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REVIEW

Toll-like receptors: an overview from invertebrates to vertebrates

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Abstract

Toll-like receptors (TLRs) are membrane glycoproteins consisting of an ectodomain, encompassing tandem LRRs (leucine-rich repeats), a membrane spanning segment and a globular cytoplasmic Toll/Interleukin-1 Receptor (TIR) domain. They detect microbes on the basis of conserved Pathogen-Associated Molecular Patterns (PAMPs). TLRs share molecular architecture and common ancestors with arthropod Toll molecules, which show a dual role, in the dorsoventral patterning of the embryo, and in the immune response to fungal infections in the adult. During the metazoan evolution Toll/TLRs modified the number and the arrangment of LRRs (protostome- and vertebrate-type), the localization of the loops interacting with different ligands, the ability to reside in the cellular or endosomal membrane and the complexity of the signaling pathway. The evolutionary mechanisms of Toll/TLR gene diversification included gene duplication, retrotranscription, high gene expansion rate within species, and alternative splicing of the transcripts. The aim of this review is to supply a schematic representation of a very complex, but still, fascinating story.

Key Words: Toll-like receptor; signaling; pathogens recognition receptors; TIR domain; leucine-rich repeat; metazoan evolution

Foreground

There is a growing interest in Toll-like receptors (TLRs), as demonstrated by the 2011 Nobel Prize in medicine awarded to BA Beutler and JA Hoffmann for their studies on TLR role in physiology and pathology.

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The founding member of the TLR family was identified as a protein involved in dorsoventral patterning of *Drosophila melanogaster* embryos (Anderson *et al*., 1985); later, it was shown also to play a role in responding to fungal infection of the adults in the same species (Lemaitre *et al*., 1996). A human homologue of the *Drosophila* Toll protein was identified as activator of adaptive immunity (Medzhitov *et al*., 1997).

At present, TLRs are the most extensively studied Pathogens Recognition Receptors (PRR) in

List of abbreviations:
CTLRR: C-Terminal LRR; GNBP: Gram negative binding proteins; HKLP: heat-killed *Legionella pneumophila*; HMM: hidden Markov model; IκB: NF-κB inhibitor; IKK: IκB kinase; IL1R: interleukin-1 receptor; IMD: immune deficiency; INF: interferon; IRAK: interleukin-1 receptor-associated kinase; LPS: lipopolysaccharide; LRR: leucine-rich repeat; LTA: lymphotoxin-alpha; MAL (synonym of TIRAP): MyD88 adaptor-like; MEKK: mytogen-activated protein kinase kinase kinase, also known as MAP3K; MPD: muramyl dipeptide; MyD88: myeloid differentiation factor 88; NF-κB: nuclear factor κ light chain enhancer of B cells; NTLRR: N-Terminal LRR; ORF open-reading frame; PAMP: pathogen-associated molecular patterns; PGRP: peptidoglycan recognition protein; PIK-1: Pelle and IL1R associated kinase; PNG: peptidoglycan; PRR: pathogens recognition receptors; TICAM: Toll-like receptor adaptor molecule; TIR: Toll/Interleukin-1 receptor; TIRAP (synonym of MAL): TIR containing adaptor molecule; TLR: Toll-like receptor; TNF: tumor necrosis factor; Tollip: Toll interacting protein; TRAM: TRIF related adaptor molecule, also known as TCAM; TRIF: TIR domain containing adaptor inducing interferon-β; SARM: sterile α- and armadillo-motif-containing protein; SLIP: LPS-interacting protein; VLR: variable lymphocyte receptor; UTR: untraslated region; WSSV: white spot syndrome virus

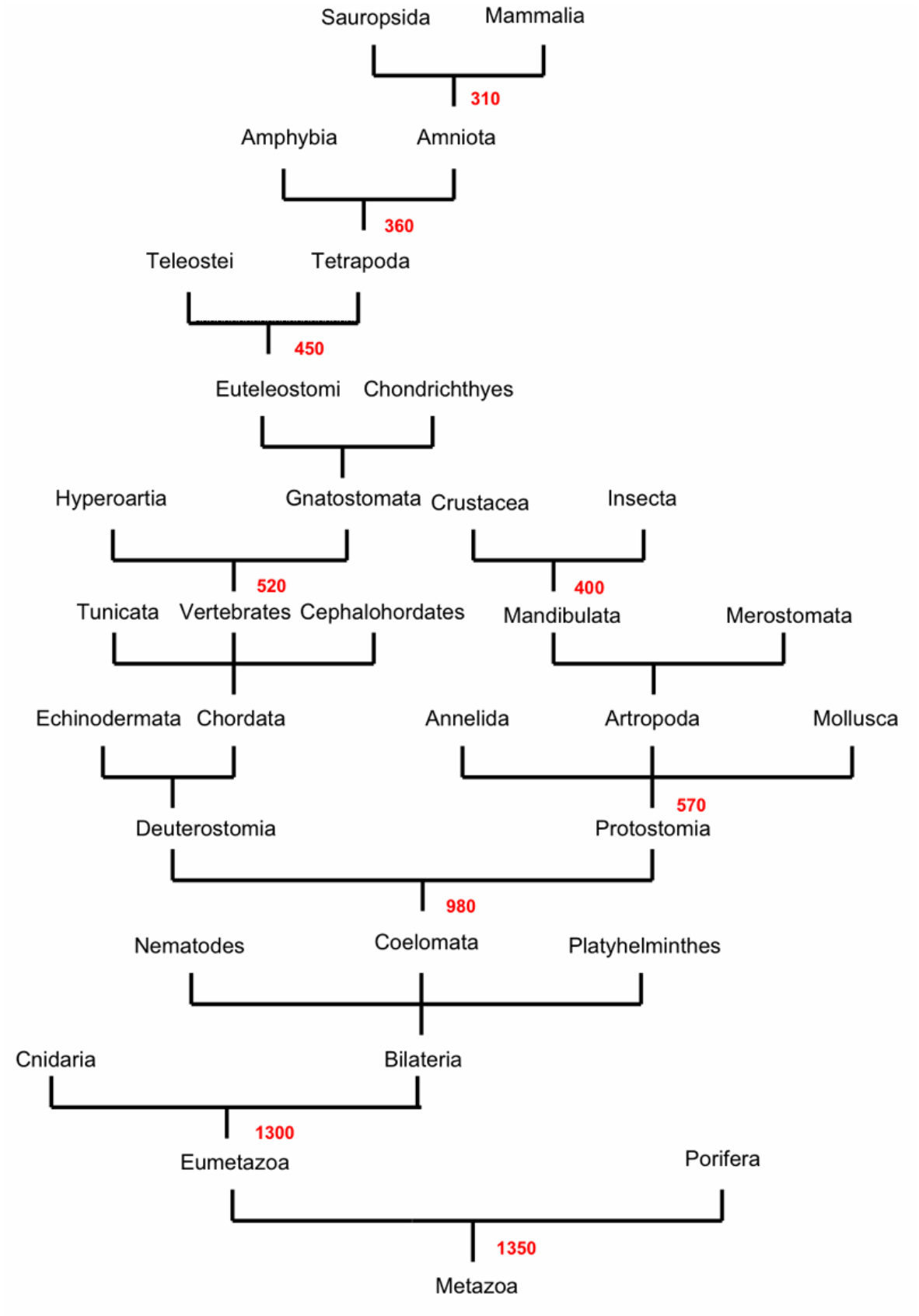

Fig. 1 Phylogenetic relationships of the reported taxa. The tree was built according to the lineages reported by NCBI taxonomy database (http://www.ncbi.nlm.nih.gov/taxonomy). Divergence times, expressed in MYA, are indicated only at the nodes analyzed by Hedges *et al.* (2004) using molecular clock methods on all available eukaryote protein sequences.

both vertebrate and invertebrate species. Several authors focus on TLRs role (Pasare and Medzhitov, 2005; Iwasaki and Medzhitov, 2010), structure (Gay and Gangloff, 2007; Jin and Lee, 2008; Botos *et al*., 2011), signaling (Barton and Medzhitov, 2003; Gay *et al*., 2011), and evolution (Leulier and Lamaitre, 2008; Werling *et al*., 2008; Satake and Sasaki, 2010; Wu *et al*., 2011). Due to the huge amounts of data present in literature, a brief excursus is here presented on all phyla in which genes encoding TLRs or their homologues have been identified (Fig. 1). We will focus special attention on TLR molecules from species that lie on the boundary between the vertebrates and invertebrates to provide a comprehensive guide to the evolution of these molecules at the emergence of the adaptive immune system. All species analyzed in the present review are listed in Table 1.

