoded by the identified contigs.

Analysis for the presence of homologues of the IL-1 and IL-18 receptor identified seven reading frames in seven contigs. Using the sequences of the human adaptor proteins MyD88, MAL, TRIF, TRAM, and SARM as a query we identified four contigs encoding homologues of these proteins. All the identified reading frames encode a clearly discernable TIR domain, but, only in the case of MyD88 and SARM, similarity of the predicted frames with the

Table 1
Structural data of predicted TLR proteins encoded in the zebrafish genome

Gene[a]	Length[b]	Exons[c]	SP[d]	TM[e]
zTLR1	765 (p)	1	0.940	−0.35
zTLR2	788	1	0.996	−0.40
zTLR3	884 (p)	4 (5)	–	−0.60
zTLR4a	741 (p)	1 (3)	–	−0.60
zTLR4b	819	3	0.999	−0.60
zTLR5a	310 (p)	–	–	−0.51
zTLR5b	208 (p)	–	–	–
zTLR7	1048	3	0.959	−0.42
zTLR8a	1014 (p)[f]	2	–	−0.43
zTLR8b	1014	2	0.985	−0.43
zTLR9	942 (p)	1	–	−0.38
zTLR18	694	4	0.993	−0.60
zTLR19	699 (p)	>2	0.803	–
zTLR20a[g]	1003	3	0.992	−0.69
zTLR20b[g]	796	2	1.000	−0.67
zTLR21	989	1	0.619	−0.37
zTLR22	948	1	0.939	−0.50

[a] The corresponding contig numbers are given in Fig. 1. All genes in the table contained a TIR domain. LRRs were identified in all genes except in the partial *zTLR5b* sequence.

[b] Total number of amino acids; (p) indicates that the predicted protein sequence is partial.

[c] Number of encoding exons; between brackets is the number of exons expected to encode the full length protein.

[d] Signal peptide probability.

[e] Guy free energy for predicted transmembrane region.

[f] On basis of sequence homology with human *TLR8* it seems likely that the two TLR homology reading frames encoded by contigs NA17057.1 and ctg10256.1 belong to the same gene that we have called *zTLR8a*. However, since there is no sequence overlap of these two contigs, the given amino acid length and exon number is speculative.

[g] Three additional, highly similar TIR domain duplications (*zTLR20c–e*) were identified on contig ctg11174.1 that contains *zTLR20a* and *b*, and a fourth duplication (*zTLR20f*) was identified on contig ctg10265.3.

