Evolutionary Origins of Toll-like Receptor Signaling

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Abstract

Toll-like receptors (TLRs) are transmembrane pattern recognition receptors that are best known for their roles in innate immunity for the detection of and defense against microbial pathogens. However, TLRs also have roles in many non-immune processes, most notably development. TLRs direct both immune and developmental programs by activation of downstream signaling pathways, often by activation of the NF-κB pathway. There are two primary TLR subtypes: 1) TLRs with multiple cysteine clusters in their ectodomain (mccTLRs) and 2) TLRs with a single cysteine cluster in their ectodomain (sccTLRs). For some time, it has been known that TLRs and the biological processes that they control are conserved in organisms from insects to mammals. However, genome and transcriptome sequencing has revealed that many basal metazoans also have TLRs and downstream NF-κB signaling components. In this review, we discuss what is known about the structure, biological function, and downstream signaling pathways of TLRs found in phyla from Porifera through Annelida. From these analyses, we hypothesize that mccTLRs emerged in the phylum Cnidaria, that sccTLRs evolved in the phylum Mollusca, and that TLRs have dual immune and developmental biological functions in organisms as ancient as cnidarians.

Key words: **Toll-like receptors, evolution, innate immunity, development, NF-kappaB.**

Review

Introduction

The innate immune system relies on host cell receptors that detect both beneficial and pathogenic microorganisms by recognizing specific microbe-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs), respectively, which include nucleic acids, proteins, lipids, and lipoproteins (Akira et al. 2006; Kawasaki and Kawai 2014; Brubaker et al. 2015). These germline-encoded MAMP receptors include Toll-like-receptors (TLRs), C-type lectin receptors, RIG-I-like receptors, and NOD-like receptors that modulate pathogen detection and host–microbiome homeostasis (Brubaker et al. 2015; Thaiss et al. 2016). Among these, TLRs are widely studied primary mediators of innate immunity in animals from insects to humans (Imler and Zheng 2004; Kawai and Akira 2007; Akira 2009; Lindsay and Wasserman 2014).

Toll-like-receptors also have roles in numerous developmental processes across species. Indeed, *Drosophila melanogaster* Toll-1 was the first TLR discovered based on its role in embryonic dorsal-ventral polarity specification (Anderson et al. 1985; Hashimoto et al. 1988). Toll-1 directs this early developmental program through activation of the transcription factor NF-κB signaling pathway, whereas the eight other Drosophila TLRs also activate NF-κB, but do so for immune responses to fungi and Gram-positive bacteria (Norris and Manley 1992; Belvin and Anderson 1996; Silverman and Maniatis 2001; Valanne et al. 2011; Lindsay and Wasserman 2014; Meyer et al. 2014). There are ten TLRs in humans and 13 in mice, and these mammalian TLRs also control NF-κB-dependent innate immune signaling (Akira et al. 2006; Kawai and Akira 2007) and specific developmental processes (Barak et al. 2014; Heiman et al. 2014).

Toll-like-receptor signaling pathways and their biological functions have been extensively studied and reviewed in flies and mammals (Akira 2009; Kawasaki and Kawai 2014; Lindsay and Wasserman 2014); however, genomic and transcriptomic sequencing of organisms from early branching phyla has uncovered prototypical TLRs, TLR-like proteins, and NF-κB pathway components in organisms that predate bilaterians (Miller et al. 2007; Leulier and Lemaitre 2008; Akira 2009; Bosch et al. 2009; Augustin et al. 2010; Gilmore and Wolenski 2012; Kawasaki and Kawai 2014; Lindsay and Wasserman 2014; Rauta et al. 2014; Zhang et al. 2015; Brennan et al. 2017; Ren et al. 2017; Williams et al. 2018). In this review, we discuss TLR structures, biological functions, and downstream NF-κB signaling pathways in phyla from Porifera through Annelida.

Structures of TLRs and NF-κB Signaling Components

Prototypical metazoan TLRs are membrane-spanning proteins found in endosomes or the plasma membrane. These TLRs contain three primary domains (fig. 1): 1) a hydrophobic leucine-rich region (LRR) ectodomain containing two to 45 leucine-rich motifs that binds MAMPs, PAMPs, or endogenous ligands (e.g., Drosophila Spätzle), 2) a transmembrane region, and 3) an intracellular Toll/interleukin-1 receptor (TIR) domain that initiates downstream signaling through adapter proteins, such as MyD88, Mal/TIRAP, TRAM, and TRIF (Akira et al. 2006; Kawai and Akira 2007; Silverman et al. 2009; Ng and Xavier 2011). In addition to NF-κB

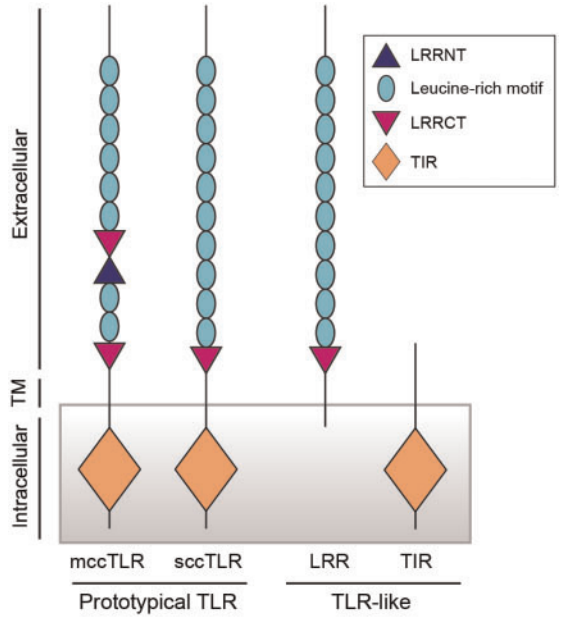

Fig. 1. A structural comparison of multiple cysteine cluster (mcc) TLRs, single cysteine cluster (scc) TLRs, and TLR-like proteins. Figure is adapted from Leulier and Lemaitre (2008). The mccTLRs and sccTLRs share a prototypical structure with an extracellular ectodomain containing leucine-rich motifs, a transmembrane domain, and an intracellular Toll/IL-1 receptor (TIR) domain. The mccTLR ectodomains have two cysteine clusters at the C-terminus of the LRR (LRRCT), and often have an internal cysteine cluster at the N-terminus of the LRR (LRRNT). In contrast, sccTLR ectodomains have only one LRRCT domain. LRR-only proteins typically have a transmembrane domain and one LRRCT, but can also have a LRRNT (not shown). Toll/IL-1 receptor (TIR) domain-only proteins commonly have transmembrane domains, but no discernable extracellular domains.

signaling, these adapter proteins can also facilitate TLR-mediated activation of MAPK and IRF3 signaling (Vasselon and Detmers 2002; Akira et al. 2006; Kawai and Akira 2007; Kawasaki and Kawai 2014).

Historically, prototypical TLRs have been classified according to the number of cysteine clusters in their ectodomains (Leulier and Lemaitre 2008). Single cysteine cluster TLRs (sccTLRs) have one cluster of cysteines at the C-terminal end of the LRR (LRRCT), and sccTLRs include all mammalian TLRs and Drosophila Toll-9 (fig. 1) (Leulier and Lemaitre 2008). More recent studies have identified sccTLRs in organisms more ancestral than arthropods (Davidson et al. 2008; Zhang et al. 2015). The second TLR subtype comprises multiple cysteine cluster TLRs (mccTLRs) that contain two LRRCTs and often a cluster of cysteines at the N-terminal end of one LRR (LRRNT) (fig. 1) (Leulier and Lemaitre 2008). The mccTLRs are found in protostomes such as Drosophila, *Caenorhabditis elegans*, and several organisms in more ancient phyla (Imler and Zheng 2004; Leulier and Lemaitre 2008; Shinzato et al. 2011; Zhang et al. 2015; Brennan et al. 2017). Generally, LRRNTs and LRRCTs contain two or four cysteine residues that provide stability and solubility to the hydrophobic LRR (Kajava 1998; Enkhbayar et al. 2004; Ng and Xavier 2011).

In the initial classification of TLR subtypes, mammalian sccTLRs were described as directly engaging PAMPs for the activation of innate immune signaling pathways, including NF-κB (Silverman and Maniatis 2001; Akira et al. 2006; Kawai and Akira 2007; Leulier and Lemaitre 2008). In contrast, the best-characterized mccTLR, Drosophila Toll-1, does not directly bind MAMPs, but rather binds a cleaved form of the fly-encoded ligand Spätzle to activate downstream TLR-dependent responses (Silverman and Maniatis 2001; Leulier and Lemaitre 2008; Valanne et al. 2011; Lindsay and Wasserman 2014). Based on Toll-1 in flies, one long-standing hypothesis has been that activation of mccTLRs by microbes occurs indirectly, relying on endogenous TLR ligands to promote downstream signal transduction (Leulier and Lemaitre 2008; Lindsay and Wasserman 2014).

In mammals, following PAMP binding, TLRs generally activate NF-κB signaling by recruiting and binding the adapter protein MyD88 at the membrane. MyD88 then recruits IL-1R-associated kinases (IRAKs), which are phosphorylated and then engage TRAF (Tumor necrosis factor receptor associated factor) ubiquitin ligases. Activated TRAFs complex with a TGF-β-activated kinase (TAK) and a TAK1-binding protein (TAB) to phosphorylate the IκB kinase (IKK). This activated IKK then phosphorylates the NF-κB inhibitor IκB to promote its degradation, allowing for nuclear translocation of NF-κB to activate the transcription of innate immune effector genes (fig. 2A) (Gilmore and Wolenski 2012).

LRR- and TIR Domain-Containing Proteins

Proteins containing LRRs are pervasive among prokaryotes and eukaryotes and are involved in numerous protein–protein interactions and biological processes (Dolan et al. 2007; Ng et al. 2011). Humans have 375 LRR-containing proteins, many of which have uncharacterized functions (Ng et al. 2011). Approximately half of these human proteins contain only an LRR with no other discernable domains, whereas other LRR proteins contain transmembrane domains or signal peptides (Ng et al. 2011). In addition to TLRs and NOD receptors, LRRs are also found in secreted proteins, membrane-spanning proteins (fig. 1), and GPI-anchored proteins in *C. elegans*, *D. melanogaster*, and mammals (Milan et al. 2001; Dolan et al. 2007; Ng and Xavier 2011).