Introduction

The TLR family mediates sensing of microbial pathogens (Beutler, 2004). For a brief description of the molecular structure, we shall remind that TLRs are type I integral membrane glycoproteins and different members of the family detect microbes on the basis of conserved Pathogen-Associated Molecular Patterns (PAMPs). They consist of an ectodomain, a membrane spanning segment and a globular cytoplasmic Toll/Interleukin-1 Receptor (TIR) domain (Fig. 2). The ectodomain is arranged in a horseshoe structure (Fig. 2), encompassing 19 - 27 tandem LRRs (leucine-rich repeats). Each LRR contains a conserved 11-residue segment, being the consensus sequence LXXLXLXXNXL, where X can be any amino acid, L is a hydrophobic residue (leucine, valine, isoleucine or phenylalanine) and N can be asparagine or cysteine (Kobe and Kajava, 2001). Each repeat consists of a β-strand and an α-helix connected by loops. Two regions, each containing several cysteines, flank the LRRs, NTLRR (N-Terminal LRR) and CTLRR (C-Terminal LRR).

A TIR domain (Fig. 2) is present in the cytoplasmic region; it has an α - β fold consisting of a central five-stranded parallel β-sheet surrounded by five α-helices. The same fold occurs also in the adaptor proteins MyD88 (Myeloid Differentiation factor 88), Mal (MyD88 adaptor-like), TRIF (TIR domain containing adaptor inducing interferon-β) TRAM (TRIF related adaptor molecule) and SARM (sterile α- and armadillo-motif-containing protein) (O'Neill and Bowie, 2007). The adaptors associate with each other or with TLRs by the respective TIR domains (Dunne *et al*., 2003). The receptor complex recruits, in succession, IRAKs (interleukin-1 receptor-associated kinases), TRAF6 (TNF receptor-associated factor 6), IKK, which phosphorylates the NFκB inhibitor IκB, which activates the transcription (Fig. 3). TLR signaling is also tuned by MicroRNAs, which target 3'UTR of transcripts that encode signaling components (O'Neill *et al*., 2011). The signaling induces production of pro-inflammatory cytokines such as interleukins, interferon, TNF, responsible for direct innate response and for triggering adaptive immune cells.

Table 1 Metazoan species whose Toll-TLR molecules have been considered in the present study

Porifera	*Amphimedon queenslandica*
	Suberites domuncula
Cnidaria	*Acropora millepora*
	Acropora palmata
	Hydra magnipapillata
	Montastraea faveolata
	Nematostella vectensis
Nematodes	*Caenorhabditis elegans*
Platyhelminthes	*Schmidtea mediterranea*
Annellida	*Capitella capitata*
	Helobdella robusta
	Hirudo medicinalis
Mollusca	*Chlamys farreri*
	Crassostrea gigas
	Euprymna scolopes
	Mya arenaria
Merostomata	*Tachypleus tridentatus*
	Carcinoscorpius rotundicauda
Insecta	*Aedes aegypti*
	Anopheles gambiae
	Apis mellifera
	Drosophila melanogaster
	Tribolium castaneum
Crustacea	*Fenneropenaeus chinensis*
	Litopaeneus vannamei
	Marsupenaeus japonicus
	Penaeus monodon
Echinodermata	*Strongylocentrotus purpuratus*
Cephalocordata	*Branchiostoma belcheri*
	Branchiostoma floridae
Tunicata	*Boltenia villosa*
	Ciona intestinalis
	Oikopleura dioica
Hyperoartia	*Lethenteron camitschaticum*
Chondrichthyes	*Callorhincus milii*
	Chiloscyllium griseum
Teleostei	*Cynoglossus semilaevis*
	Cyprinus carpio
	Danio rerio
	Gobiocypris rarus
	Ictalurus punctatus
	Oncorhynchus mykiss
	Paralichthys olivaceus
	Pseudosciaena crocea
	Salmo salar
	Takifugu rubripes
Amphybia	*Xenopus laevis*
	Xenopus tropicalis
Reptiles	*Anolis carolinensis*
Aves	*Accipiter cooperii*
	Amazona albifrons
	Carpodacus mexicanum
	Dromaius novaehollandiae
	Falco naumanni
	Gallus gallus
	Oceanodroma leucorhpoa
	Picoides pubescens
	Taeniopygia guttata

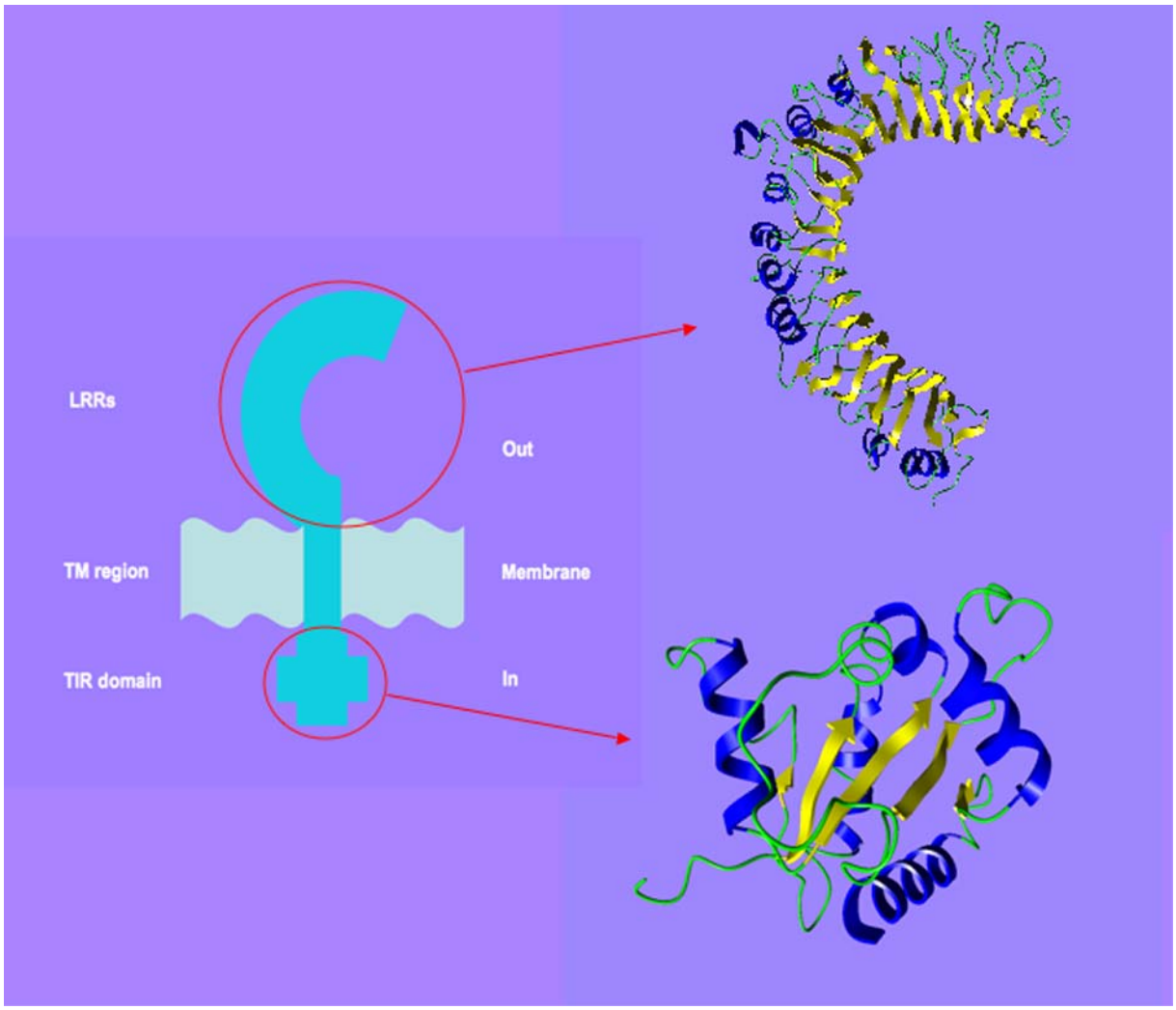

Fig. 2 TLR molecule. On the right side the molecular structure of the mammalian TLR. At the top the ectodomain of mouse TLR2 (PDB ID: 3A7C), at the bottom the human TIR domain (PDB ID: 1FYX).