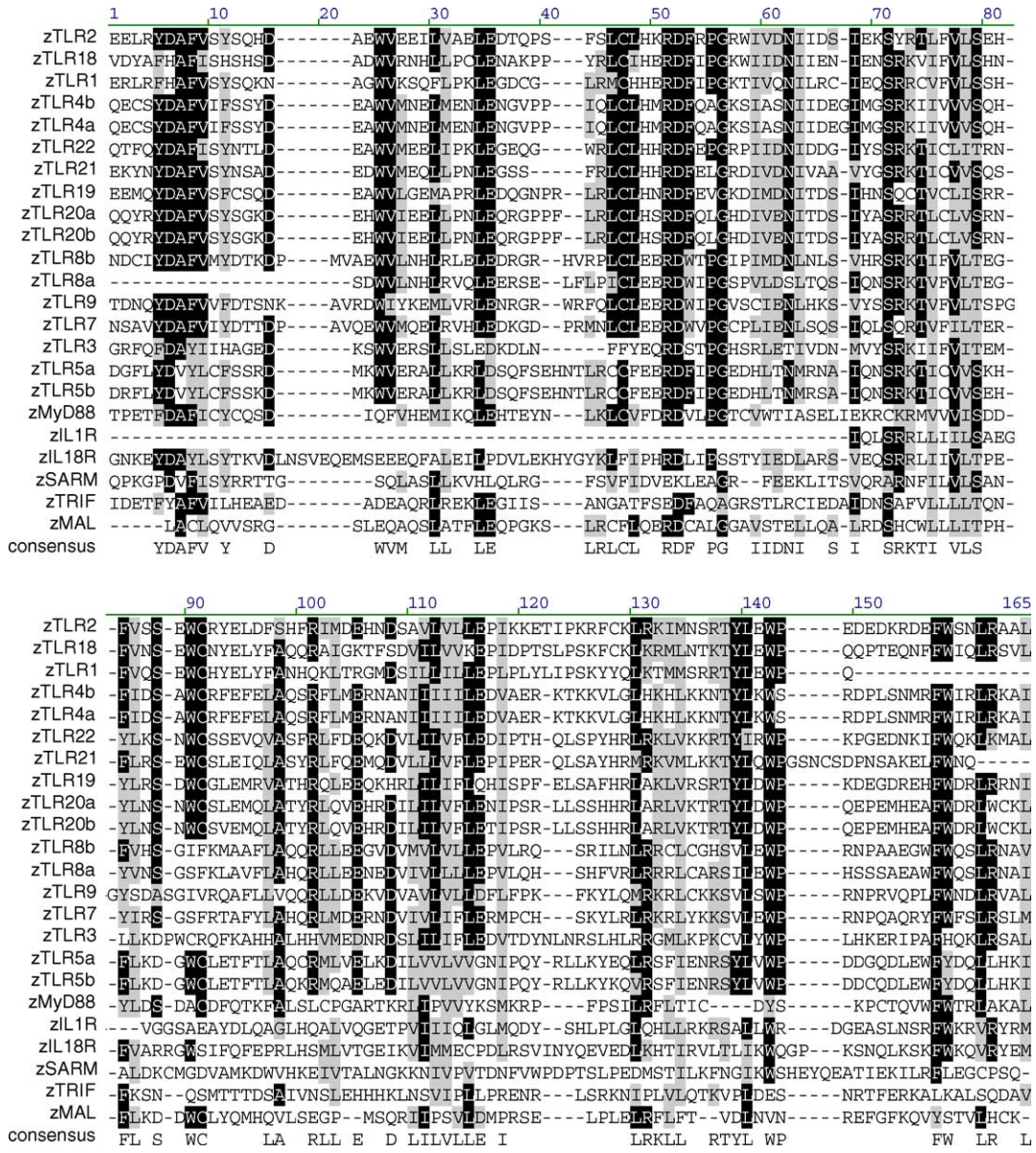

Fig. 2. Amino acid alignment of the TIR domains of the TLR, IL-1R/IL-18R and adaptor proteins of zebrafish. Identical or strongly conserved residues are indicated with black boxes and similar residues are boxed grey. The TIR domain sequences of *zTLR8a*, *zIL-1R* and *zMAL* are incomplete at the N-terminal end and those of *zTLR1* and *zTLR21* at the C-terminus because these regions were present in low quality sequence regions in the corresponding contigs.

human counterparts was sufficient to confirm the identity of a full length reading frame with certainty. A dendrogram representing the sequence similarities between all TIR domain-containing proteins identified in the zebrafish genome and indicating their corresponding contig numbers is given in Fig. 1 and a sequence alignment of the TIR domains is shown in Fig. 2.

3.2. Detailed homology analysis of the TIR domain-containing proteins

The predicted TLR, IL-1R and IL-18R proteins and the four homologues to the adaptor family were further analysed for their similarity with the human TIR domain-containing proteins using the ClustalW algorithm. The results indicated the presence of one or more counterparts for the human *TLR1*, *TLR2*, *TLR3*, *TLR4*, *TLR5*, *TLR7*, *TLR8*, *TLR9*, *IL-1R* and *IL-18R* genes and one copy of the adaptor genes *MyD88*, *MAL*, *TRIF* and *SARM*. The TIR domains of these proteins were taken as a basis for subsequent phylogenetic analysis as shown in Fig. 3. A phylogeny reconstruction based on the full length and partial sequences produced a similar result (not shown). In some cases when the sequences were highly duplicated (i.e. four duplicates of the *TLR20a* and *20b* homologues) or when the protein sequence predictions were rather inaccurate (i.e. in the case of five other IL-1R and IL-18R