Genes encoding proteins with TIR domains have been identified in the phyla Porifera and Cnidaria, however, many of these predicted TIR proteins lack an LRR ectodomain (Miller et al. 2007; Bosch et al. 2009; Gauthier et al. 2010; Poole and Weis 2014; Baumgarten et al. 2015). These TIR-only proteins contain primarily a TIR domain and, in some cases, a transmembrane domain (fig. 1) (Bosch et al. 2009; Poole and Weis 2014). Therefore, it is likely that these TIR-only proteins function in signal transduction pathways, but do not bind MAMPs/PAMPs. Interestingly, the mammalian TLR adapter proteins Mal and TRAM closely resemble the TIR-only proteins found in basal invertebrates: that is, Mal and TRAM have defined C-terminal TIR domains, as well as an N-terminal sequence that is required for membrane association

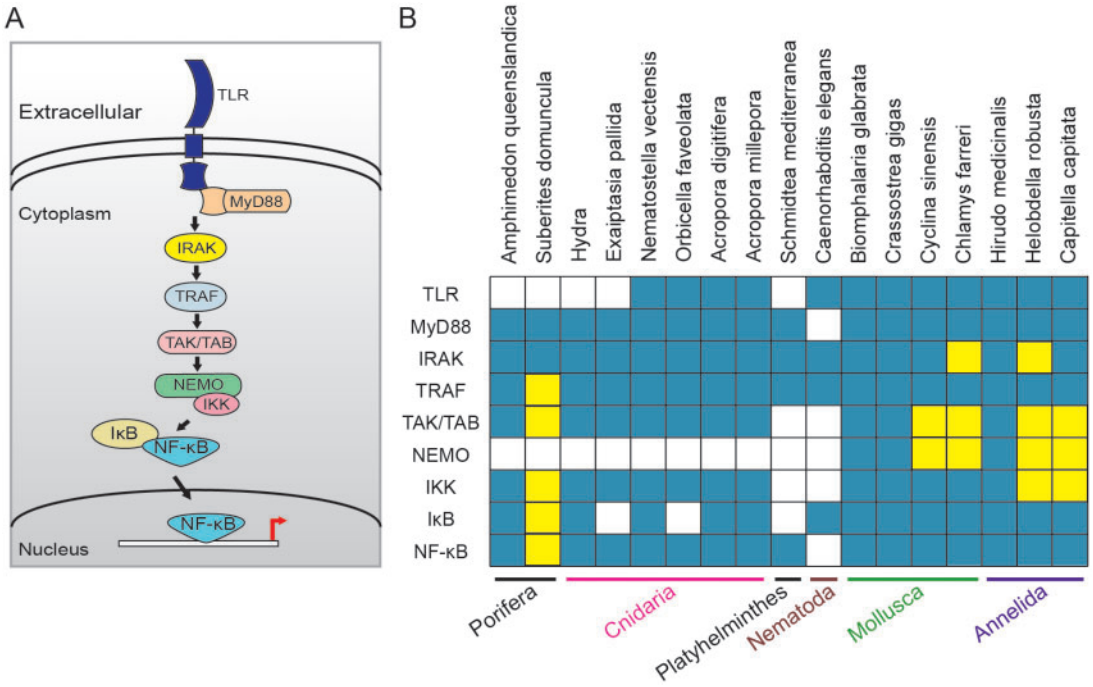

Fig. 2. TLR-to-NF-κB pathway components expressed in organisms in phyla Porifera through Annelida. Figure is adapted from Gilmore and Wolenski (2012). (*A*) General structure of a conserved TLR signaling pathway to activate NF-κB. (*B*) Listed are the members of the conserved TLR-to-NF-κB signaling pathway found in the indicated organisms from most basal (left) to most recent branching (right). Blue boxes indicate the presence of a pathway component and white boxes indicate the absence of a pathway component. Yellow boxes indicate pathway components that have not been identified in the literature, but are hypothesized to be present based on the completeness of TLR-to-NF-κB pathways in other organisms of the same phylum.

(Ve et al. 2015). Thus, mammalian TLR adapter proteins may have evolved from ancient TIR-domain only proteins.

Prototypical TLRs Are Not Found in Poriferans

The phylum Porifera is one of the earliest extant metazoan taxa and comprises sponges (Worheide et al. 2012). Although complete genome information is limited among poriferans, no prototypical TLRs have been identified in any member of this phylum (Gauthier et al. 2010; Hentschel et al. 2012). One of the most widely studied poriferans at the molecular level is the demosponge *Amphimedon queenslandica*, which expresses two TIR domain-containing proteins with N-terminal IL-1R-like immunoglobulin (Ig) domains (Gauthier et al. 2010; Srivastava et al. 2010; Hentschel et al. 2012). *Amphimedon queenslandica* also has an LRR-containing protein that has Ig- and EGF (Epidermal Growth Factor)-like domains (Gauthier et al. 2010). The sponge *Suberites domuncula* expresses a TIR-only protein (Sd-TLR) that has a transmembrane domain, but lacks an LRR (Wiens et al. 2007).

Despite the absence of prototypical TLRs in poriferans, *A. queenslandica* and *S. domuncula* have genes encoding several TLR-to-NF-κB pathway proteins including MyD88 and NF-κB homologs, suggesting that MyD88-dependent downstream signaling to NF-κB from TIR-only proteins occurs in poriferans (fig. 2*B*) (Gauthier et al. 2010; Gilmore and Wolenski 2012; Song et al. 2012). Nevertheless, the biological roles and molecular details of downstream signaling pathways directed by TIR-only proteins in *A. queenslandica* have yet to be determined. Several TLR-to-NF-κB pathway proteins (IgTIR1, Tollip, Pellino, and NF-κB) are expressed in a tissue-specific manner in early *A. queenslandica* development, suggesting that this pathway functions in development (table 1) (Gauthier et al. 2010). In *S. domuncula*, Sd-TLR has been studied in the context of immunity (table 1) (Wiens et al. 2005; Wiens et al. 2007). The Sd-TLR is expressed in outer tissues and the canal filtration system, which is in constant contact with microbes (Wiens et al. 2007). Treatment of *S. domuncula* with a synthetic lipoprotein analog [Pam3Cys-Ser-(Lys)4] induces the expression of a caspase-like protease that is hypothesized to function in innate immune-activated apoptosis (Wiens et al. 2007). Furthermore, incubation of this synthetic lipoprotein with recombinant Sd-TLR prior to tissue exposure abolishes the increase in protease expression, suggesting that 1) Sd-TLR is capable of binding Pam3Cys-Ser-(Lys)4 and 2) Sd-TLR activates a pro-apoptotic pathway in response to microbial-like molecules (Wiens et al. 2007). However, it is unclear how Sd-TLR interacts with Pam3Cys-Ser-(Lys)4 given that Sd-TLR lacks an LRR which is required for PAMP binding in prototypical TLRs (Wiens et al. 2007). Additionally, *S. domuncula* likely has a MyD88-dependent signaling pathway that responds to microbes because LPS treatment of tissues induced both the expression of MyD88 and its direct interaction with an LPS-interacting protein (Wiens et al. 2005).

Table 1. Summary of Structures and Biological Roles of TLR Genes in Organisms in Phyla Porifera through Annelida and in Arthropoda.

Organism	TLR Type	Putative Biological Role	References
Porifera			
Amphimedon queenslandica	LRR, TIR	Development	Gauthier et al. (2010)
Suberites domuncula	TIR	Immunity	Weins et al. (2005, 2007)
Cnidaria			
Nematostella vectensis	mccTLR	Immunity, development	Brennan et al. (2017)
Orbicella faveolata	mccTLR, TIR	—	Williams et al. (2018)
Acropora digitifera	sccTLR, mccTLR, TIR	—	Poole and Weis (2014), Shinzato et al. (2011)
Acropora millepora	mccTLR, TIR	—	Poole and Weis (2014), Palmer and Traylor-Knowles (2012)
Hydra	LRR, TIR	Immunity	Bosch et al. (2009)
Exaiptasia pallida	TIR	—	Baumgarten et al. (2015), Poole and Weis (2014)
Platyhelminthe			
Schmidtea mediterranea	LRR, TIR	Regeneration	Peiris et al. (2014)
Rotifer			
Adineta vaga	LRR, TIR	—	Flot et al. (2013)
Nematoda			
Caenorhabditis elegans	mccTLR, LRR, TIR	Development, neural function	Brandt and Ringstad (2015), Gissendanner and Kelley (2013), Lui and Shen (2011), Irazoqui et al. (2010), Pujol et al. (2001)
Caenorhabditis briggsae	mccTLR	—	Stein et al. (2003)
Caenorhabditis brenneri	mccTLR	—	Coghlan et al. (2008)
Caenorhabditis japonica	mccTLR	—	Coghlan et al. (2008)
Mollusca			
Biomphalaria glabrata	sccTLR, mccTLR	Immunity	Adema et al. (2017), Pila et al. (2016, 2017)
Crassostrea gigas	sccTLR, mccTLR, TIR	Immunity	Zhang et al. (2011, 2013, 2015)
Cyclina sinensis	sccTLR	Immunity	Ren et al. (2016, 2017)
Chlamys farreri	mccTLR, LRR	Immunity	Wang et al. (2011, 2015, 2016, 2017), Qui et al. (2007)
Annelida			
Hirudo medicinalis	sccTLR	Neuroimmunity, neurogenesis	Tasiemski and Salzet (2017), Cuvillier-Hot et al. (2011), Schikorski et al. (2009)
Helobdella robusta	sccTLR, mccTLR	—	Simakov et al. (2013), Davidson et al. (2008)
Capitella capitata	sccTLR, mccTLR	—	Davidson et al. (2008)
Arthropoda			
Drosophila melanogaster	sccTLR, mccTLR, LRR	Immunity, development, neurogenesis, neuronal function	Lindsay and Wasserman (2014), Pare et al. (2014), McIlroy et al. (2013), Wayburn and Volk (2009), Kurusu et al. (2008)

LRR, leucine-rich region only protein; mcc, multiple cysteine cluster; scc, single cysteine cluster; TIR, Toll/IL-1 receptor domain only protein; TLR, Toll-like receptor.