The majority of TLRs associate to form homodimers. However, there are some exceptions, one is represented by TLR2 that dimerizes with TLR1, TLR6 (Takeuchi *et al*., 2002), or TLR10 (Govindaraj *et al*., 2010) to provide different PAMP specificity. C-terminal ectodomains dimerize while N-termini are oriented in opposite directions. This architecture is crucial for both the ligand binding and the assembly with the adaptors.

TLR functions are well known in mammals and all knowledge accumulated so far is based on interpretation of the general role TLRs play in non-mammalian species.

Some TLRs are expressed on the cell membrane and their ectodomain recognizes extracellular PAMPs, while others are expressed in endosomes to detect internalized PAMPs. TLRs are expressed not only in immune cells (dendritic cells, monocytes, macrophages, B lymphocytes) but also in non-immune cells, including fibroblasts, endothelial cells, adipocytes, epithelial cells and glial cells.

The TLR ligands can be categorized into lipid, protein and nucleic acid components. TLR ligand specificity is due to different modes of LRRs assembly. Moreover, ligand- interacting residues have been demonstrated to be present on both the concave and the convex side of the horseshoe (Jin and Lee, 2008).

So far, at least 23 vertebrate TLRs have been identified, based on amino acid similarity, genomic structure, and ligand properties. They can be grouped into six major families (Roach *et al*., 2005) as reported in Table 2.

Porifera

Porifera represents the most ancient metazoan phylum showing nucleotide sequences reminiscent of TLR. Two species, *Suberites domuncula* (Wiens *et al.*, 2005, 2007) and *Amphimedon queenslandica* (Gauthier, 2010), belonging to this phylum, have been investigated in an attempt to trace back the evolutionary origin of TLR. Both are demosponges.

The Muller group has identified in *S. domuncula* several proteins resembling some Toll/TLR-pathway components, including a LPS-interacting protein (SLIP) whose predicted ectodomain lacks LRRs; it dimerizes and bind a MyD88 homologue but it appears significantly atypical in that it lacks a clear death domain (Wiens *et al*., 2005). As sponge MyD88 and SLIP are co-immunoprecipitated by the reciprocal antibodies, they clearly can interact *in vitro*.

Subsequently, the same group has reported the cloning of three major elements of the sponge innate immune response: TLR-like, IRAK and caspase, highly homologous with vertebrate orthologs. In particular, a TIR domain, highly homologous to mammalian TIR, is present in the TLR-like cytoplasmic region. In the ectodomain no clear LRR has been detected (Wiens *et al*., 2007). It has been hypothesized that *S. domuncula* TLR corresponds to a short splice variant of a longer transcript as reported in vertebrate TLRs (Iwami *et al*., 2000; Wells *et al*., 2006).

The availability of the whole-genome sequence of the sponge *A. queenslandica* has allowed the identification of two related receptors, AmqIgTIRs, which comprise at least three extracellular IL1R-like immunoglobulin domains and an intracellular TIR domain. The remainder of the TLR/IL1R pathway is mostly conserved and includes genes known to interact with TLRs and IL1Rs in bilaterians, such as Tollip (Toll interacting protein) and MyD88 (Gauthier *et al.*, 2010). However, the ambiguous status of the sponge MyD88 related protein means that it is unclear whether sponges have a canonical Toll/TLR signaling pathway.

Because *A. queenslandica* and basal eumetazoans encode similar proteins with extracellular IL1R-like Ig domains and an intracellular TLR-like TIR domain, it can be suggested that a similar receptor existed in the last common metazoan ancestor.

This implicates that in eumetazoans the genes encoding TIR domains with TLR features and those encoding LRR-containing domains were combined together yielding the ancestral gene of the canonical Toll/TLR family. This is in line with the extensive exon-shuffling event occurred in metazoan and eumetazoan lineages (Srivastava, 2010).

Cnidaria

The phylum Cnidaria provides crucial insights into the early evolution of animals because it is the likely sister group of the superphylum Bilateria. Although the literature on cnidarian immunity is wide, the distinction between historecognition and host-response/disease is unclear in this group of primitive organisms (Rinkevich, 2011). Results on Cnidaria molecules resembling TLRs have been reviewed by Hemmrich *et al.* (2007) and by Dunn (2009).

Data collected in five different cnidarian species are reviewed below. The species investigated are as follows: *Hydra magnipapillata* belonging to Hydrozoa; *Nematostella vectensis, Acropora millepora, Acropora palmata* and *Montastraea faveolata* belonging to Anthozoa.

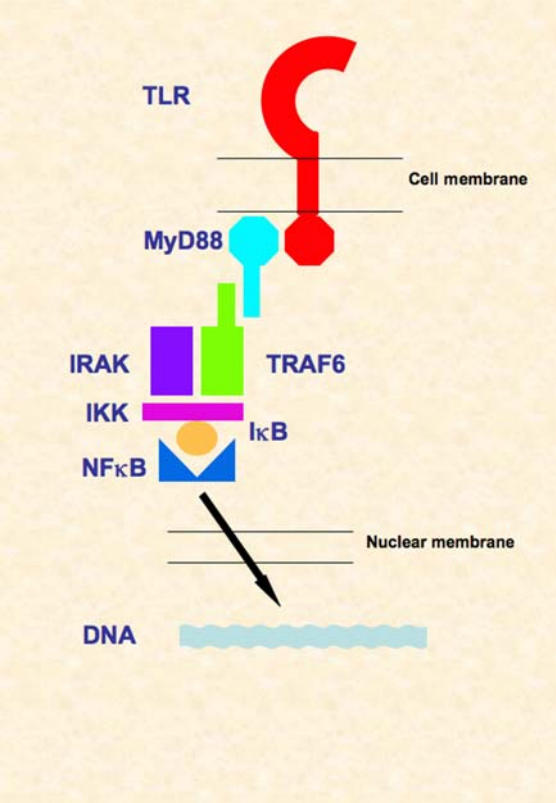

Fig. 3 TLR signaling pathway in mammals.

Extensive searching the *Hydra* predicted protein collection, using the available hidden Markov models (HMMs) has not succeeded in identifying proteins having the canonical Toll/TLR structure. On the other hand, four TIR domain-containing proteins have been found. Two of them show sequence features related to MyD88 including the typical death domain. The two other *Hydra* TIR domain-containing proteins show relatively short ectodomains lacking LRRs. These proteins are known as HyTRR-1 and HyTRR-2 and are expressed on the epithelial cells (Miller *et al*., 2007; Augustin *et al*., 2010). By functional studies it has been demonstrated that they are capable of mounting an immune response through a non-conventional signaling pathway (Bosch *et al*., 2009). Sequence comparison has provided further evidence that these TIR-domain sequences cluster with TIR domains of other animal TLRs, rather than with intracellular TIR domain adaptors, suggesting that they are TLR-related molecules (Zheng *et al*., 2005). In addition, no NF-κB homologues have been identified in *Hydra* (Miller *et al*., 2007).