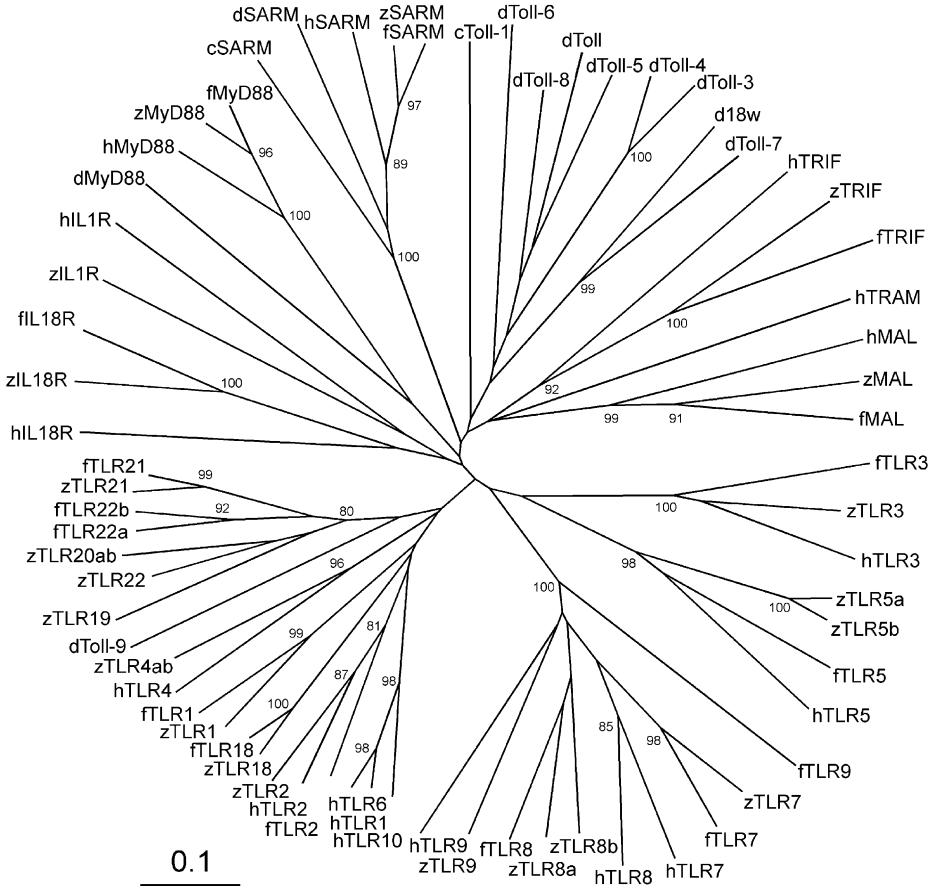

Fig. 3. Unrooted phylogenetic tree of vertebrate and invertebrate TLR, IL-1R/IL-18R and TIR domain adaptor proteins. The tree was constructed by neighbor-joining analysis based on an alignment of the amino acid sequences of the TIR domains. The numbers indicate the occurrence of nodes during bootstrap analysis. The bootstrap values are given as percentages of 10,000 reiterations and only values above 80 are shown. The scale bar indicates the branch length. c: *Caenorhabdites elegans*; d: *Drosophila*; f: *fugu rubripes*; h: human; z: zebrafish.

homologues) the results are not represented. Based on the phylogenetic analysis there was no ambiguity for the naming of the predicted genes *zTLR2*, *zTLR3*, *zTLR4a*, *zTLR4b*, *zTLR5a*, *zTLR5b*, *zIL-1R*, *zIL-18R*, *zMyD88*, *zMAL*, *zTRIF* and *zSARM*. The naming of the *zTLR3*, *zTLR4*, *zMyD88* and *zSARM* genes was further supported by the conservation of internal intron–exon boundaries. The genes designed *zTLR7*, *zTLR8a*, *zTLR8b*, *zTLR9* form a defined group with the human *TLR7*, *8* and *9* genes and were named following the nomenclature proposed for the reported homologues of these genes in the *F. rubripes* genome (Oshiumi et al., 2003b). For the predicted genes designated *zTLR1* and *zTLR18*, the naming was more difficult since there was no clear difference in relatedness to any of the sequences in the group of *hTLR1*, *hTLR6* or *hTLR10*. We therefore decided to adopt the nomenclature for the reported *F. rubripes* homolog of the *zTLR1* open reading frame. We also identified a homolog of the *zTLR18* gene prediction in scaffold s027305 of the *F. rubripes* genome that was not previously reported by Oshiumi et al. (2003b). In addition to the close homologues of the human *TLR* family, several gene predictions form a clearly discernable subgroup that cannot be found in the human genome. In contrast, the predicted protein group is more closely related to the Toll-9 protein of *D. melanogaster*. Also in the *F. rubripes* genome database, three gene predictions with homology to this class can be found. Two of these were previously reported as *fTLR21*, and *fTLR22* by Oshiumi et al. (2003b). The third one that we called *fTLR22b*, is present in scaffold s003381 of the *F. rubripes* genome database. Based on further ClustalW analyses and comparisons of predicted intron–exon boundaries in this subgroup of zebrafish and *Fugu* TLR homologues (data not shown) we have designated the zebrafish homologues *zTLR21* and *zTLR22*. The other members of this group were arbitrarily designated *zTLR19*, *zTLR20a* and *zTRL20b*.

3.3. Expression analysis of zebrafish TLRs, IL-1R/IL-18R and TIR domain adaptor genes

To determine which of the predicted *TLR* genes were expressed, we chose to design primers for RT-PCR analysis immediately upstream or downstream of the TIR domains or within the lesser conserved regions of the TIR domain. By RT-PCR analysis using total RNA from adult zebrafish, we

could confirm that nearly all the predicted *TLR* genes are expressed, except for the genes *zTLR19* and *zTLR20b*. For the two *zTLR4* genes that have identical TIR domains, primers were designed in a more upstream region to be able to distinguish between their expression. Using both *zTLR4* primer pairs, extension products were generated, indicating that both *zTLR4* copies are expressed. One of the two genes for which expression could not be detected, *zTLR19*, must also be an expressed gene, because an EST (AL923233) corresponding to this gene is present in the database. A new set of primers was designed corresponding to this EST, with which expression could eventually be detected, but only after two successive rounds of amplification. It is possible that *zTLR19* is expressed in a specific tissue, since the EST was isolated from a library of mixed tissues. Very few other ESTs of *TLR* genes are found in the database, including ESTs corresponding to *zTLR3* (BG304206, BF158452), *zTLR4b* (CD760215) and an EST corresponding to *zTLR21* (fv45e11.x1). The z*TLR20b* gene for which expression could never be detected is present on the contig 11174.1 that contains multiple duplications of the TIR domain (*zTLR20a-e*), and therefore *zTLR20b* might represent a pseudogene. Another *zTLR20* duplication (*zTLR20f*) was identified on contig 10265.3. Although all of these duplicated *zTLR20* genes are nearly identical, primers specific for the 3′-ends of some of them could be designed. Using these primers, it could be demonstrated that at least two *zTLR20* homologues (*zTLR20a* and *f*) are expressed, while expression of two others (*zTLRb* and *c*) was not detected. RT-PCR analysis also confirmed the expression of the predicted z*IL-1R* and z*IL-18R* genes, and of all the predicted TIR domain adaptor genes.