Cnidarian TLRs Vary in Structure and Abundance

The phylum Cnidaria consists of corals, sea anemones, hydroids, and jellyfish, and has attracted recent interest due to the negative effects of climate change and disease on cnidarian survival (Daly et al. 2007; Goldstone 2008; Vidal-Dupiol et al. 2011; Palmer and Traylor-Knowles 2012; Pinzon et al. 2015). Studies aimed at understanding cnidarian immunity have identified prototypical TLRs and TLR-like genes in many of these organisms (table 1) (Daly et al. 2007; Miller et al. 2007; Bosch et al. 2009; Baumgarten et al. 2015). Overall, the numbers and types of TLR-like proteins vary greatly among cnidarians. For example, the reef-building coral *Acropora digitifera* encodes three mccTLRs, one sccTLR with a short ectodomain, and 13 TIR-only proteins (Shinzato et al. 2011; Poole and Weis 2014). Its close relative *A. millepora* encodes ten TIR domain-containing proteins, but only one mccTLR (Palmer and Traylor-Knowles 2012; Poole and Weis 2014). In contrast, the sea anemone *Nematostella vectensis* and the stony coral *Orbicella faveolata* each encodes a single mccTLR, whereas TIR-only proteins have only been identified in *O. faveolata* (Miller et al. 2007; Brennan et al. 2017; Williams et al. 2018). In general, there appears to be an expansion of TIR-only protein encoding genes in corals, which has been suggested to contribute to pathogen resistance and microbial symbiosis (Poole and Weis 2014).

Phylogenetic analysis clusters the *N. vectensis*, *O. faveolata*, *A. digitifera*, and *A. millepora* TLRs into a cnidarian-specific clade and with mccTLRs from mollusks and nematodes (fig. 3). Even though the *A. digitifera* TLR2 is an sccTLR, it still clusters with cnidarian mccTLRs (fig. 3). Analysis of the *A. digitifera* TLR2 amino acid sequence reveals that it has a short ectodomain with only four leucine-rich repeat motifs. As such, we hypothesize that *A. digitifera* TLR2 resulted from the truncation of an mccTLR, which could explain its sequence similarity to mccTLRs in *A. digitifera* and *A. millepora* but not to sccTLRs in other phyla (fig. 3).

The diversity of TLR structure within the phylum Cnidaria is further substantiated by proteins in *Hydra* and the sea anemone *Exaiptasia pallida*, neither of which contains a prototypical TLR (Bosch et al. 2009; Poole and Weis 2014). Rather, *Hydra* has two LRR domain-containing proteins, HyLRR-1 and HyLRR-2, and two transmembrane TIR-only proteins (Bosch et al. 2009).

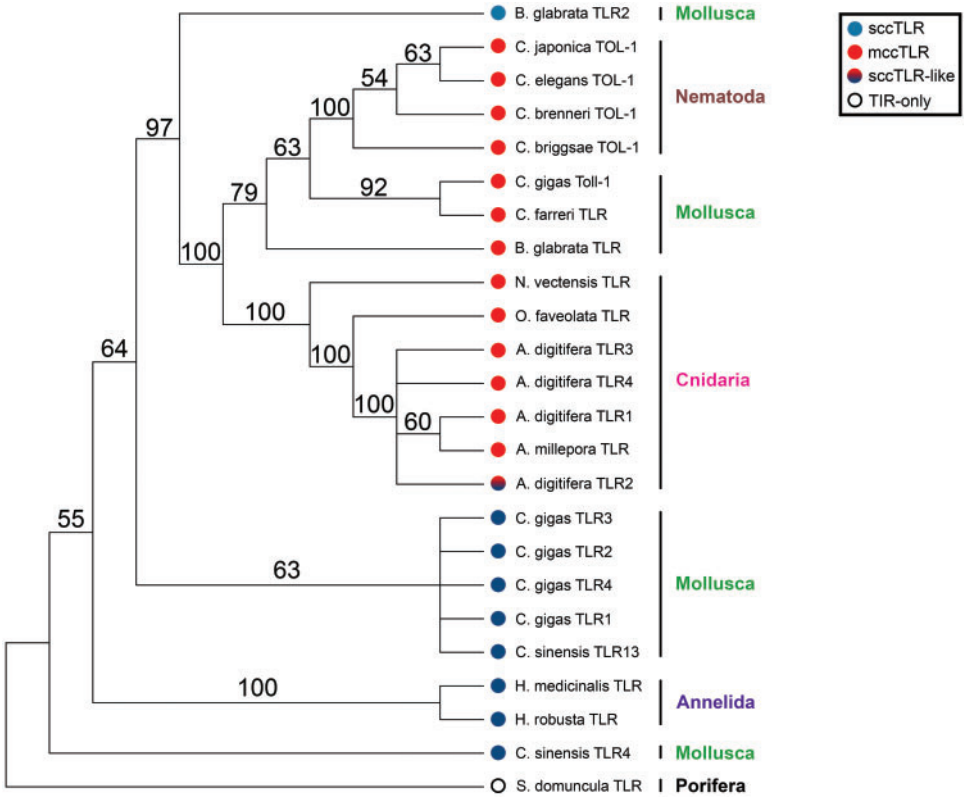

Fig. 3. Phylogenetic analysis of TLRs in organisms in phyla Porifera through Annelida. Maximum likelihood analysis was performed using full-length sequences of TLRs from the cnidarians *Nematostella vectensis*, *Orbicella faveolata*, *Acropora digitifera*, *Acropora millepora*, the nematodes *Caenorhabditis elegans*, *Caenorhabditis japonica*, *Caenorhabditis brenneri*, *Caenorhabditis briggsae*, the mollusks *Biomphalaria glabrata*, *Crassostrea gigas*, *Cyclina sinensis*, *Chlamys farreri*, and the annelids *Hirudo medicinalis* and *Helobdella robusta*. Phylogeny was rooted with the poriferan *Suberites domuncula* TLR. Branches indicate bootstrap support values. Blue circles indicate sccTLRs, red circles indicate mccTLRs, a blue and red circle indicates an sccTLR-like protein, and an open circle indicates a TIR-only protein. Phyla are labeled to the right of the branches. Bayesian analysis of the included TLRs shows clustering that is consistent with maximum-likelihood analysis (supplementary fig. S1, Supplementary Material online).

The *Hydra* LRR proteins are structurally different from the ectodomains found in prototypical TLRs because the Hydra LRR proteins contain non-TLR moieties such as EGF domains or coiled–coiled motifs (Bosch et al. 2009; Augustin et al. 2010). In *E. pallida*, sequences encoding two TIR domain-only proteins have been identified, however, these sequences are overlapping and may encode the same protein (Poole and Weis 2014; Baumgarten et al. 2015). In contrast to *Hydra*, LRR-only proteins have not been identified in *E. pallida* (Poole and Weis 2014; Baumgarten et al. 2015). Given that prototypical TLRs are not found in phyla more ancestral than Cnidaria and that some, but not all, cnidarians have TLRs, we hypothesize that prototypical TLRs originated within this phylum.

Cnidarian TLRs: Evidence for Directing Immunity and Development

Although the phylum Cnidaria diverged from bilateria over 500 MYA, nearly complete innate immune signaling pathways are present in most cnidarians (Hemmrich et al. 2007). Homologs to the TLR-to-NF-κB pathway are present in *N. vectensis*, *A. digitifera*, *Hydra*, *O. faveolata*, and *E. pallida*; however, these pathways appear to lack the IKK scaffold NEMO and independent IκB homologs have not been found in *O. faveolata* or *E. pallida* (fig. 2*B*) (Bosch et al. 2009; Gilmore and Wolenski 2012; Baumgarten et al. 2015; Brennan et al. 2017; Mansfield et al. 2017; Williams et al. 2018).

Several studies have suggested that cnidarian TLRs and TLR-like proteins are upstream activators of microbial-responsive NF-κB pathways (Bosch et al. 2009; Brennan et al. 2017; Williams et al. 2018). For example, LPS treatment of *O. faveolata* tissue induces the expression of several NF-κB pathway genes, and the *O. faveolata* mccTLR (Of-TLR) and the *N. vectensis* mccTLR (Nv-TLR) can both interact with the human TLR adapter MYD88 (Brennan et al. 2017; Williams et al. 2018). Furthermore, Nv-TLR can induce NF-κB signaling in human cells when Nv-TLR-expressing cells are exposed to a heat-inactivated preparation of the pathogenic coral bacterium *Vibrio coralliilyticus* or to *Salmonella typhimurium* flagellin (Brennan et al. 2017). These results suggest that Nv-TLR directly detects PAMPs, a result that opposes convention that PAMP-activated endogenous ligands activate mccTLRs (Leulier and Lemaitre 2008; Brennan et al. 2017).

Tissue-specific transcriptome analysis revealed that the *N. vectensis* TLR-to-NF-κB pathway components are expressed in a motile multicellular structure called the nematosome (Brennan et al. 2017). Nematosomes are composed of phagocytic cells that are capable of engulfing

V. coralliilyticus and phylum-specific cells (cnidocytes) known for their role in defense processes (Wolenski et al. 2013; Babonis et al. 2016; Brennan et al. 2017). Thus, it has been suggested that nematosomes play a role in anemone immunity by activation of TLR-to-NF-κB signaling in response to pathogens (Babonis et al. 2016; Brennan et al. 2017).