Instead, different data have been obtained in anthozoan species. A TLR gene (*NvTLR-1*) is present in the genome of the starlet sea anemone, *Nematostella vectensis* (Putnam *et al*., 2007; Sullivan *et al*., 2007), a basal cnidarian, but not in the genomes of other anthozoans, such as the coral species *Acropora millepora* and *Montastraea faveolata* (Schwarz *et al*., 2008). Using HMM-based

search methods five TIR-containing proteins have been identified in *N. vectensis* (Miller *et al*., 2007). NvTLR-1 is clearly related to members of the Toll/TLR family. The typical TLR architecture consists of only a C-terminal cys-rich motif flanking the LRRs proximal to the membrane, instead *N. vectensis* NvTLR-1 is predicted to contain the NTLRR and an additional CTLRR within the LRRs. Moreover, a phylogenetic analysis of TIR-containing proteins has grouped the NvTLR-1 TIR with its fly and human counterparts. Interestingly, three more *N. vectensis* TIR-containing proteins contain multiple (two or three) immunoglobulin domains, resembling the structure of mammalian IL-1Rs. In addition, a single MyD88 homologue (NvMyD88) and several kinases involved in the Toll-TLR signaling have been identified (Sullivan *et al*., 2007).

Searching the datasets of nucleotide sequences from *A. palmata* and *A. millepora* has shown that the respective TIRs are very similar to those contained in the *Nematostella* IL-1R-like proteins (Miller *et al*., 2007).

Finally, characterization of *M. faveolata* EST dataset has led to identification of partial sequences of genes involved in immune response such as MAPK, NF-κB, and TIR-containing proteins (Schwarz *et al*., 2008).

Nematodes and Platyhelminthes

Nematodes are bilaterian animals belonging to Pseudocoelomata and are very abundant on Earth. Analysis of the *Caenorhabditis elegans* genome identified a gene encoding a TLR (Tol-1), other genes encoding proteins involved in Toll-TLR signaling pathway (TRF-1, PIK-1, IκB) and one more TIR-containing protein (TIR-1), which is homologous to human SARM1. However, homologues of MyD88 or NF-κB have not been found in the *C. elegans* genome (Irazoqui *et al*., 2010). The predicted Tol-1 ectodomain contains 22 LRRs, with one interspersed NTLRR and two CTLRRs, followed by a putative transmembrane region and a cytosolic TIR domain (Pujol *et al*., 2001). This architecture is similar to that of NvTLR-1, but the number of LRRs is larger in the nematode species than in cnidarian species. Tol-1 is preferentially expressed in the nervous system of adult animals. The developmental role of Tol-1 in embryogenesis has been ascertained by analysis of mutans, but there is no evidence for a role in resistance to a number of pathogens. Since MyD88 and NF-κB are absent and the only TIR-containing protein seems to be not involved in immune signaling, other signaling pathways, like p38 mitogen-activated protein kinase (MAPK) cascade probably assume immune functions (Kim *et al.*, 2002). These additional immune pathways might have evolved in primitive metazoans and have been maintained throughout metazoan evolution, functioning in concert with TIR pathways.

Although very important because of infecting millions of people, Plathelminthes remain scarcely investigated at molecular level. An important contribution comes from the work of Sanchez *et al*. (2002), which have characterized about 3,000 non-redundant cDNA from a clonal line of the planarian

Table 2 Vertebrate TLR families. Letters preceeding some TLRs are as: x, *Xenopus*; t, teleost; c, chicken; m, mouse. The remaining TLRs are shared by all vertebrate species

family	
1	TLR1, TLR2, TLR6, TLR10, xTLR14, tTLR14, t TLR18, cTLR15,
3	TLR3
4	TLR4
5	TLR5
7	TLR7, TLR8, TLR9
11	mTLR11, mTLR12, mTLR13, cTLR21, xTLR21, xTLR22,
21	tTLR13, tTLR19, tTLR20, tTLR21, tTLR22, tTLR23

Schidtea mediterranea. In this library a single 620-nt long mRNA sequence (AY066289) was found to be similar to mammalian TLR4. Because of the low value of similarity, this datum is scarcely useful.

Annelida

Annelida belongs to Protostoma. The leech *Helobdella robusta* and the polychaete *Capitella capitata* (Davidson *et al*., 2008) have been analyzed for the presence of TLRs: 16 TLR contigs were detected in the genome of *H. robusta* and 105 in *C. capitata*. *H. robusta* TLR sequences do not seem to be orthologous to those of *C. capitata*; the majority of *C. capitata* TLR sequences are very similar to each other and seem to result from a recent gene duplication event.

Both species show a basic TLR domain structure (extracellular LRRs, transmembrane segment, TIR domain), but *H. robusta* TLRs present the extracellular LRR clusters with tandem CTLRR and NLRR (protostome-type) whereas all *C. capitata*, but one, TLRs present a structure similar to mammalian TLRs (vertebrate-type).

In *C. capitata*, but not in *H. robusta* genome, the putative orthologs of TLR signaling pathway proteins (MEKK, IκB e and NF-κB) have also been identified whereas MyD88 and TRAF homologues have been found in both genomes.

Recently, a cDNA library of *Hirudo medicinalis* has been analyzed (Cuvillier-Hot *et al*., 2011) and the complete sequence of HmTLR1 has been determined; the ectodomain presents 6 LRRs preceded by one NTLRR. HmTLR1 shows great homologies with *Mus musculus* and *Monodelphis domestica* TLR13, while no significant homology with molecules identified in other annelids has been noticed. The transcripts are preferentially expressed in neurons as well as in microglial cells and the protein, similarly to mouse TLR3, has been localized in endosomal compartment of neuronal cells.

To explore the biological functions of HmTLR1, gene expression has been quantified during the regeneration process and following microbial challenges. By regeneration tests, a differentiation role of HmTLR1 gene has been excluded; on the

other hand, after *H. medicinalis* exposition to different microbes, the levels of HmTLR1 transcripts have been observed to differently increase, demonstrating the capability of distinguishing microbial components (Cuvillier-Hot *et al*., 2011).

Mollusca

From an evolutionary point of view mollusks are placed between the two traditional model organisms *D. melanogaster*, in which Toll protein and Toll signaling pathway have been identified for the first time, and *C. elegans*, in which little evidence for the existence of TLR signaling pathway have been accumulated so far. The molluscan species investigated in this context are the bivalvian *Chlamys farreri, Mya arenaria, Crassostrea gigas*, and the cephalopod *Euprymna scolopes.*

In *C. farreri* most of the Toll-TLR signaling pathway components have been demonstrated to occur: CfToll-1 (Qiu *et al*., 2007a), CfMyd88 (Qiu *et al*., 2007b), CfTRAF6 (Qiu *et al*., 2009), CfNFκB and CfIκB, indicating the possibility of the presence of a Toll-TLR signaling pathway in mollusks. The sequence features of these five key genes involved in TLR signaling pathway in scallop *C. farreri* have been characterized (Wang *et al*., 2011a). The expression levels of CfTLR, CfMyD88 (Limei *et al*., 2007), CfTRAF6 (Limei *et al*., 2009), CfIκB and CfNFκB increase after LPS stimulation and decrease after RNAi suppression. An interaction between recombinant CfTLR-TIR domain and recombinant CfMyD88 has been proved by ELISA assay.

Zhang *et al*. (2011) have cloned the TLR gene in *C. gigas* (CgToll-1) and have found its expression to be affected by bacterial challenge. A phylogenetic analysis of the molecules involved in Toll-TLR signaling indicates that *C. gigas* downstream genes IκB (Escoubas *et al*., 1999; Montagnani *et al*., 2008) and Rel (Montagnani *et al*., 2004) cluster with protostome orthologs, while upstream genes, MyD88, TRAF6 (Gueguen *et al*., 2003; Roberts *et al*., 2009) and IRAK cluster with deuterostome orthologs. CgToll-1 is a typical single-pass transmembrane protein including signal peptide, 19 LRRs containing an interspersed NTLRR, a transmembrane domain and a TIR domain. Moreover, among 20 potential *N*-linked glycosylation sites in CgToll-1, nine are anchored at the convex surface of LRRs accounting for a high degree of putative *N*-linked glycosylation in this region (Zhang *et al*., 2011).