To find further experimental support for the sequence predictions, we set out to clone the full length sequences of the *zTLR2*, *zTLR4b* and *zMyD88* genes. In agreement with our prediction, the *zTLR2* gene was found to be encoded by a single exon, similar as the human *TLR2* gene. Furthermore, it was confirmed that the gene structures of *zTLR4b* and human *TLR4* are highly conserved, each consisting of three encoding exons (Fig. 4). Finally, the *zMyD88* and human *MyD88* genes also demonstrate identical structures with five

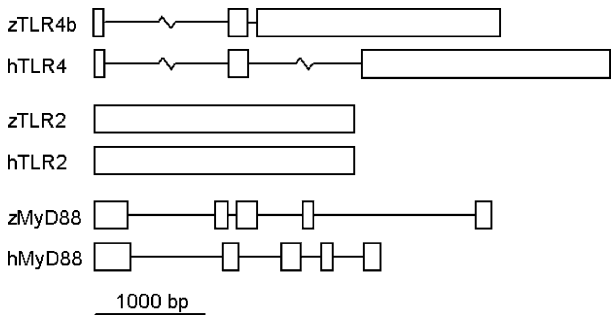

Fig. 4. Conserved gene structures of the zebrafish and human *TLR4*, *TLR2* and *MyD88* genes. Coding exon regions are depicted with boxes and introns with lines. Introns larger than 2000 bp in the *TLR4* genes are not drawn to scale.

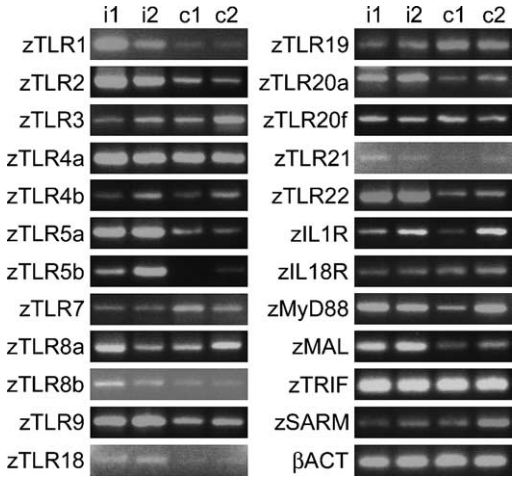

Fig. 5. RT-PCR analysis of zebrafish *TLR*, *IL-1R/IL-18R* and TIR domain adaptor gene expression. RNAs used for amplification were from adult male zebrafish infected (i1 and i2) by intraperitoneal inoculation with *M. marinum* strain M (fish i1) or strain E11 (fish i2) or from healthy fish inoculated with control fluid (c1 and c2). β-Actin (β-ACT) was used as a control for constitutive expression.

encoding exons (Fig. 4) and precisely conserved intron–exon boundaries.

3.4. Expression analysis in Mycobacterium-infected zebrafish

To investigate if the expression of *TLR* genes was responsive to microbial infection, we compared expression levels between two fish infected with *M. marinum* and two healthy control fish (Fig. 5). The infected fish were harvested for RNA isolation 8 weeks after intraperitoneal injection with a *Mycobacterium* suspension, when they showed clear symptoms of tuberculosis disease. RT-PCR analysis demonstrated that the homologues of the human *TLR1* (*zTLR1* and *18*), *TLR2*, *TLR5* and *TLR9* genes were expressed at higher levels in the infected fish compared with the controls. Furthermore, the infected fish also showed up-regulated expression levels of the fish-specific *zTLR20a* and *zTLR22* genes. Similar expression levels between infected and control fish were observed for the β-actin constitutive control gene, the homologues of the human *TLR3*, *TLR4*, *TLR7* and *TLR8* genes and the fish-specific *zTLR19*, *zTLR20f* and *zTLR21* genes. Expression levels of the *zIL-1R* and *zIL-18R* genes were also unaffected by *Mycobacterium* infection. Finally, the zebrafish homologue of *MAL* showed increased expression in the infected fish, while the expression levels of the other putative TIR domain adaptor genes, *zMyD88*, *zTRIF* and *zSARM*, were not responsive to *Mycobacterium* infection.