Similar to what has been shown for Nv-TLR, flagellin (from *S. typhimurium*) can stimulate a composite protein containing the *Hydra* HyLRR-2 fused to a human TIR domain to activate NF-κB signaling in human cells (Bosch et al. 2009). Thus, flagellin may be a PAMP that is recognized by cnidarian LRR-containing proteins to initiate innate immune signaling. The involvement of a TLR/MyD88-dependent pathway in *Hydra* immunity is further supported by the findings that in vivo knockdown of a TIR-only protein reduced antimicrobial peptide production (Bosch et al. 2009) and that MyD88-deficient *Hydra* showed increased susceptibility to lethal infection with the bacterium *Pseudomonas aeruginosa* (Franzenburg et al. 2012).

Cnidarian TLRs also appear to have roles in early development. Specifically, knockdown of Nv-TLR results in abnormal *N. vectensis* embryonic development (Brennan et al. 2017). Nevertheless, the ligand or downstream signaling pathway responsible for this Nv-TLR-directed developmental program is not known as it is hypothesized to be independent of NF-κB (Wolenski et al. 2013; Brennan et al. 2017). Overall, research to date suggests that cnidarian TLRs have roles in early embryonic development and NF-κB-dependent innate immune signaling (table 1).

TLRs in Platyhelminthes and Rotifers Are Largely Uncharacterized

Turbellarians (phylum Platyhelminthes, genus Planaria) are useful models for studying tissue regeneration, however, their immune systems are largely unexplored (Peiris et al. 2014). Components of a simplified TLR-to-NF-κB signaling pathway have been identified in the fresh water planarian *Schmidtea mediterranea*, however, prototypical TLRs have not been found (fig. 2*B*) (Leulier and Lemaitre 2008; Forsthoefel et al. 2012; Peiris et al. 2014). *Schmidtea mediterranea* does, however, have several LRR-only genes and TIR domain-only genes (table 1) (Peiris et al. 2014). During *S. mediterranea* head regeneration, it has been shown that transcripts for TLR-like proteins, MyD88, IRAK, and TRAF are upregulated, suggesting that a TLR-directed pathway is required for regeneration or for preventing infection during the regeneration process (table 1) (Peiris et al. 2014). Additionally, NF-κB likely has a role in *S. mediterranea* intestinal function due to its expression in intestinal phagocytes and because knockdown of NF-κB resulted in dissociation of intestinal tissues and ultimate lysis of *S. mediterranea* (Forsthoefel et al. 2012). Nevertheless, no studies have directly characterized biological roles for TLR-like proteins and downstream NF-κB signaling in *S. mediterranea*.

The phylum Rotifera consists of aquatic microinvertebrates that primarily reproduce asexually (Flot et al. 2013; Weisse et al. 2013; Snell 2014). Like turbellarians, the genome of the rotifer *Adineta vaga* has putative LRR-containing genes and TIR domain-containing genes, but no prototypical TLRs have been identified (table 1) (Flot et al. 2013). In fact, to our knowledge no studies have characterized immune pathways, including NF-κB, or immune responses in rotifers.

Given that turbellarians and rotifers harbor separate LRR- and TIR domain-only proteins, the recognition of and downstream signaling in response to microbes within these organisms may occur via the interaction of independent LRR and TIR domain proteins, similar to what is found in the cnidarian *Hydra* (Bosch et al. 2009). The apparent loss of TLRs and many downstream NF-κB signaling components in these organisms may be due to a loss of metazoan genes in turbellarians and rotifers (Gladyshev et al. 2008; Zarowiecki and Berriman 2015). Unlike most metazoan genomes, rotifer genomes have also been subject to extensive horizontal gene transfer and have genes with bacterial, fungal, and plant origins (Gladyshev et al. 2008). The lack of immune studies in phyla Platyhelminthes and Rotifera provides exciting research opportunities for understanding how these organisms recognize and respond to microbes.

Nematode TLRs Direct NF-κB-Independent Developmental Programs

The phylum Nematoda consists of both parasitic and free-living roundworms that inhabit a wide range of environments (Blaxter 2011). To date, research on free-living nematodes has been dominated by genetic studies on the model organism *Caenorhabditis elegans*. *C. elegans* has genes encoding a prototypical mccTLR (TOL-1), a TIR-only protein, and several LRR-only proteins (Pujol et al. 2001; Pradel et al. 2007; Leulier and Lemaitre 2008; Irazoqui et al. 2010; Liu and Shen 2011; Mancuso et al. 2012; Gissendanner and Kelley 2013;Brandt and Ringstad 2015). We also identified single mccTLRs in the genomes of the related nematodes *Caenorhabditis briggsae*, *Caenorhabditis brenneri*, and *Caenorhabditis japonica* (Stein et al. 2003; Coghlan et al. 2008; Harris et al. 2010); however, no studies have characterized the biological roles or downstream pathways of these TLRs (table 1). Furthermore, phylogenetic analysis of the *C. elegans*, *C. briggsae*, *C. brenneri*, and *C. japonica* TLRs with TLRs from other phyla discussed in this review revealed clustering of the Caenorhabditis TLRs into nematode-specific branches and with mccTLRs from all other phyla (fig. 3). Thus, there is high TLR sequence conservation among Caenorhabditis TLRs.

The *C. elegans* genome has lost many TLR-to-NF-κB signaling pathway components including MyD88, IKK, and NF-κB (fig. 2*B*) (Pujol et al. 2001; Irazoqui et al. 2010). Therefore, TOL-1 cannot direct NF-κB-dependent biological programs (Irazoqui et al. 2010). TOL-1 is required for an early developmental program, and a deletion in the TOL-1 LRR region caused severe and lethal physical abnormalities (Pujol et al. 2001). However, it is unclear what downstream pathways are activated by TOL-1 for these early developmental programs (Pujol et al. 2001; Irazoqui et al. 2010). TOL-1 also has a role in immunity by promoting pathogen avoidance and the development and function of microbial metabolite-sensing

neurons (BAG neurons) (Pujol et al. 2001; Pradel et al. 2007; Irazoqui et al. 2010; Brandt and Ringstad 2015). Interestingly, *C. elegans* pathogen-avoidance behaviors rely on the proper function of BAG neurons to sense nearby pathogens, and it is hypothesized that a TOL-1-to-p38 MAPK pathway is responsible for BAG neuron function and development (Brandt and Ringstad 2015; Galbadage et al. 2016). TOL-1 has also been implicated more directly in microbial defense in that *C. elegans* with a TIR domain mutant of TOL-1 were more susceptible to some bacterial infections and had reduced expression of an antimicrobial molecule (Pujol et al. 2001; Pradel et al. 2007; Tenor and Aballay 2008; Irazoqui et al. 2010; Rangan et al. 2016; Battisti et al. 2017). Of note, no ligands for *C. elegans* TOL-1 signaling have been identified (Pujol et al. 2001; Tenor and Aballay 2008; Brandt and Ringstad 2015;).

Although studies in *C. elegans* have described roles for TOL-1 in development and neural function (table 1), the indirect immune role for TOL-1 clearly differs from those found in Drosophila and mammalian TLR-mediated innate immunity because TOL-1 does not signal to NF-κB (Pujol et al. 2001; Irazoqui et al. 2010; Brandt and Ringstad 2015). In contrast, the TIR-only protein (TIR-1) is hypothesized to mediate *C. elegans* innate immunity through activation of MAPK signaling pathways and has been shown to promote resistance to pathogen infection by regulating antimicrobial peptide production (Couillault et al. 2004; Irazoqui et al. 2010). Although information on other Caenorhabditis nematode TLRs is lacking, we hypothesize that their biological roles are similar to those found in *C. elegans* given their close phylogenetic relationships (fig. 3).

TLR Gene Expansion in Mollusks

The phylum Mollusca comprises aquatic and terrestrial invertebrates including bivalves, cephalopods, and gastropods. In particular, bivalves (i.e., clams, scallops, and oysters) are sessile filter feeders that are constantly exposed to abiotic and biotic stressors, including parasites (Philipp et al. 2012; Zhang et al. 2015). Compared with more basal phyla, many molluscan genomes exhibit an expansion of TLR-encoding genes of both sccTLR and mccTLR subtypes (table 1) (Philipp et al. 2012; Zhang et al. 2015; Ertl et al. 2016). It is hypothesized that this expansion and divergence of TLRs contributes to molluscan immunity and their ability to adapt to stressors (Philipp et al. 2012; Zhang et al. 2015).

This expansion of genes encoding prototypical TLRs is observed in the gastropod snail *Biomphalaria glabrata*, which encodes 25 sccTLRs and two mccTLRs (Adema et al. 2017). Similarly, the Pacific oyster *Crassostrea gigas* has 83 TLR genes: four mccTLRs, five sccTLRs, and 74 TLRs that are variants of sccTLRs and mccTLRs (Zhang et al. 2015). Four TIR-only proteins have also been identified in *C. gigas* (Zhang et al. 2015). In the clam *Cyclina sinensis*, two sccTLRs have been identified (Ren et al. 2016) and in the scallop *Chlamys farreri*, a single mccTLR and several LRR-only proteins have been identified (Qiu et al. 2007; Wang et al. 2016; Wang et al. 2017). Given the overall expansion of TLRs in mollusks, additional TLR genes are likely encoded by *C. sinensis* and *C. farreri*.

Phylogenetic analysis of select molluscan TLRs indicates that TLRs cluster based on their ectodomain subtype. That is, mccTLRs from *C. gigas* (Toll-1), *C. farreri* (Toll-1), and *B. glabrata* (TLR) cluster more similarly with mccTLRs found in cnidarians and nematodes than with sccTLRs from the mollusks *C. gigas* and *C. sinensis* (fig. 3). In fact, branching between the *C. gigas* sccTLRs and the *C. sinensis* TLR13 is not resolved, which may due be to low sequence divergence brought about during duplication of molluscan TLRs (fig. 3). Interestingly, the *B. glabrata* sccTLR (TLR2) does not cluster with other molluscan sccTLRs, which may be due to an LRR N-terminal domain in *B. glabrata* TLR2 that is not found in other molluscan sccTLRs (fig. 3). The *B. glabrata* TLR2 provides an example of a molluscan TLR that may have diverged during TLR gene expansion.