Although widely distributed, CgToll-1 is preferentially expressed in hemolymph; this feature resembles that of CfToll (Qiu *et al*., 2007a). Furthermore, it has been reported that CgToll-1 up-regulates the expression of MyD88 in *C. gigas* (Tirape *et al*., 2007). Haemocytes have been demonstrated to have a crucial role in mollusk defense system, and a high expression level of CgToll-1 in these cells suggests the key role of CgToll-1 in *C. gigas* immune response.

Studies performed on immune gene expression levels in *Mya arenaria* haemocytes, have revealed that genes encoding homologues of TLR2 and IRAK4 are significantly regulated following *in vivo* infection with *Vibrio splendidus* (Mateo *et al*., 2010).

Finally, Goodson *et al*. (2005) have described seven component of the Toll-TLR pathway by screening an EST library from the juvenile light organ of the cephalopod *E. scolopes*. *E. scolopes* TLR architecture is consistent with that of mammals and *Drosophila*.

Merostomata

Merostomata is an arthropod class including the order Xiphosura and the extinct order Eurypterida. A TLR, named tToll, has been identified in the haemocytes of the horseshoe crab *Tachypleus tridentatus* (family Limulidae). The architecture and length of tToll are similar to those of *Drosophila* Toll1 (Inamori *et al*., 2000; 2004). tToll consists of 22-25 residue-long LRRs flanked by cys-rich motives and containing NTLRR and CTLRR; a putative transmembrane domain is placed near the ectodomain and a TIR domain is present in the C-terminal cytoplasmic region. Twenty potential *N*-linked glycosylation sites are localized in the ectodomain. tToll and *Drosophila* Toll1 ectodomains share 23 % identity, whereas tToll TIR shows 39 % sequence identity with *Drosophila* Toll1 and 31 % with human TLR2 and TLR4.

The structural relationship with *Drosophila* Toll1 and the absence of insertions in the LRRs suggest that, similarly to *Drosophila* Toll1, tToll does not function as PAMP-binding receptor, and that its real ligand might be a protein resembling *Drosophila* Späetzle (Kurata *et al.,* 2006). Coagulogen, the final target of the coagulation cascade of horseshoe crabs, whose structure is similar to Späetzle, could be the candidate molecule. Its cleaved form, coagulin, could promote tToll dimerization and, in turn, the activation of intracellular signaling.

In another merotostome species, *Carcinoscorpius rotundicauda,* the TLR adaptor SARM has been identified and shown to share many signature motives with vertebrate and invertebrate SARMSs (Belinda *et al*., 2008).

Insecta

Insecta is an arthropod class with more than a million of species, and includes the most diverse group of animals. Studies performed on the model species *Drosophila melanogaster*, has opened the way for knowledge of fundamental mechanisms of embryo development and immune response in insects. At present Toll-like nucleotide sequences have been determined in 24 different insect species. After Toll identification (Anderson *et al*., 1985), additional Toll family members (Toll2-9) have been recognized in the *D. melanogaster* genome (Hoffmann, 2003; Valanne *et al*., 2011). Gradually, the dual function in embriogenesis and immune response has been ascertained (Ferrandon *et al*., 2007). All Tolls, but Toll9, contain 1 - 4 additional cys-rich motives interspersed in the LRR region (Imler and Hoffmann, 2001). Tolls do not bind any PAMPs, however require accessory proteins. Persephone or PGRPs and GNBPs are the molecules recognizing fungi or Gram-positive

bacteria, respectively (Gobert *et al*., 2003; Pal and Wu, 2009). In *Drosophila* molecules unrelated to Tolls, called IMD (immune deficiency) mediate the resistence to Gram-negative bacteria (Lemaitre *et al*., 1995; De Gregorio *et al*., 2002). Persephone, which is a protease, directly cleaves a protein called Späetzle, which is able to bind Toll and activate the signaling cascade. PGRPs, and GNBPs activate proteolytic cascades, finally cleaving Späetzle. Tolls have been demonstrated to dimerize (Hu *et al*., 2004) and the molecular structure of the Toll-Späetzle complex has been investigated by molecular modeling and electron microscopy; the complex shows a stoichiometry and architecture totally unrelated to that of TLR-ligand complexes, being the Toll binding sites at the N-terminal end of each monomer (Gangloff *et al*., 2008). Adaptor proteins, including MyD88, are involved in the signaling mechanism; however, antimicrobial peptides rather than cytokines are the immune defense molecules induced by the signaling. Toll genes have also been searched in sequenced genomes of other species that belong to the class Insecta. The genomes of five insect species revealed the presence of a different number of Toll encoding genes: 5 in *Apis mellifera* (Evans *et al*., 2006), 9 in *Tribolium castaneum* (Zou *et al*., 2007), 11 including two pseudogenes, in *Bombyx mori* (Cheng *et al*., 2008), 10 in *Anopheles gambiae* (Cristophides *et al*., 2002), 12 in *Aedes aegypti* (Waterhouse *et al*., 2007). A comparison of the insect Tolls suggests a species-specific diversification process.

Crustacea

Crustacea is another arthropod class that is distinct from insecta by the possession of two-parted limbs. TLRs have been found in four crustacean species, all belonging to the family Penaeidae. LvToll1, the first crustacean Toll identified in *Litopenaeus vannamei,* (Yang *et al*., 2007), has a typical protostome-like TLR structure with an ectodomain composed by 16 LRRs, a transmembrane domain and an intracellular TIR domain. A sequence comparison of LvToll TIR with that of insect Toll shows high similarities, between 54.3 and 59.9 %.

Other two novel TLRs have been identified more recently in the same species (Wang *et al*., 2011b); LvToll2 and LvToll3 share 43.2 % and 25.4 % identity with LvToll1, respectively. The cellular localization of the three LvTolls is different: LvToll1 and LvToll3 are present in both membrane and cytoplasm, while LvToll2 is restricted to the cytoplasm. They are constitutively expressed in many tissues including gill, stomach, intestine, nerve, muscle, pyloric caecum, spermary, and epidermis. Upon challenges with *Vibrio alginolyticus* and WSSV, the three LvTolls show a different response: LvToll1 is up regulated with both challenges; LvToll2 is up regulated only with WSSV challenge; finally, LvToll3 is up regulated with both challenges as LvToll1, but at different times. They also may be involved in phagocytosis (Wang *et al*., 2011b).

A partial sequence of a similar Toll-related gene has also been identified in a *Penaeus monodon* gill cDNA library. It is expressed, with no significant differences, in gut, gill and hepatopancreas haemocytes and compound eye (Arts *et al*., 2007).

In 2008 TLRs have been described in two additional crustacean species, *Fenneropenaeus chinensis* (Yang *et al*., 2008) and *Marsupenaeus japonicus* (Mekata *et al*., 2008). FcToll shows a structure and expression pattern similar to those of the previously identified shrimp Tolls. MjToll shows high identity (96.9 %) with PmToll and low identity with other crustacean homologues (59.0 %). The MjToll gene is constitutively expressed in the same tissues as LvToll1 and its expression is increased by peptidoglycans.

Echinodermata

Echinodermata is a phylum of deuterostomes that separated from Chordata about 600 MYA (Ayala *et al*., 1998). A survey of the genome of the purple sea urchin *Strongylocentrotus purpuratus* (family Strongylocentrotidae), a member of the phylum Echinodermata, has revealed the presence of a large number (4 - 5 % of the identified genes) of vertebrate immune gene homologues (Hibino *et al*., 2006; Rast *et al*., 2006; Buckley and Smith, 2007; Buckley *et al*., 2008). 222 Toll-like receptor gene models have been identified; these TLRs, are grouped into two categories, a greatly expanded multigene family, including 211 genes comprised of seven subfamilies, and a very small set of 11 divergent genes.