4. Discussion

Based on the recently released shotgun sequence of the zebrafish genome, we have described here the annotation

of the Toll-like and interleukin families of cell surface receptors that both signal via a conserved intracellular TIR domain. Furthermore, we have annotated the genes of signalling adaptor proteins that also contain a TIR domain. We show that the zebrafish genome contains at least 17 expressed *TLR* genes, as well as expressed homologues of *IL-1R*, *IL-18R* and four different TIR domain adaptor genes.

Previously it was suggested that early vertebrates contained few *TLRs* and that the *TLR4* gene, which is the LPS sensor in mammals, might have emerged as a distinct entity only 180 million years ago (Du et al., 2000). Furthermore, a recent analysis of the *TLR* family in the genome of the pufferfish, *F. rubripes*, suggested that *TLR4* is absent from the fish lineage (Oshiumi et al., 2003b). These hypotheses are contradicted by our data showing that the zebrafish contains two copies of the *TLR4* gene in a tandem duplication. The full length gene corresponding to one of the *zTLR4* copies was cloned, based on which we could conclude that *zTLR4* and *hTLR4* display a conserved intron–exon structure. Therefore, our data confirm evolutionary calculations indicating that *TLR4* diverged from other *TLRs* during early vertebrate evolution, more than 400 million years ago (Beutler and Rehli, 2002). The apparent absence of *TLR4* in *Fugu* may be explained by a specific loss of this gene in the *Fugu* lineage or by incompleteness of the *Fugu* genome shotgun sequence. Although the effects of LPS have not been studied as extensively in fish as in mammals, recent reports indicate that teleost fish also display LPS responsiveness (MacKenzie et al., 2003; Milston et al., 2003). Therefore, it is possible that an ancestral *TLR4* gene was already implicated in LPS sensing.

The zebrafish genome also contains a conserved homologue of *TLR2*, which like its human counterpart is encoded by a single exon. In contrast, the *TLR2* gene of *Fugu* contains an intron in the TIR domain and possibly additional introns in other parts of the protein. In zebrafish, we found no evidence for the presence of intron–exon patterns deviating from those of the human counterparts. In fact, the analyses of the cloned *zTLR2*, *zTLR4* and *zMyD88* genes and the predictions of *zTLR3* and *zSARM* indicate that the gene structures of *TLR* and TIR domain adaptor genes of zebrafish and human are highly conserved and that prototypes of these genes must therefore have been present in their common ancestor.

Our phylogenetic analysis supports that zebrafish and *Fugu* contain one or two clearly defined counterparts of each of the *TLR3* and *TLR5* genes. Therefore, a previous suggestion that a common mammalian ancestral gene of *TLR3* and *TLR5* developed 150 million years ago does not hold true (Du et al., 2000), but a more ancient precursor of each gene must have existed during early vertebrate life. The phylogeny analysis further demonstrates that the *TLR7*, *TLR8* and *TLR9* genes form a defined group. However, there is little support for the branching pattern within this group, making it difficult to assign orthologies. In assigning names to the zebrafish *TLRs* of this group we have looked at the closest homologue in *Fugu* and followed the nomenclature that was proposed for this species. Also the fish counterparts of the genes in the mammalian *TLR1*, *TLR6* and *TLR10* group cannot be unambiguously assigned based on the phylogeny. The closest homologues in zebrafish are represented by a gene that we named *zTLR1* because of its close resemblance to *fTLR1* and by another gene that we named *zTLR18*. We also identified the homologue of this gene in *Fugu* and named it *fTLR18* accordingly. In addition, the phylogeny reconstruction indicates the presence of a fish-specific *TLR* cluster, consisting of *zTLR19*, *zTLR20a/b*, *zTLR21* and *zTLR22*, and their homologues of *Fugu*. This cluster is closely related to the *Drosophila Toll-9* gene, which is the only member of the *Drosophila Toll* family that groups with the vertebrate *TLRs*.

Within the *TLR* family of zebrafish, relativity recent gene duplications seem to have occurred for *zTLR4*, *5*, *8* and *20*. In the case of *zTLR20*, multiple duplications of the TIR domain are present on the same contig and it is well possible that some of these represent pseudogenes. The occurrence of duplications is common in teleost fish genomes, where gene families generally have expanded in comparison with the corresponding families in mammalian genomes (Robinson-Rechavi et al., 2001). In the human genome, the *TLR7* and *TLR8* genes on the X-chromosome also appear to have resulted from a duplication of an ancestral gene. Furthermore, in the vicinity of the human *TLR2* gene we found an duplication of the TIR domain that has not been described.

In conclusion, the phylogeny analysis clearly demonstrates that the radiation of *TLRs* took place in the common ancestor of all vertebrates, strongly suggesting that the evolution of specific functions of different *TLRs* in the innate immune response would have occurred in parallel with the evolution of the adaptive immune system of vertebrates. Also the radiation of interleukin receptors appears to have occurred during early vertebrate life. Furthermore, we have shown here that zebrafish and *Fugu* contain conserved homologues of four of the TIR domain adaptor proteins that have been identified in the human genome (MyD88, MAL, TRIF and SARM), suggesting that the signalling pathways employed by the different *TLR* and *IL-R* receptors of mammals and teleosts are likely to be highly conserved. The identification in the zebrafish genome of homologues of the five members of the nuclear factor κB family of transcription factors that are central targets of mammalian *TLR* signalling, further supports this notion (unpublished results).