Molluscan TLR-to-NF-κB pathways are more complex than those in more basal phyla (fig. 2*B*) (Wang et al. 2011; Zhang et al. 2011, 2013, 2015; Zhang and Coultas 2011; Philipp et al. 2012; Pila et al. 2016, 2017; Ren et al. 2016, 2017; Adema et al. 2017). In fact, multiple homologs for TLR-to-NF-κB pathway intermediates have been identified in *C. gigas*, suggesting gene expansion of not only molluscan TLRs but also of downstream signaling components (Zhang et al. 2015).

Roles for Molluscan TLRs in Immunity

Several studies have demonstrated that molluscan TLRs activate MyD88-dependent NF-κB signaling (Wang et al. 2011; Zhang et al. 2013). Specifically, the independent expression of four *C. gigas* sccTLRs in HEK293 cells was able to activate human NF-κB signaling, and this activation increased upon coexpression of the *C. gigas* MyD88 (Zhang et al. 2013). Additionally, the TIR domain of the *C. farreri* mccTLR, Cf-Toll-1, can interact with *C. farreri* MyD88 (Wang et al. 2011).

Molluscan TLRs also have roles in responses to pathogens. In particular, infection of *C. gigas* with the pathogenic bacterium *Vibrio anguillarum* induced the expression of the mccTLR Cg-Toll-1 (Zhang et al. 2011). In *C. farreri*, LPS treatment induced expression of Cf-Toll-1, Cf-MyD88, Cf-TRAF6, Cf-IκB, and Cf-NF-κB (Wang et al. 2011). Additionally, Cf-Toll-1-deficient *C. farreri* were more susceptible to lethal infection with the bacterium *Listonella anguillara* (Wang et al. 2011). Infection of a resilient strain of *B. glabrata* with the parasitic platyhelminth *S. mansoni* induced the expression of its mccTLR, Bg-TLR. Moreover, knockdown of Bg-TLR in infection-resistant *B. glabrata* rendered them more susceptible to *S. mansoni* infection (Pila et al. 2016, 2017).

Molluscan TLRs and NF-κB pathway components are expressed in hemocytes, which are circulating phagocytic immune cells that detect and engulf parasites or pathogens (Wang et al. 2011; Zhang et al. 2011, 2013; McAnulty and Nyholm 2016; Pila et al. 2016, 2017; Ren et al. 2016, 2017; Adema et al. 2017). In *B. glabrata*, Bg-TLR is highly expressed in hemocytes, and knockdown of Bg-TLR impaired hemocyte phagocytic activity (Pila et al. 2016, 2017). Treatment of *C. gigas* hemocytes with heat-inactivated *Vibrio parahaemolyticus* induced the expression of four sccTLRs (Zhang et al. 2011, 2013).

Studies also support a pathogen-responsive TLR-to-NF-κB pathway in the hemocytes of *C. sinensis*. Knockdown of *C. sinensis* TLR13 caused a decrease in expression of all other NF-κB pathway components in hemocytes (Ren et al. 2017). Furthermore, infection of *C. sinensis* hemocytes with the pathogenic bacteria *V. anguillarum* or *Micrococcus luteus* induced the expression of *C. sinensis* TLR13, TLR4, MyD88, IRAK4, TRAF6, IKKα, IκB, and NF-κB, and this transcriptional response to *V. anguillarum* or *M. luteus* was ablated upon knockdown of TLR13 (Ren et al. 2016, 2017). TLR-dependent expression of NF-κB pathway components is also found in *C. farreri* where Cf-Toll-1 positively regulates the expression of numerous NF-κB pathway components in hemocytes (Wang et al. 2011). Interestingly, LRR-only genes have also been identified and characterized in *C. farreri*. Four *C. farreri* LRR-only genes were upregulated in hemocytes upon exposure to several pathogenic bacteria, including *V. anguillarum*, and several TLR ligands (Wang et al. 2016, 2017). Taken together, these studies indicate that molluscan hemocytes express TLR-to-NF-κB pathway components that are generally transcriptionally upregulated in response to pathogens.

Studies in *C. farreri* have also shown that molluscan TLRs can directly detect pathogens. That is, the Cf-Toll-1 ectodomain can be stimulated with numerous known TLR ligands [Pam3CSK4, β-glucan, peptidoglycan, poly (I:C), imiquimod, and CpG] to activate NF-κB signaling in human cells, indicating that this mccTLR directly detects MAMPs/PAMPs (Wang et al. 2015). A *C. farreri* LRR-only protein has also been shown to bind LPS, PGN, beta-glucan, and poly (I:C) in biochemical assays (Wang et al. 2016). Thus, the ability of *C. farreri* to detect and respond to microbes is not limited to full-length TLRs.

Based on these studies, it is clear that molluscan TLRs are primarily expressed in hemocytes and have NF-κB-dependent roles in microbial detection and immunity (table 1). However, TLRs might also have roles in molluscan development, as it has been reported that three *C. gigas* TLRs are upregulated during the gastrula and early trochophore stages of embryonic development (Zhang et al. 2015). Further studies are required to elucidate the developmental roles of molluscan TLRs.

Annelid TLRs Have Roles in Neuroimmunity and Neurogenesis

The phylum Annelida consists of segmented worms and leeches that are models for neural regeneration; however, annelid neural repair and immunity may share common signaling components, including TLRs (Schikorski et al. 2009; Cuvillier-Hot et al. 2011; Rodet et al. 2015; Tasiemski and Salzet 2017). One well-characterized annelid model organism is the medicinal leech Hirudo medicinalis, which encodes four TLRs, one of which is an sccTLR (Schikorski et al. 2009; Cuvillier-Hot et al. 2011; Tasiemski and Salzet 2017). Sixteen TLRs have also been identified in the leech *Helobdella robusta*, eight of which are mccTLRs (Davidson et al. 2008), and we identified an sccTLR in *H. robusta* gene models (Simakov et al. 2013). In the polychaete worm *Capitella capitata*, gene duplication is hypothesized to be responsible for the 105 putative TLR-like protein homologs present in its genome (Davidson et al. 2008). Among these 105 putative TLRs, *C. capitata* encodes 22 sccTLRs and a single mccTLR (Davidson et al. 2008). Phylogenetic analysis of an *H. medicinalis* sccTLR (Hm-TLR1) and an *H. robusta* sccTLR demonstrated phylum-specific clustering and clustering with sccTLRs in mollusks (fig. 3).

Annelid TLR-to-NF-κB pathways appear to be more complex than those found in most other basal phyla (fig. 2*B*), however, no studies have shown that annelid TLRs can act as activators of NF-κB (Schikorski et al. 2009; Macagno et al. 2010; Cuvillier-Hot et al. 2011; Rodet et al. 2015; Tasiemski and Salzet 2017). Most functional studies on annelid TLRs have been performed using the *H. medicinalis* sccTLR, Hm-TLR1, which is expressed in neurons and microglial cells and has been shown to have a role in immunity (Schikorski et al. 2009; Cuvillier-Hot et al. 2011). For example, exposure of *H. medicinalis* nerve cords to several bacterial molecules (including LPS) caused an increase in Hm-TLR1 expression in these nerve cords, and treatment of partially severed nerve cords with bacteria caused an accumulation of Hm-TLR1 protein at the lesion site during neuroregeneration (Schikorski et al. 2009; Cuvillier-Hot et al. 2011). Evidence suggests that the *H. medicinalis* neuronal response to LPS involves a MyD88-dependent pathway given that treatment of neurons with LPS induced Hm-MyD88 to immediately disperse from the plasma membrane (Rodet et al. 2015). Interestingly, the regeneration of severed nerve cords occurs more rapidly in the presence of bacteria, which is hypothesized to be a result of Hm-TLR1 inducing the expression of the inflammatory cytokine p43/EMAPII (Schikorski et al. 2009; Cuvillier-Hot et al. 2011). Given what is known about Hm-TLR1, it is hypothesized that annelid TLRs are important for neural immunity to promote rapid neuroregeneration (table 1) (Schikorski et al. 2009; Cuvillier-Hot et al. 2011; Rodet et al. 2015).

Conclusions and Prospects

In this review, we have compiled information on the structures, phylogeny, expression, biological roles, and downstream signaling pathways of Toll-like receptors in basal phyla. Prototypical TLRs have not been identified in poriferans or in single-celled eukaryotes, including choanoflagellates (Song et al. 2012). Our analyses indicate that prototypical TLRs first appeared as multiple cysteine cluster (mcc) TLRs within the phylum Cnidaria. Nevertheless, not all cnidarians have prototypical TLRs, and some cnidarians have separate LRR and TIR domain proteins that can interact to induce downstream signaling. As such, we hypothesize that prototypical TLRs emerged from the fusion of LRR and TIR domain-only genes. Moreover, it appears that mccTLRs are the primary TLR subtype from Cnidaria to Nematoda. With the expansion of TLR genes in Mollusca, we believe that sccTLRs likely emerged in mollusks. Consistent with this hypothesis, molluscan mccTLRs are more similar to mccTLRs in more ancestral phyla than they are to molluscan or annelid sccTLRs,

supporting that ectodomain composition determines phylogenetic clustering (fig. 3).

Drosophila and mammalian TLRs direct numerous biological processes, most notably for innate immunity and development. Specifically, the roles for the Drosophila Tolls in immunity, development, neurogenesis, and neural function are observed in TLRs in more basal organisms, indicating that these biological functions are conserved (table 1). Evidence suggests that in Cnidaria and Porifera, TLRs have roles in both immunity and development. In nematodes, the lack of a direct immune function for TLRs may be due to loss of downstream NF-κB pathway genes in these organisms. In more derived phyla, such as mollusks and annelids, complex NF-κB pathways and multiple TLRs with roles in immunity have been studied. Moreover, annelid TLRs have putative functions in neuroimmunity and neurogenesis, which are processes that are also directed by mammalian TLRs (Barak et al. 2014; Heiman et al. 2014). It is important to note, however, that mollusk and annelid TLRs may also have developmental roles that have not yet been discovered.