The expanded TLR genes are intronless and code for proteins with a vertebrate-type structure; three of the small set have a typical protostome-like structure and their TIR domains exhibit a protostome-like sequence and a shorter C-terminal β-strand. Protostome-like sequences have not been identified in other deuterostomes, suggesting that they were present in the common ancestor of modern Bilateria and then were lost in vertebrate lineage (Rast *et al.,* 2006). The remaining five TLR genes of the small group code for an unusual short ectodomain and seems to have affinities with the protostome-type genes (Hibino *et al*., 2006).

The majorirty of the sea urchin TLR genes are more similar to each other than to those of other animals: this suggests that a TLR gene expansion occurred in *S. purpuratus*. The majority of the differences among these genes fall in the ectodomain probably accounting for the diversification of immune recognition specificity. Differences consist of: individual amino acid substitutions, small insertion/deletions, insertions of long sequences between or within LRR motives or insertions of additional LRRs. In protostomes long insertions are less frequent than in vertebrates in which they modify ligand specificity (Bell *et al*., 2003). The hypervariability is confined to particular LRRs. The presence of recently duplicated genes, the occurrence of many pseudogenes (25 - 30 %) and the regionalized hypervariability suggest that the sea urchin TLR genes undergo a dynamic

evolution characterized by a high gene turnover rate (Rast *et al*., 2006).

Expanded TLR genes are absent or weakly expressed in embryos prior to the end of gastrulation whereas their expression increases in early pluteus. Protostome-like TLR genes are absent in embryos whereas are predominatly expressed in coelomocytes and in tube feet in the adult (Hibino *et al*., 2006).

In addition, the survey of *S. purpuratus* genome has identified 26 genes coding for potential TLR adaptor proteins: a MyD88 ortholog and three more genes with a MyD88-like domain, an orthologue of SARM, 14 SARM-related genes, and 7 genes encoding cytoplasmic TIR domain proteins. The expansion of TLR genes has occurred in parallel with a modest expansion of TLR adaptor signaling protein genes. The presence of homologues of TLR signal transduction proteins suggests that the engagement of TLRs may lead to the activation of NF-κB, already known since isolated and characterized in *S. purpuratus* (Pancer *et al*., 1999).

Cephalochordata

The phylum Chordata comprises cephalochordates (amphioxus), urochordates (tunicates), and vertebrates. These groups diverged from a common ancestor during or prior to the Cambrian explosion (Holland *et al*., 2008). By recent phylogenetic studies cephalochordates have been recognized as the basal group of the phylum Chordata, since vertebrates and urochordates have diverged later (Bourlat *et al*., 2006; Delsuc *et al*., 2006). This indicates that cephalochordates represent the oldest still existing lineage of the phylum Chordata.

Sequencing the genome of the cephalochordate *Branchiostoma floridae* has allowed a better understanding of the basal chordate evolution. A recent insight into *B. floridae* genome has revealed that its TLR system possesses an unprecedented degree of arrangement since including an expanded vertebrate-type family, consisting at least of 36 TLRs, a protostome-type group of 12 elements and 40 TIR-containing adaptors. Rapid tandem gene duplication has been suggested to be the mechanism generating the majority of *B. floridae* vertebrate-type TLRs (Huang *et al*., 2008). Based on phylogenetic analysis of the TIR domains of amphioxus and vertebrate TLRs, surprisingly protostome-type *B. floridae* TLRs has been shown to cluster with the vertebrate TLR4 lineage; on the other hand, 33 variable-type *B. floridae* TLRs show a paraphyletic relationship with vertebrate TLR11 lineage, 19 of them comprising a distinct clade designated as SC75. In this clade there are two pseudogenes and 12 intronless genes, probably generated by retrotranscription events. In the expanded group, the TIR domain is highly conserved (identity higher than 85%), whereas the ectodomain is more variable. The occurrence of positively selected positions has been demonstrated in LRRs. The evolution of SC75 clade resembles that of the *S. purputatus* variable-type TLRs (Huang *et al*., 2008).

The search for elements of the Toll-TLR signaling pathway in the amphioxus genome has also been carried out: 4 MyD88-like, 10 SARM-like, one TIRAP-like and one TICAM2-like gene have been identified, whereas no homologue of TICAM1 has been detected.

A single TLR gene, *bbtTLR1*, which is inserted into an intron in the reverse orientation, has been identified, cloned and characterized in the Chinese amphioxus *Branchiostoma belcheri tsingtauense* (Yuan *et al*., 2009). The genomic region containing *bbtTLR1* has also been demonstrated to be highly polymorphic. Its structure is of vertebrate-type showing one NTLRR, 22 LRRs and one CTLRR in the ectodomain, a transmembrane domain, and a TIR domain in the cytoplasmic region. *bbtTLR1* shares 77 % identity with *bfTLR1*. Most of the bbTLR1 LRRs show the canonical LRR motif except LRR6 and 7; two large insertions are present next to LRR3 and 10; in addition, 10 putative *N*-glycosilation sites have been detected.

bbtTLR1 expression is detectable in the villi of the gut epithelial cells, midgut diverticulum, in the connective tissues and coelome cells: it is predominantly expressed in certain regions that represent the frontline of host defense. It was also demonstrated that bbtTRL1 is a surface receptor expressed on cell membrane.

The expression of its transcript can be greatly upregulated by LPS and Gram-negative bacteria (*Vibrio vulnificus*) and scarcely by LTA, PGN and Gram-positive bacteria (*Staphylococcus aureus*), and is unaffected by Glucans and poly I:C.

A MyD88 homologue, bbtMyD88, has been characterized and its involvement in NF-κB activation has been demonstrated (Yuan *et al*., 2009).

Tunicata

Urochordates are considered the invertebrate group more closely related to vertebrates. The presence of TLR-like molecules in Urochordates has been investigated in two ascidian species *Boltenia villosa* (family Piuridae) (Davidson and Swalla, 2002), and *Ciona intestinalis* (family Cionidae) (Azumi *et al*., 2003; Sasaki *et al*., 2009; Nonaka and Satake, 2011) and in one appendicularian species, *Oikopleura dioica* (family Oikopleuridae) (Denoeud *et al*., 2010)

Davidson and Swalla (2002) have isolated in a *B. villosa* cDNA library a gene, *BvLRR*, resambling *D. melanogaster* Toll and showing distinct peaks of expression during larval or post-larval development.

Azumi *et al*. (2003) by screening the draft genome sequence of *C. intestinalis* have identified only 3 TLR gene models, homologous to TLR4, 6, and 7, respectively, and several genes involved in the TLR signaling, including MyD88, IRAK, TRAF, IκB, and NFκB.

Sasaki *et al*. (2009) have investigated the structures, localization, ligand recognition, activity and cytokine production of two TLRs of *C. intestinalis,* CiTLR1 and CiTLR2. Both deduced protein sequences show the typical TLR architecture consisting of an intracellular TIR domain, a transmembrane domain, and multiple

extracellular LRRs. In particular, CiTLR1 exhibits 7 putative LRRs and only one CTLRR (vertebrate-type), while CiTLR2 displays 13 LRRs and three CTLRRs, which are features found in the protostome-type TLR.

The overall amino acid sequence of CiTLRs shares no significant sequence homology with human TLRs: TIR domains of CiTLR1 and CiTLR2 are more similar to human TLR4 and TLR6, and the overall sequences are most homologous to human TLR7 and TLR8, respectively.

In juveniles *CiTLR1* and *CiTLR2* genes are expressed intensively in stomach, intestine and in haemocytes. In the adult both genes are equally expressed in the stomach; in anterior and middle intestine CiTLR1 expression predominates over that of CiTLR2. Unlike mammalian TLRs, which have been found to be exclusively either on plasma membrane or in endosomes, CiTLR1 and CiTLR2 present both localizations, even if CiTLR2 is more intensively expressed in endosomes than on cell membrane.