To investigate the expression of the *zTLR* genes in response to a microbial infection, we used adult zebrafish suffering from an infection with *M. marinum*, the causative agent of fish tuberculosis. *M. marinum* is genetically closely related to the human pathogen, *Mycobacterium tuberculosis* (Tonjum et al., 1998). Infection of zebrafish with *M. marinum* leads to the aggregation of macrophages and lymphocytes into granulomatous structures characteristic of tuberculosis. *M. marinum* also causes a superficial granulomatous skin infection in humans. Recently, it has been

shown that clusters of *M. marinum*-infected macrophages, which probably represent granuloma focal points, can already be observed in zebrafish embryos whose adaptive immune system has not yet developed (Davis et al., 2002).This result indicates that the innate immunity is sufficient to initiate granuloma formation. The mycobacterial cell wall is a complex structure containing peptidoglycan, lipoproteins, arabinogalactan, and a large number of lipids and glycolipids, such as the lipoarabinomannans. The interaction of these specialised macromolecules with the innate immune system of the host is known to involve the members of the TLR receptor family (Brightbill et al., 1999; Means et al., 1999b; Heldwein and Fenton, 2002). We have shown here that mycobacterium infection of zebrafish results in upregulated expression levels of the *zTLR2* gene and of the *zTLR1* and *zTLR18* genes that both are closely related to human *TLR1*. These results are in perfect agreement with the known functions of human TLR1 and TLR2 in sensing lipoprotein (TLR2 in association with TLR1 (Takeuchi et al., 2002)), peptidoglycan (TLR2 (Schwandner et al., 1999; Takeuchi et al., 1999)) and glycolipids (TLR2 (Means et al., 1999a; Underhill et al., 1999)) from mycobacteria. An induced *TLR2* expression was also observed in murine macrophages infected with *M. avium* (Wang et al., 2000) and the expression of a goldfish *TLR* was induced in macrophages exposed to *M. chelonei* (Stafford et al., 2003). The observed inductions of *zTLR1*, *zTLR2* and *zTLR18* in response to *M. marinum* suggest that recognition mechanisms of microbial patterns were already established in the common vertebrate ancestor and that the functions of receptors in the TLR1 and TLR2 groups may be conserved between mammals and teleosts. Mycobacterial infection also induced the *zTLR20a* and *zTLR22* genes, suggesting that receptors of the fish-specific *TLR* cluster may also function in sensing microbial infections.

While the functions of human TLR1 and TLR2 have been implicated in mycobacterial infections, TLR4 has mainly been associated with the recognition of gram-negative bacteria. However, there is evidence that TLR4 is redundantly involved with TLR2 in the response to *M. tuberculosis*, but the *M. avium* response seems to be mediated only by TLR2 (Reiling et al., 2002; Abel et al., 2002; Means et al., 1999b). No induction of *zTLR4* was observed in our *M. marinum* infection experiment. Also, we observed no induction of *zTLR3*,7 and *8a/b*, the counterparts of human TLRs specifically involved in sensing of nucleotide derivates and associated with viral rather than bacterial infections. However, we did observe induction of the *zTLR9* gene that also belongs to the nucleotide-sensing group. This induction is not surprising, since *TLR9* has been implicated in the recognition of unmethylated CpG dinucleotide motifs that are commonly found in viral as well as bacterial genomes. The observed induction of *zTLR5a* and *5b*, two homologues of the human *TLR5* gene that has been implicated in the recognition of bacterial flagellin, is less readily explained, since *M. marinum* does not produce flagella. It is possible that the *zTLR5a/b* genes were induced as a result of a secondary infection or that their ligand specificities differ from those of their human counterparts. Alternatively, the induction of *zTLR5a/b* could be unrelated to the ligand specificity. For example, the CpG-sensing *TLR9* gene was found to be induced following LPS stimulation of murine macrophages (An et al., 2002), indicating that microbial products can also induce other *TLRs* than those specific for their own recognition. Finally, no induction of *zIL-1R* and *zIL-18R* was observed, but because we did not analyse all members of the zebrafish IL-R receptor family, it is possible that other *IL-1R/IL-18R* homologues exist that are responsive to mycobacterial infection.

Recent work of Davis et al. (2002) and Van der Sar et al. (2003), who exploited the optical transparency of zebrafish embryos for in vivo imaging of the effects of bacterial infections on the behaviour of macrophages, already provided excellent examples of the added value of zebrafish as an immunological model system. The presence of highly conserved families of Toll-like and interleukin receptors and associated TIR domain adaptor proteins in zebrafish, and the here reported induction of a subset of these genes in response to mycobacterial infection, further underscore the usefulness of zebrafish for studies of the vertebrate innate immune system.