Several basal TLRs have been shown to activate NF-κB signaling, but mainly in reconstituted mammalian cell culture systems. Therefore, future studies should directly address whether TLR-to-NF-κB signaling occurs in animals in these basal phyla. Studies in cnidarians, mollusks, and annelids have shown that pathogen treatment increases the expression of genes in the TLR-to-NF-κB pathway, suggesting that some basal organisms respond to infection through transcriptional upregulation of innate immune pathways. This transcriptional upregulation of TLR-to-NF-κB pathway components in basal phyla differs from the rapid posttranscriptional nuclear translocation of NF-κB that occurs in response to pathogen activation of TLRs in flies and mammals.

Some evidence suggests that invertebrate TLR/LRR proteins (e.g., cnidarian) can detect bacterial flagellin and that some mollusk TLRs can detect multiple ligands. Nevertheless, it is still not generally known whether basal TLRs directly engage MAMPs/PAMPs, host-encoded ligands, or both for downstream signaling and biological effect. As such, the types of ligands that activate TLR-directed developmental and immune signaling pathways in basal phyla remain to be determined.

Further studies aimed at identifying the activating ligands of basal TLRs, as well as the involvement of these TLR–ligand interactions in both immune and nonimmune processes will provide important insights into organism-specific biology and the evolution of TLR structure, signaling, and function in more complex organisms. Moreover, such knowledge may be relevant to understanding diseases and environmentally induced changes in the physiology of important invertebrates (e.g., corals) or how some invertebrates (e.g., snails) provide havens for parasites that cause human disease.

Materials and Methods

Phylogenetic Analysis

For comparative analysis of full-length TLRs, we used the amino acid sequences of TLRs from *S. domuncula*, *N. vectensis*, *O. faveolata*, *A. digitifera*, *A. millepora*, *C. elegans*, *C. japonica*, *C. brenneri*, *C. briggsae*, *B. glabrata*, *C. gigas*, *C. sinensis*, *C. farreri*, *H. medicinalis*, *and H. robusta*. The amino acid sequences of these TLRs and their references are included in supplementary table S1, Supplementary Material online. The current state of genomes of organisms utilized in this phylogenetic analysis is included in supplementary table S2, Supplementary Material online. For organisms without a sequenced genome, transcriptomes or cDNA libraries were reported (supplementary table S2, Supplementary Material online). Clustal Omega (Sievers et al. 2011) was used to align the TLR sequence data set using default parameters, and phylogenetic comparison was performed using maximum likelihood analysis bootstrapped 1000 times using PAUP* and rooted with *S. domuncula* TLR (Swofford 2002).

Identification of TLR Subtype

For subtyping of TLRs not annotated in the literature, we analyzed the amino acid sequences of these TLRs for the number of cysteine clusters in their ectodomain using the LRRfinder program (http://www.lrrfinder.com/lrrfinder.php). This program identifies conserved leucine-rich repeats, leucine-rich repeat N-terminal domains (LRRNTs), and leucine-rich repeat C-terminal domains (LRRCTs) and their amino acid coordinates using position-specific scoring matrices. Single cysteine cluster TLRs were identified by the presence of only one LRRCT. Multiple cysteine cluster TLRs were identified by the presence of two LRRCTs, with one LRRCT interrupting the leucine-rich repeat region. Conserved LRRNT sequences were rarely identified in the TLRs surveyed. These amino acid sequences were then manually inspected for cysteine clusters within conserved LRRCTs.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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References

Adema CM, Hillier LW, Jones CS, Loker ES, Knight M, Minx P, Oliveira G, Raghavan N, Shedlock A, Do Amaral LR, et al. 2017. Whole genome analysis of a schistosomiasis-transmitting freshwater snail. *Nat Commun.* 8:15451.

Akira S. 2009. Pathogen recognition by innate immunity and its signaling. *Proc Jpn Acad Ser B Phys Biol Sci.* 85(4): 143–156.

Akira S, Uematsu S, Takeuchi O. 2006. Pathogen recognition and innate immunity. *Cell* 124(4): 783–801.

Anderson KV, Bokla L, Nusslein-Volhard C. 1985. Establishment of dorsal-ventral polarity in the *Drosophila* embryo: the induction of polarity by the Toll gene product. *Cell* 42(3): 791–798.

Augustin R, Fraune S, Bosch TC. 2010. How *Hydra* senses and destroys microbes. *Semin Immunol.* 22(1): 54–58.

Babonis LS, Martindale MQ, Ryan JF. 2016. Do novel genes drive morphological novelty? An investigation of the nematosomes in the sea anemone *Nematostella vectensis*. *BMC Evol Biol*. 16(1): 114.
Barak B, Feldman N, Okun E. 2014. Toll-like receptors as developmental tools that regulate neurogenesis during development: an update. *Front Neurosci*. 8:272.
Battisti JM, Watson LA, Naung MT, Drobish AM, Voronina E, Minnick MF. 2017. Analysis of the *Caenorhabditis elegans* innate immune response to *Coxiella burnetii*. *Innate Immun*. 23(2): 111–127.
Baumgarten S, Simakov O, Esherick LY, Liew YJ, Lehnert EM, Michell CT, Li Y, Hambleton EA, Guse A, Oates ME, et al. 2015. The genome of *Aiptasia*, a sea anemone model for coral symbiosis. *Proc Natl Acad Sci U S A*. 112(38): 11893–11898.
Belvin MP, Anderson KV. 1996. A conserved signaling pathway: the *Drosophila* toll-dorsal pathway. *Annu Rev Cell Dev Biol*. 12:393–416.
Blaxter M. 2011. Nematodes: the worm and its relatives. *PLoS Biol*. 9(4): e1001050.
Bosch TC, Augustin R, Anton-Erxleben F, Fraune S, Hemmrich G, Zill H, Rosenstiel P, Jacobs G, Schreiber S, Leippe M, et al. 2009. Uncovering the evolutionary history of innate immunity: the simple metazoan *Hydra* uses epithelial cells for host defence. *Dev Comp Immunol*. 33(4): 559–569.
Brandt JP, Ringstad N. 2015. Toll-like receptor signaling promotes development and function of sensory neurons required for a *C. elegans* pathogen-avoidance behavior. *Curr Biol*. 25(17): 2228–2237.
Brennan JJ, Messerschmidt JL, Williams LM, Matthews BJ, Reynoso M, Gilmore TD. 2017. Sea anemone model has a single Toll-like receptor that can function in pathogen detection, NF-κB signal transduction, and development. *Proc Natl Acad Sci U S A*. 114(47): E10122–E10131.
Brubaker SW, Bonham KS, Zanoni I, Kagan JC. 2015. Innate immune pattern recognition: a cell biological perspective. *Annu Rev Immunol*. 33:257–290.
Chapman JA, Kirkness EF, Simakov O, Hampson SE, Mitros T, Weinmaier T, Rattei T, Balasubramanian PG, Borman J, Busam D, et al. 2010. The dynamic genome of *Hydra*. *Nature* 464(7288): 592–596.
Coghlan A, Fiedler TJ, McKay SJ, Flicek P, Harris TW, Blasiar D, Stein LD. 2008. nGASP: the nematode genome annotation assessment project. *BMC Bioinformatics* 9:549.
Couillault C, Pujol N, Reboul J, Sabatier L, Guichou JF, Kohara Y, Ewbank JJ. 2004. TLR-independent control of innate immunity in *Caenorhabditis elegans* by the TIR domain adaptor protein TIR-1, an ortholog of human SARM. *Nat Immunol*. 5(5): 488–494.
Cuvillier-Hot V, Boidin-Wichlacz C, Slomianny C, Salzet M, Tasiemski A. 2011. Characterization and immune function of two intracellular sensors, HmTLR1 and HmNLR, in the injured CNS of an invertebrate. *Dev Comp Immunol*. 35(2): 214–226.
Daly M, Brugler M, Cartwright P, Collins A, Dawson M, Fautin D, France S, McFadden C, Opresko D, Rodriguez E, et al. 2007. The phylum Cnidaria: a review of phylogenetic patterns and diversity 300 years after Linnaeus. *Zootaxa* 1668:127–182.
Davidson CR, Best NM, Francis JW, Cooper EL, Wood TC. 2008. Toll-like receptor genes (TLRs) from *Capitella capitata* and *Helobdella robusta* (Annelida). *Dev Comp Immunol*. 32(6): 608–612.
Dolan J, Walshe K, Alsbury S, Hokamp K, O'Keeffe S, Okafuji T, Miller SF, Tear G, Mitchell KJ. 2007. The extracellular leucine-rich repeat superfamily; a comparative survey and analysis of evolutionary relationships and expression patterns. *BMC Genomics* 8:320.
Enkhbayar P, Kamiya M, Osaki M, Matsumoto T, Matsushima N. 2004. Structural principles of leucine-rich repeat (LRR) proteins. *Proteins* 54(3): 394–403.
Ertl NG, O'Connor WA, Papanicolaou A, Wiegand AN, Elizur A. 2016. Transcriptome analysis of the sydney rock oyster, *Saccostrea glomerata*: insights into molluscan immunity. *PLoS One* 11(6): e0156649.
Flot JF, Hespeels B, Li X, Noel B, Arkhipova I, Danchin EG, Hejnol A, Henrissat B, Koszul R, Aury JM, et al. 2013. Genomic evidence for ameiotic evolution in the bdelloid rotifer *Adineta vaga*. *Nature* 500(7463): 453–457.
Forsthoefel DJ, James NP, Escobar DJ, Stary JM, Vieira AP, Waters FA, Newmark PA. 2012. An RNAi screen reveals intestinal regulators of branching morphogenesis, differentiation, and stem cell proliferation in planarians. *Dev Cell* 23(4): 691–704.
Franzenburg S, Fraune S, Kunzel S, Baines JF, Domazet-Loso T, Bosch TC. 2012. MyD88-deficient *Hydra* reveal an ancient function of TLR signaling in sensing bacterial colonizers. *Proc Natl Acad Sci U S A*. 109(47): 19374–19379.
Galbadage T, Shepherd TF, Cirillo SL, Gumienny TL, Cirillo JD. 2016. The *Caenorhabditis elegans* p38 MAPK Gene plays a key role in protection from mycobacteria. *Microbiologyopen* 5(3): 436–452.
Gauthier ME, Du Pasquier L, Degnan BM. 2010. The genome of the sponge *Amphimedon queenslandica* provides new perspectives into the origin of Toll-like and interleukin 1 receptor pathways. *Evol Dev*. 12(5): 519–533.
Gilmore T, Wolenski F. 2012. NF-κB: where did it come from and why?. *Immunol Rev*. 246(1): 14–35.
Gissendanner CR, Kelley TD. 2013. The *C. elegans* gene pan-1 encodes novel transmembrane and cytoplasmic leucine-rich repeat proteins and promotes molting and the larva to adult transition. *BMC Dev Biol*. 13(1): 21.
Gladyshev EA, Meselson M, Arkhipova IR. 2008. Massive horizontal gene transfer in bdelloid rotifers. *Science* 320(5880): 1210–1213.
Goldstone JV. 2008. Environmental sensing and response genes in cnidaria: the chemical defensome in the sea anemone *Nematostella vectensis*. *Cell Biol Toxicol*. 24(6): 483–502.
Grohme MA, Schloissnig S, Rozanski A, Pippel M, Young GR, Winkler S, Brandl H, Henry I, Dahl A, Powell S, et al. 2018. The genome of *Schmidtea mediterranea* and the evolution of core cellular mechanisms. *Nature* 554(7690): 56–61.
Harris TW, Antoshechkin I, Bieri T, Blasiar D, Chan J, Chen WJ, De La Cruz N, Davis P, Duesbury M, Fang R, et al. 2010. WormBase: a comprehensive resource for nematode research. *Nucleic Acids Res*. 38(Database issue): D463–D467.
Hashimoto C, Hudson KL, Anderson KV. 1988. The Toll gene of *Drosophila*, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell* 52(2): 269–279.
Heiman A, Pallottie A, Heary RF, Elkabes S. 2014. Toll-like receptors in central nervous system injury and disease: a focus on the spinal cord. *Brain Behav Immun*. 42:232–245.
Hemmrich G, Miller DJ, Bosch TC. 2007. The evolution of immunity: a low-life perspective. *Trends Immunol*. 28(10): 449–454.
Hentschel U, Piel J, Degnan SM, Taylor MW. 2012. Genomic insights into the marine sponge microbiome. *Nat Rev Microbiol*. 10(9): 641–654.
Imler JL, Zheng L. 2004. Biology of Toll receptors: lessons from insects and mammals. *J Leukoc Biol*. 75(1): 18–26.
Irazoqui JE, Urbach JM, Ausubel FM. 2010. Evolution of host innate defence: insights from *Caenorhabditis elegans* and primitive invertebrates. *Nat Rev Immunol*. 10(1): 47–58.
Kajava AV. 1998. Structural diversity of leucine-rich repeat proteins. *J Mol Biol*. 277(3): 519–527.
Kawai T, Akira S. 2007. Signaling to NF-kappaB by Toll-like receptors. *Trends Mol Med*. 13(11): 460–469.
Kawasaki T, Kawai T. 2014. Toll-like receptor signaling pathways. *Front Immunol*. 5:461.
Kruse M, Muller IM, Muller WE. 1997. Early evolution of metazoan serine/threonine and tyrosine kinases: identification of selected kinases in marine sponges. *Mol Biol Evol*. 14(12): 1326–1334.
Kurusu M, Cording A, Taniguchi M, Menon K, Suzuki E, Zinn K. 2008. A screen of cell-surface molecules identifies leucine-rich repeat proteins as key mediators of synaptic target selection. *Neuron*. 59(6): 972–985.
Leulier F, Lemaitre B. 2008. Toll-like receptors–taking an evolutionary approach. *Nat Rev Genet*. 9(3): 165–178.
Li Y, Sun X, Hu X, Xun X, Zhang J, Guo X, Jiao W, Zhang L, Liu W, Wang J, et al. 2017. Scallop genome reveals molecular adaptations to semi-sessile life and neurotoxins. *Nat Commun*. 8(1): 1721.
Lindsay SA, Wasserman SA. 2014. Conventional and non-conventional *Drosophila* Toll signaling. *Dev Comp Immunol*. 42(1): 16–24.
Liu OW, Shen K. 2011. The transmembrane LRR protein DMA-1 promotes dendrite branching and growth in *C. elegans*. *Nat Neurosci*. 15(1): 57–63.