CiTLR1 and CiTLR2 interact with multiple PAMPs, which are differentially recognized by vertebrate TLRs; CiTLR1 induces a response to zymosan, while CiTLR2 elicits a prominent dose-dependent response to poly I:C, (specific ligand for human TLR3), to heat-killed *Legionella pneumophila* (HKLP) (specific ligand for human TLR2) and flagellin (specific ligand for human TLR5). It is ascertained that CiTLRs, like vertebrate TLRs, directly recognize their PAMPs, without requiring association of additional specific proteins. Notably, both CiTLR show equipotent NF-κB activation in response to the same ligand. Finally, up regulation of TNF-α in response to CiTLR ligands has been demonstrated to occur in stomach and intestine.

Also in the pelagic appendicularian *O. dioica* the genome has been surveyed for pathogen sensors (Denoeud *et al*., 2010): only one TLR-like protein has been identified and search for MyD88, SARM, TIRAP and TICAM has been unsuccessful.

Hyperoartia

Hyperoartia is a class of jawless fish (agnathans), that diverged from gnathostomes about 520 MYA (Hedges *et al*., 2004). In the surviving jawless fish which are the lowest class of vertebrates including lamprey and hagfish, an exclusive adaptive immune system comprises variable lymphocyte receptors (VLRs) containing LRR subunits (Pancer *et al*., 2004). Two TLRs, named laTLR14a and laTLR14b, were initially identified in *Lampreta japonica* (synonym: *Lethenteron camitschaticum,* Petromyzontidae) by PCR-based cloning using primers designed on sequences of TLR2 from various species (Ishii *et al*., 2007). Both laTLRs contain 8 LRRs, a transmembrane region, and a cytoplasmic TIR domain. The two laTLRs show 56% homology with each other, and the TIRs are similar to those of the human TLR2 subfamily, probably orthologs of fish TLR14. An 85-kDa protein has been identified in a human HEK293 transfectant by using in immunoblotting a polyclonal Ab specific for laTLR14b. FACS, histochemical, and confocal analyses have shown that laTLR14b is expressed in the cells, preferentially in gills, gut, and leukocytes. Further investigation is required to determine whether the lamprey TLRs are localized on macrophages/monocytes. These cells should be different from lamprey lymphocytes, which have been shown to be VLR-positive cells. It has been demonstrated that by artificial dimerization of laTLR14b, NF-κB, as well as INF-β promoter can be activated; however the pattern of PAMPs recognition by these laTLRs remains unknown.

More recently, advances in whole genome sequencing and annotation have allowed the identification of 16 genes predicted to encode TLRs from the latest *Petromyzon marinus* (synonym: *Lethenteron camitschaticum*) genome database (Pre-Ensemble lamprey Genome Browser) and NCBI trace archive. It should be reminded that the predicted protein sequences of the lamprey TLRs and their respective TIR domains have been subjected to comparative analyses using the NCBI non-redundant protein database and BLASTP search. The repertoire of predicted lamprey TLRs has been determined and phylogenetic analyses indicate that the repertoire consists of both fish- and mammalian-type TLRs (Kasamatsu *et al*., 2010). At present, three types of TLRs belonging to the TLR2 subfamily have been found in the lamprey, which correspond to TLR24 (pmTLR2a-d), TLR14 (pmTLR14a-c), and the ortholog of jawed vertebrate TLR14 (TLR14d), forming clearly distinct clusters in a phylogenetic tree (Kasamatsu *et al*., 2010). Similarly, two TLR7/8 and three TLR21 genes have been identified in the lamprey genome, able to recognize foreign RNA molecules and unmethylated CpG DNAs, respectively. The TLR2 subfamily, TLR3, TLR5, TLR7/8, and TLR21/22 are conserved in the lamprey and teleosts, suggesting that lampreys and jawed vertebrates share the same TLR family with both mammalian- and fish-type TLRs. Since both types may have arisen together with vertebrates, they may represent the origin of the TLR repertoire in vertebrates.

Finally by the genome analysis, four other proteins have been identified as TLR adaptor-like proteins since they are similar to MyD88, TICAM or SARM.

Chondrichthyes

Data about chondrichthyan TLR are surprisingly scarce. The only datum we found is a nucleotide sequence of *Chiloscyllium griseum* (family Hemiscylliidae), 270-nt long, which has been registered in GenBank as TLR2 under the accession number JF792813. BLAST search has shown a high homology of *C. griseum* TLR2 with mammalian TLR2a (E value: $3e^{-84}$); a SMART analysis of the deduced amino acid sequence has revealed the occurrence of a partial TIR domain. The partial sequence length does not allow to speculate about chondrichthyan TLR.

In *Callorhinchus milii* genome two gene models encoding TICAM and one TIRAP, all supposed to be TLR signaling components, have been identified (Wu *et al*., 2011).

Teleostei

The nucleotide sequences assigned to TLR genes have been determined in 31 teleost species belonging to 8 different families (Table 3). Data available at present have been obtained by 7 sequenced genome or transcriptomes (*Danio rerio, Takifugu rubripes*, *Tetraodon nigroviridis, Oryzias latipes and Gasterosteus aculeatus*, *Oncorhynchus mykiss*, and *Salmo salar*). The determined sequences can be attributed to 16 different TLR types, 8 out of 16 being teleost specific. With respect to family 1, which includes mammalian TLR1, 2, 6, and 10 (Roach *et al*. 2005), orthologs of mammalian TLR6 and TLR10 are absent but a further member of the same family, TLR14, is present and shares some features with TLR1, 6 and 10. Both families 3 and 7 (including TLR7, 8 and 9) share teleost and mammalian orthologs. Concerning the family 4, the majority of teleost species, but the most anciently diverged Otocephala taxon, has lost TLR4; and whenever present, as in *D. rerio* and *Gobiocyris rarus*, it does not recognize the mammalian agonist, LPS (Sepulcre *et al.,* 2009). The family 21 comprises the other members specific for the teleost lineage (TLR13, 19, 20, 21, 22 and 23) (Palti, 2011). In addition, paralogous or duplicated TLR genes, probably resulting from the third or fourth round of the whole genome duplication event, have been identified in *D. rerio* (Jault *et al*., 2004; Meijer *et al*., 2004), *O. mykiss* (Palti, 2010), *Cyprinus carpio* (Kongchum *et al*., 2011).

A unique feature of teleost TLRs is the presence, in addition to TLR5, of a soluble TLR5 molecule (TLR5S), which lacks the transmenbrane and TIR domains in *O. mykiss* (Tsujita *et al*., 2004, 2006) and *T. rubripes* (Oshiumi *et al*., 2003) genomes. In *O. mykiss* TLR5S has been demonstrated to possess an adjuvant role in the response to bacteria via physical binding to flagellin (Tsujita *et al*., 2006). Soluble forms of TLRs are usually absent in mammalian genomes and those previously identified (Iwami *et al*., 2000; LeBouder *et al*. 2003) are probably generated by alternative splicing. TLR5S has also been identified in transcripts of *S. salar* (Tsoi *et al*., 2006) and *Ictalurus punctatus* (Baoprasertkul *et al*., 2007a). Its expression pattern generally differs from that of the membrane form and is increased upon infection of catfish specimens with *Edwardsiella ictaluri* (Bilodeau and Waldbieser, 2005).

Functional data have been obtained in many teleost species. In *O. mykiss* increased TLR3 trascriptional activity was demonstrated after poly (I:C) or infectious hematopoietic virus treatments (Rodriguez *et al.,* 2005). In the same species *Areomonas salmonicida* induced higher expression of TLR22 (Rebl *et al*., 2007) and Vaccine adjuvant R848 amplified TLR7 expression (Palti *et al.* , 2010). Exposure of *I. punctatus* to the Gram-negative bacterium *Edwardsiella ictaluri* activated the TLR3 and TICAM production (Baopraserkul *et al.,* 2006), and modestely down-regulated TLR2 (Baopraserkul *et al.,* 2007b).