Acknowledgements

The authors thank Kim Thompson (Institute of Aquaculture, Stirling, UK) for *M. marinum* strain E11, Wim Schouten and Astrid van der Sar for assistance with the zebrafish infection experiments and Robert Geisler (Max-Planck-Institut für Entwicklungsbiologie, Tuebingen, Germany) for zebrafish of the Tuebingen line.

References

Abel, B., Thieblemont, N., Quesniaux, V.J., Brown, N., Mpagi, J., Miyake, K., Bihl, F., Ryffel, B., 2002. Toll-like receptor 4 expression is required to control chronic *Mycobacterium tuberculosis* infection in mice. J. Immunol. 169, 3155–3162.

Aderem, A., Ulevitch, R.J., 2000. Toll-like receptors in the induction of the innate immune response. Nature 406, 782–787.

An, H., Xu, H., Yu, Y., Zhang, M., Qi, R., Yan, X., Liu, S., Wang, W., Guo, Z., Qin, Z., Cao, X., 2002. Up-regulation of TLR9 gene expression by LPS in mouse macrophages via activation of NF-kappaB, ERK and p38 MAPK signal pathways. Immunol. Lett. 81, 165–169.

Barton, G.M., Medzhitov, R., 2002. Toll-like receptors and their ligands. Curr. Top. Microbiol. Immunol. 270, 81–92.

Barton, G.M., Medzhitov, R., 2003. Toll-like receptor signaling pathways. Science 300, 1524–1525.

Belvin, M.P., Anderson, K.V., 1996. A conserved signaling pathway: the *Drosophila* toll-dorsal pathway. Annu. Rev. Cell Dev. Biol. 12, 393–416.

Beutler, B., Rehli, M., 2002. Evolution of the TIR, tolls and TLRs: functional inferences from computational biology. Curr. Top. Microbiol. Immunol. 270, 1–21.

Bilak, H., Tauszig-Delamasure, S., Imler, J.L., 2003. Toll and Toll-like receptors in *Drosophila*. Biochem. Soc. Trans. 31, 648–651.

Brightbill, H.D., Libraty, D.H., Krutzik, S.R., Yang, R.B., Belisle, J.T., Bleharski, J.R., Maitland, M., Norgard, M.V., Plevy, S.E., Smale, S.T., Brennan, P.J., Bloom, B.R., Godowski, P.J., Modlin, R.L., 1999. Host defence mechanisms triggered by microbial lipoproteins through toll-like receptors. Science 285, 732–736.

Dabbagh, K., Lewis, D.B., 2003. Toll-like receptors and T-helper-1/T-helper-2 responses. Curr. Opin. Infect. Dis. 16, 199–204.

Davis, J.M., Clay, H., Lewis, J.L., Ghori, N., Herbomel, P., Ramakrishnan, L., 2002. Real-time visualization of mycobacterium–macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. Immunity 17, 693–702.

Du, X., Poltorak, A., Wei, Y., Beutler, B., 2000. Three novel mammalian toll-like receptors: gene structure, expression, and evolution. Eur. Cytokine Netw. 11, 362–371.

Fitzgerald, K.A., Palsson-McDermott, E.M., Bowie, A.G., Jefferies, C.A., Mansell, A.S., Brady, G., Brint, E., Dunne, A., Gray, P., Harte, M.T., McMurray, D., Smith, D.E., Sims, J.E., Bird, T.A., O'Neill, L.A., 2001. Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. Nature 413, 78–83.

Guy, H.R., 1985. Amino acid side-chain partition energies and distribution of residues in soluble proteins. Biophys. J. 47, 61–70.

Heldwein, K.A., Fenton, M.J., 2002. The role of Toll-like receptors in immunity against mycobacterial infection. Microbes. Infect. 4, 937–944.

Horng, T., Barton, G.M., Medzhitov, R., 2001. TIRAP: an adapter molecule in the Toll signaling pathway. Nat. Immunol. 2, 835–841.

Janeway Jr., C.A., Medzhitov, R., 2002. Innate immune recognition. Annu. Rev. Immunol. 20, 197–216.

Jault, C., Pichon, L., Chluba, J., 2003. Toll-like receptor gene family and TIR-domain adapters in *Danio rerio*. Mol. Immunol. this issue, 10.1016/ j.molimm.2003.10.001.

Jebanathirajah, J.A., Peri, S., Pandey, A., 2002. Toll and interleukin-1 receptor (TIR) domain-containing proteins in plants: a genomic perspective. Trends Plant Sci. 7, 388–391.

Kaisho, T., Akira, S., 2003. Regulation of dendritic cell function through Toll-like receptors. Curr. Mol. Med. 3, 373–385.

MacKenzie, S., Planas, J.V., Goetz, F.W., 2003. LPS-stimulated expression of a tumor necrosis factor-alpha mRNA in primary trout monocytes and in vitro differentiated macrophages. Dev. Comp. Immunol. 27, 393–400.

Means, T.K., Lien, E., Yoshimura, A., Wang, S., Golenbock, D.T., Fenton, M.J., 1999a. The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors. J. Immunol. 163, 6748–6755.