Macagno ER, Gaasterland T, Edsall L, Bafna V, Soares MB, Scheetz T, Casavant T, Da Silva C, Wincker P, Tasiemski A, et al. 2010. Construction of a medicinal leech transcriptome database and its application to the identification of leech homologs of neural and innate immune genes. *BMC Genomics* 11:407.

Mancuso VP, Parry JM, Storer L, Poggioli C, Nguyen KC, Hall DH, Sundaram MV. 2012. Extracellular leucine-rich repeat proteins are required to organize the apical extracellular matrix and maintain epithelial junction integrity in *C. elegans*. *Development* 139(5): 979–990.

Mansfield KM, Carter NM, Nguyen L, Cleves PA, Alshanbayeva A, Williams LM, Crowder C, Penvose AR, Finnerty JR, Weis VM, et al. 2017. Transcription factor NF-kappaB is modulated by symbiotic status in a sea anemone model of cnidarian bleaching. *Sci Rep*. 7(1): 16025.

McAnulty SJ, Nyholm SV. 2016. The role of hemocytes in the Hawaiian Bobtail Squid, *Euprymna scolopes*: a model organism for studying beneficial host-microbe interactions. *Front Microbiol*. 7:2013.

McIlroy G, Foldi I, Aurikko J, Wentzell JS, Lim MA, Fenton JC, Gay NJ, Hidalgo A. 2013. Toll-6 and Toll-7 function as neurotrophin receptors in the *Drosophila melanogaster* CNS. *Nat Neurosci*. 16(9): 1248–1256.

Meyer SN, Amoyel M, Bergantinos C, de la Cova C, Schertel C, Basler K, Johnston LA. 2014. An ancient defense system eliminates unfit cells from developing tissues during cell competition. *Science* 346(6214): 1258236.

Milan M, Weihe U, Perez L, Cohen SM. 2001. The LRR proteins capricious and Tartan mediate cell interactions during DV boundary formation in the *Drosophila* wing. *Cell* 106(6): 785–794.

Miller DJ, Hemmrich G, Ball EE, Hayward DC, Khalturin K, Funayama N, Agata K, Bosch TC. 2007. The innate immune repertoire in cnidaria–ancestral complexity and stochastic gene loss. *Genome Biol*. 8(4): R59.

Moya A, Huisman L, Ball EE, Hayward DC, Grasso LC, Chua CM, Woo HN, Gattuso JP, Foret S, Miller DJ. 2012. Whole transcriptome analysis of the coral *Acropora millepora* reveals complex responses to CO(2)-driven acidification during the initiation of calcification. *Mol Ecol*. 21(10): 2440–2454.

Ng A, Xavier RJ. 2011. Leucine-rich repeat (LRR) proteins: integrators of pattern recognition and signaling in immunity. *Autophagy* 7(9): 1082–1084.

Ng AC, Eisenberg JM, Heath RJ, Huett A, Robinson CM, Nau GJ, Xavier RJ. 2011. Human leucine-rich repeat proteins: a genome-wide bioinformatic categorization and functional analysis in innate immunity. *Proc Natl Acad Sci U S A*. 108(Suppl 1): 4631–4638.

Norris JL, Manley JL. 1992. Selective nuclear transport of the *Drosophila* morphogen dorsal can be established by a signaling pathway involving the transmembrane protein Toll and protein kinase A. *Genes Dev*. 6(9): 1654–1667.

Palmer CV, Traylor-Knowles N. 2012. Towards an integrated network of coral immune mechanisms. *Proc Biol Sci*. 279(1745): 4106–4114.

Pan B, Ren Y, Gao J, Gao H. 2015. De novo RNA-Seq analysis of the venus clam, *Cyclina sinensis*, and the identification of immune-related genes. *PLoS One* 10(4): e0123296.

Pare AC, Vichas A, Fincher CT, Mirman Z, Farrell DL, Mainieri A, Zallen JA. 2014. A positional Toll receptor code directs convergent extension in *Drosophila*. *Nature* 515(7528): 523–527.

Peiris TH, Hoyer KK, Oviedo NJ. 2014. Innate immune system and tissue regeneration in planarians: an area ripe for exploration. *Semin Immunol*. 26(4): 295–302.

Philipp EE, Kraemer L, Melzner F, Poustka AJ, Thieme S, Findeisen U, Schreiber S, Rosenstiel P. 2012. Massively parallel RNA sequencing identifies a complex immune gene repertoire in the lophotrochozoan *Mytilus edulis*. *PLoS One* 7(3): e33091.

Pila EA, Li H, Hambrook JR, Wu X, Hanington PC. 2017. Schistosomiasis from a snail's perspective: advances in snail immunity. *Trends Parasitol*. 33(11): 845–857.

Pila EA, Tarrabain M, Kabore AL, Hanington PC. 2016. A novel Toll-like receptor (TLR) influences compatibility between the Gastropod *Biomphalaria glabrata*, and the Digenean Trematode *Schistosoma mansoni*. *PLoS Pathog*. 12(3): e1005513.

Pinzon JH, Kamel B, Burge CA, Harvell CD, Medina M, Weil E, Mydlarz LD. 2015. Whole transcriptome analysis reveals changes in expression of immune-related genes during and after bleaching in a reef-building coral. *R Soc Open Sci*. 2(4): 140214.

Poole AZ, Weis VM. 2014. TIR-domain-containing protein repertoire of nine anthozoan species reveals coral-specific expansions and uncharacterized proteins. *Dev Comp Immunol*. 46(2): 480–488.