The ortholog molecules involved in the TLR signaling cascades have also been identified in teleosts (Takano *et al*., 2010). In the genome of *D. rerio* MyD88, MAL, TICAM, TRIF and SARM have been identified (Jault *et al*. 2004; Meijer *et al.* 2004). The functionality of teleost MyD88 has been confirmed by infecting *D. rerio* with the pathogen *Salmonella enterica* (van der Sar *et al*., 2006) or *Paralichthys olivaceus* with *Ewardsiella tarda* (Takano *et al*., 2006). Additional MyD88 sequences are known from *Pseudosciaena crocea, Cynoglossus semilaevis, S. salar* (Rebl *et al*., 2010). Takano *et al*. (2010) discussed the presence of other elements (TIRAP, TICAM, SARM, IRAK, IKK, TRAF) of the teleost TLR signaling pathway.

NF-κB proteins have been characterized in *D. rerio* (Phelan *et al*., 2005) and *P. olivaceus* (Yazawa *et al*., 2005) demonstrating that teleost NF-κB has a critical role in the transcription occurring downstream of signaling cascade.

Amphybia

The developmental and immunological studies on *Xenopus laevis* and *Xenopus (Silurana) tropicalis* have been reviewed by Robert and Otha (2009). The presence in *X. laevis* of a signaling pathway primed by a Spätzle/Toll in dorsoventral patterning has been demonstrated by Armstrong *et al*. (1998) and a mammalian MyD88 homologue has been cloned (Prothmann *et al*., 2000). More recently the availability of *X. tropicalis* genome database has provided a useful tool to deeply investigate the TLR pattern composition. In the draft genome, Roach (2005) has identified several TLR genes. Subsequently, Ishii *et al*. (2007) have searched the last genome version (JGI 4.1) for TLR by BLASTP analysis using the TIR domain of *T. rubripes* TLRs and have found 19 proteins. Nineteen out of 23 complete sequences have shown high scores of identity with mammalian TLRs; the remaining 4 have been annotated as *X. tropicalis* MyD88 molecules. TLR2 and 6 are duplicated. An ortholog for each teleost TLR has been found, except for *T. rubripes* TLR23; among TLRs present in mammals but absent in teleosts, orthologs of mammalian TLR6, 12 and 13, have been found, while the search for TLR4, 10 and 11 has failed. A particular attention has been focused on TLR4 whose presence in teleosts is controversial; its gene seems to be buried in a DNA region where prediction tools indicate no exons. The size and number of the LRR of each *X. tropicalis* TLR are similar to those of the human TLR counterpart. Moreover the same authors have demonstrated that *X. tropicalis* TLRs are costitutively expressed in the tadpole as well as in the adult (Ishii *et al*., 2007).

Sauropsida

The phylum Sauropsida includes reptiles and birds. While TLR (or presumptive TLR) sequences from 17 different bird species are present in databanks, the search for reptile sequences unveiled only one species, *Anolis carolinensis*. Sequences of this species have been annotated as molecules resembling mammalian TLR2, 3, 4, 5, 6, 7 and 13. At present there are not articles in the literature that describe these genes.

On the contrary, a lot of papers deal with bird TLRs. It should be reminded that avian immune system differs from that of mammals in some aspects. It presents different immune organs, such as the Bursa of Fabricius, it lacks organized lymph nodes, neutrophils and eosinophils, but have heterophils. Birds have also a different repertoire of cytokines, chemokines, defensins and integrins (Kaiser, 2007).

Knowledge of the avian immunology has considerably expanded by the assembly of the *Gallus gallus* genome (Consortium, 2004). Bird genome analysis has been extended to a species diverged from *G. gallus* lineage about 100 MYA, the Zebra finch (*Taeniopygia guttata*, family Passeriformes) in an attempt to obtain a more complete definition of the TLR types (Brownlie and Allan, 2011). The existence of ten avian TLRs have been confirmed by several authors (Smith *et al*., 2004; Yilmaz *et al*., 2005; Boyd *et al*., 2007; Temperley *et al*., 2008; Alcaide and Edwards, 2011). Avian TLR1 and TLR2 are duplicated and also TLR7 exists in two copies in *T. guttata*. Five out of ten avian TLRs (TLR2a, 2b, 4, 5, and 7) have clear mammalian orthologs. TLR15, belonging to the vertebrate family 1, appears to be specific within the avian species whereas TLR21 is related to teleost and amphybian TLR21.

Alcaide and Edward (2011) have conducted the characterization of TLRs in seven distant non-model species *Falco naumanni*, (Falconidae), *Carpodacus mexicanum* (Fringillidae), *Oceanodroma leucorhoa* (Hydrobatidae), *Accipiter cooperii* (Accipitridae), *Amazona albifrons* (Psittacidae), *Picoides pubescens* (Picidae), and *Dromaius novaehollandiae* (Casuariidae). By using tests of selection, these authors have found that avian TLRs are dominated by purifying selection, but patterns of positive selection acts on specific amino acid residues; many of positively selected positions can be mapped to putative ligand-binding regions, suggesting that variations are linked to species-specific differences in PAMPs recognition.

A Systems Biology approach has been used to identify orthologous relationships between human and *G. gallus* TLRs and TLR-interacting molecules: annotations of chicken sequences were generated both manually and by computer, using a human Reactome Knowledgebase, previously integrated by TLR signaling genes. In particular 33 out of 38 proteins of the human TLR3 pathway were annotated integrating manual and computational analysis (Gillespie *et al.*, 2011).

Mammalia

It is extremely complex to summarize current knowledge on mammalian TLRs and this is not the main objective of this review. We will limit to indicate only several recent reviews covering parts of the topic that appear to be more important. Casanova *et al*. (2011) reviewed the role of both TLRs and IL1Rs in host defense from multiple points of view. Evolutionary genetic studies have shown that human intracellular TLRs (TLR3, 7, 8, and 9) evolved under stronger negative selection than surface-expressed TLRs (Barreiro *et al*., 2009). Epidemiological studies have revealed the association between infection diseases and variants in TLR genes. Clinical investigations have demonstrated that rare mutations affecting the TLR3-TRIF pathway underlie *Herpes simplex* encephalitis (Zhang *et al*., 2007). Also in domestical animals variation in amino acid sequence in TLR ectodomains could be associated with infectious diseases (Huenishi *et al*, 2011; Jungi *et al*., 2011). On the other hand, the viral evolution to escape the TLR recognition by manipulating their genomic content is a fascinating area that deserves more attention (Barton, 2007).

Concluding commentaries

In the last decade the number of genes encoding TLR or TLR-like molecules, identified from different species is extremely increased. However no TLR signaling pathways have been completely clarified in any animal lower than *D. melanogaster.* The evolutionary analysis of the available TLR sequences allows identification of orthologous relationships only in neighboring species; indeed sequences from phylogenetically distant species never occur in the same clade. This suggests that each lineage evolved independently by gene duplication.

The evolution of TLR architecture seems to be shaped by structural conservation and divergence. In the cytoplasmic region the TIR domain was highly conserved allowing to use in separate lineages, basically common signaling mechanisms and analogous regulatory modules. On the other hand, the ectodomain underwent significant variations. In more recently diverged species, the most relevant novelty was the loss of the additional cysteine clusters intermingled with the LRRs (protostome-type). The number of LRRs appears to increase and their sequences seem to converge in the “typical motif”. Different ligand-binding sites were shaped in the ectodomain through molecular evolution: while *Drosophila* Toll dimer accommodates two ligand molecules on the N-terminal region of each monomer, all vertebrate TLRs interact with a single ligand molecule and both monomers cooperate in the binding. Furthermore a specific feature of the vertebrate TLR evolution is the ability to sense PAMPs directly, without using a cytokine intermediate as the insect Spätzle.

By reviewing the presence of TLRs in different species it appears clear that evolution used TLR molecules for different functions, ranging from immune response to developmental signaling and cell adhesion. While studies at genomic level have been performed on a large number of species covering the majority of the most important phyla, unfortunately at present investigations on the TLR functions are limited to a few species, particularly in the protostome lineage. This hampers the definition of a convincing scenario for the emergence of the TLR-mediated immune response. Whether the TLR cooptation in immunity in insects and vertebrates represents a convergent evolution is still an unsolved question.

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