Means, T.K., Wang, S., Lien, E., Yoshimura, A., Golenbock, D.T., Fenton, M.J., 1999b. Human toll-like receptors mediate cellular activation by *Mycobacterium tuberculosis*. J. Immunol. 163, 3920–3927.

Medzhitov, R., 2001. Toll-like receptors and innate immunity. Nat. Rev. Immunol. 1, 135–145.

Milston, R.H., Vella, A.T., Crippen, T.L., Fitzpatrick, M.S., Leong, J.A., Schreck, C.B., 2003. In vitro detection of functional humoral immunocompetence in juvenile chinook salmon (*Oncorhynchus tshawytscha*) using flow cytometry. Fish Shellfish Immunol. 15, 145–158.

O'Neill, L.A., Fitzgerald, K.A., Bowie, A.G., 2003. The Toll-IL-1 receptor adaptor family grows to five members. Trends Immunol. 24, 286–290.

Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T., Seya, T., 2003a. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. Nat. Immunol. 4, 161–167.

Oshiumi, H., Tsujita, T., Shida, K., Matsumoto, M., Ikeo, K., Seya, T., 2003b. Prediction of the prototype of the human Toll-like receptor gene family from the pufferfish, *Fugu rubripes*, genome. Immunogenetics 54, 791–800.

Puttinaowarat, S., Thompson, K.D., Adams, A., 2000. Mycobacteriosis: detection and identification of aquatic *Mycobacterium* species. Fish Vet. J. 5, 6–21.

Ramakrishnan, L., Falkow, S., 1994. *Mycobacterium marinum* persists in cultured mammalian cells in a temperature-restricted fashion. Infect. Immun. 62, 3222–3229.

Reiling, N., Holscher, C., Fehrenbach, A., Kroger, S., Kirschning, C.J., Goyert, S., Ehlers, S., 2002. Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with *Mycobacterium tuberculosis*. J. Immunol. 169, 3480–3484.

Robinson-Rechavi, M., Marchand, O., Escriva, H., Bardet, P.L., Zelus, D., Hughes, S., Laudet, V., 2001. Euteleost fish genomes are characterized by expansion of gene families. Genome Res. 11, 781–788.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. E 4, 406–425.

Schwandner, R., Dziarski, R., Wesche, H., Rothe, M., Kirschning, C.J., 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. J. Biol. Chem. 274, 17406–17409.

Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., Kimoto, M., 1999. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J. Exp. Med. 189, 1777–1782.

Stafford, J.L., Ellestad, K.K., Magor, K.E., Belosevic, M., Magor, B.G., 2003. A toll-like receptor (TLR) gene that is up-regulated in activated goldfish macrophages. Dev. Comp. Immunol. 27, 685–698.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., Akira, S., 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 11, 443–451.

Takeuchi, O., Sato, S., Horiuchi, T., Hoshino, K., Takeda, K., Dong, Z., Modlin, R.L., Akira, S., 2002. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J. Immunol. 169, 10–14.

Tonjum, T., Welty, D.B., Jantzen, E., Small, P.L., 1998. Differentiation of *Mycobacterium ulcerans*, *M. marinum*, and *M. haemophilum*: mapping of their relationships to *M. tuberculosis* by fatty acid profile analysis, DNA–DNA hybridization, and 16S rRNA gene sequence analysis. J. Clin. Microbiol. 36, 918–925.

Underhill, D.M., Ozinsky, A., Smith, K.D., Aderem, A., 1999. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. Proc. Natl. Acad. Sci. U.S.A. 96, 14459–14463.

Van der Sar, A.M., Musters, R.J.P., Van Eeden, F.J.M., Appelmelk, B.J., Vandenbroucke-Grauls, C.M.J.E., Bitter, W., 2003. Zebrafish embryos as a model host for the real time analysis of *Salmonella typhimurium* infections. Cell. Microbiol. 5, 601–611.

Wang, T., Lafuse, W.P., Zwilling, B.S., 2000. Regulation of toll-like receptor 2 expression by macrophages following *Mycobacterium avium* infection. J. Immunol. 165, 6308–6313.

Xu, Y., Tao, X., Shen, B., Horng, T., Medzhitov, R., Manley, J.L., Tong, L., 2000. Structural basis for signal transduction by the Toll/interleukin-1 receptor domains. Nature 408, 111–115.

Yamamoto, M., Sato, S., Mori, K., Hoshino, K., Takeuchi, O., Takeda, K., Akira, S., 2002. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. J. Immunol. 169, 6668–6672.

Yoder, J.A., Nielsen, M.E., Amemiya, C.T., Litman, G.W., 2002. Zebrafish as an immunological model system. Microbes. Infect. 4, 1469–1478.

Zuany-Amorim, C., Hastewell, J., Walker, C., 2002. Toll-like receptors as potential therapeutic targets for multiple diseases. Nat. Rev. Drug Discov. 1, 797–807.