Pradel E, Zhang Y, Pujol N, Matsuyama T, Bargmann CI, Ewbank JJ. 2007. Detection and avoidance of a natural product from the pathogenic bacterium *Serratia marcescens* by *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A*. 104(7): 2295–2300.

Pujol N, Link EM, Liu LX, Kurz CL, Alloing G, Tan MW, Ray KP, Solari R, Johnson CD, Ewbank JJ. 2001. A reverse genetic analysis of components of the Toll signaling pathway in *Caenorhabditis elegans*. *Curr Biol*. 11(11): 809–821.

Qiu L, Song L, Xu W, Ni D, Yu Y. 2007. Molecular cloning and expression of a Toll receptor gene homologue from Zhikong scallop, *Chlamys farreri*. *Fish Shellfish Immunol*. 22(5): 451–466.

Rangan KJ, Pedicord VA, Wang YC, Kim B, Lu Y, Shaham S, Mucida D, Hang HC. 2016. A secreted bacterial peptidoglycan hydrolase enhances tolerance to enteric pathogens. *Science* 353(6306): 1434–1437.

Rauta PR, Samanta M, Dash HR, Nayak B, Das S. 2014. Toll-like receptors (TLRs) in aquatic animals: signaling pathways, expressions and immune responses. *Immunol Lett*. 158(1-2): 14–24.

Ren Y, Ding D, Pan B, Bu W. 2017. The TLR13-MyD88-NF-kappaB signalling pathway of *Cyclina sinensis* plays vital roles in innate immune responses. *Fish Shellfish Immunol*. 70:720–730.

Ren Y, Pan H, Pan B, Bu W. 2016. Identification and functional characterization of three TLR signaling pathway genes in *Cyclina sinensis*. *Fish Shellfish Immunol*. 50:150–159.

Rodet F, Tasiemski A, Boidin-Wichlacz C, Van Camp C, Vuillaume C, Slomianny C, Salzet M. 2015. Hm-MyD88 and Hm-SARM: two key regulators of the neuroimmune system and neural repair in the medicinal leech. *Sci Rep*. 5:9624.

Schikorski D, Cuvillier-Hot V, Boidin-Wichlacz C, Slomianny C, Salzet M, Tasiemski A. 2009. Deciphering the immune function and regulation by a TLR of the cytokine EMAPII in the lesioned central nervous system using a leech model. *J Immunol*. 183(11): 7119–7128.

Shinzato C, Shoguchi E, Kawashima T, Hamada M, Hisata K, Tanaka M, Fujie M, Fujiwara M, Koyanagi R, Ikuta T, et al. 2011. Using the *Acropora digitifera* genome to understand coral responses to environmental change. *Nature* 476(7360): 320–323.

Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Soding J, et al. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol*. 7:539.

Silverman N, Maniatis T. 2001. NF-kappaB signaling pathways in mammalian and insect innate immunity. *Genes Dev*. 15(18): 2321–2342.

Silverman N, Paquette N, Aggarwal K. 2009. Specificity and signaling in the *Drosophila* immune response. *Invertebrate Surviv J*. 6(2): 163–174.

Simakov O, Marletaz F, Cho SJ, Edsinger-Gonzales E, Havlak P, Hellsten U, Kuo DH, Larsson T, Lv J, Arendt D, et al. 2013. Insights into bilaterian evolution from three spiralian genomes. *Nature* 493(7433): 526–531.

Snell TW. 2014. Rotifers as models for the biology of aging. *Intl Rev Hydrobiol*. 99(1-2): 84–95.

Song X, Jin P, Qin S, Chen L, Ma F. 2012. The evolution and origin of animal Toll-like receptor signaling pathway revealed by network-level molecular evolutionary analyses. *PLoS One* 7(12): e51657.

Srivastava M, Simakov O, Chapman J, Fahey B, Gauthier ME, Mitros T, Richards GS, Conaco C, Dacre M, Hellsten U, et al. 2010. The *Amphimedon queenslandica* genome and the evolution of animal complexity. *Nature* 466(7307): 720–726.

Stein LD, Bao Z, Blasiar D, Blumenthal T, Brent MR, Chen N, Chinwalla A, Clarke L, Clee C, Coghlan A, et al. 2003. The genome sequence of *Caenorhabditis briggsae*: a platform for comparative genomics. *PLoS Biol*. 1(2): E45.

Swofford D. 2002. Phylogenetic analysis using parsimony (*and other methods). Sunderland (MA): Sinauer Associates.
Tasiemski A, Salzet M. 2017. Neuro-immune lessons from an annelid: the medicinal leech. *Dev Comp Immunol*. 66:33–42.
Tenor JL, Aballay A. 2008. A conserved Toll-like receptor is required for *Caenorhabditis elegans* innate immunity. *EMBO Rep*. 9(1): 103–109.
Thaiss CA, Zmora N, Levy M, Elinav E. 2016. The microbiome and innate immunity. *Nature* 535(7610): 65–74.
The C. elegans Sequencing Consortium 1998. Genome sequence of the nematode *C. elegans*: a platform for investigating biology. *Science* 282:2012–2018.
Valanne S, Wang JH, Ramet M. 2011. The *Drosophila* Toll signaling pathway. *J Immunol*. 186(2): 649–656.
Vasselon T, Detmers PA. 2002. Toll receptors: a central element in innate immune responses. *Infect Immun*. 70(3): 1033–1041.
Ve T, Williams SJ, Kobe B. 2015. Structure and function of Toll/interleukin-1 receptor/resistance protein (TIR) domains. *Apoptosis* 20(2): 250–261.
Vidal-Dupiol J, Ladriere O, Meistertzheim AL, Foure L, Adjeroud M, Mitta G. 2011. Physiological responses of the scleractinian coral *Pocillopora damicornis* to bacterial stress from *Vibrio coralliilyticus*. *J Exp Biol*. 214(Pt 9): 1533–1545.
Wang M, Wang L, Guo Y, Sun R, Yue F, Yi Q, Song L. 2015. The broad pattern recognition spectrum of the Toll-like receptor in mollusk Zhikong scallop *Chlamys farreri*. *Dev Comp Immunol*. 52(2): 192–201.
Wang M, Wang L, Guo Y, Yi Q, Song L. 2016. An LRR-only protein representing a new type of pattern recognition receptor in *Chlamys farreri*. *Dev Comp Immunol*. 54(1): 145–155.
Wang M, Wang L, Jia Z, Wang X, Yi Q, Zhao L, Song L. 2017. The versatile functions of LRR-only proteins in mollusk *Chlamys farreri*. *Dev Comp Immunol*. 77:188–199.
Wang M, Yang J, Zhou Z, Qiu L, Wang L, Zhang H, Gao Y, Wang X, Zhang L, Zhao J, et al. 2011. A primitive Toll-like receptor signaling pathway in mollusk Zhikong scallop *Chlamys farreri*. *Dev Comp Immunol*. 35(4): 511–520.
Wayburn B, Volk T. 2009. LRT, a tendon-specific leucine-rich repeat protein, promotes muscle-tendon targeting through its interaction with Robo. *Development* 136(21): 3607–3615.
Weisse T, Laufenstein N, Weithoff G. 2013. Multiple environmental stressors confine the ecological niche of the rotifer *Cephalodella acidophila*. *Freshw Biol*. 58(5): 1008–1015.
Wiens M, Korzhev M, Krasko A, Thakur NL, Perovic-Ottstadt S, Breter HJ, Ushijima H, Diehl-Seifert B, Muller IM, Muller WE. 2005. Innate immune defense of the sponge *Suberites domuncula* against bacteria involves a MyD88-dependent signaling pathway. Induction of a perforin-like molecule. *J Biol Chem*. 280(30): 27949–27959.
Wiens M, Korzhev M, Perovic-Ottstadt S, Luthringer B, Brandt D, Klein S, Muller WE. 2007. Toll-like receptors are part of the innate immune defense system of sponges (demospongiae: Porifera). *Mol Biol Evol*. 24(3): 792–804.
Williams LM, Fuess LE, Brennan JJ, Mansfield KM, Salas-Rodriguez E, Welsh J, Awtry J, Banic S, Chacko C, Chezian A, et al. 2018. A conserved toll-like receptor-to-NF-kappaB signaling pathway in the endangered coral *Orbicella faveolata*. *Dev Comp Immunol*. 79:128–136.
Wolenski FS, Bradham CA, Finnerty JR, Gilmore TD. 2013. NF-κB is required for cnidocyte development in the sea anemone *Nematostella vectensis*. *Dev Biol*. 373(1): 205–215.
Worheide G, Dohrmann M, Erpenbeck D, Larroux C, Maldonado M, Voigt O, Borchiellini C, Lavrov DV. 2012. Deep phylogeny and evolution of sponges (phylum Porifera). *Adv Mar Biol*. 61:1–78.
Zarowiecki M, Berriman M. 2015. What helminth genomes have taught us about parasite evolution. *Parasitology* 142(S1): S85–S97.
Zhang L, Li L, Guo X, Litman GW, Dishaw LJ, Zhang G. 2015. Massive expansion and functional divergence of innate immune genes in a protostome. *Sci Rep*. 5:8693.
Zhang L, Li L, Zhang G. 2011. A *Crassostrea gigas* Toll-like receptor and comparative analysis of TLR pathway in invertebrates. *Fish Shellfish Immunol*. 30(2): 653–660.
Zhang SM, Coultas KA. 2011. Identification and characterization of five transcription factors that are associated with evolutionarily conserved immune signaling pathways in the schistosome-transmitting snail *Biomphalaria glabrata*. *Mol Immunol*. 48(15–16): 1868–1881.
Zhang Y, He X, Yu F, Xiang Z, Li J, Thorpe KL, Yu Z. 2013. Characteristic and functional analysis of toll-like receptors (TLRs) in the lophotrocozoan, *Crassostrea gigas*, reveals ancient origin of TLR-mediated innate immunity. *PLoS One* 8(10): e76464.