MyD88-deficient *Hydra* reveal an ancient function of TLR signaling in sensing bacterial colonizers

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Toll-like receptor (TLR) signaling is one of the most important signaling cascades of the innate immune system of vertebrates. Studies in invertebrates have focused on the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*, and there is little information regarding the evolutionary origin and ancestral function of TLR signaling. In *Drosophila*, members of the Toll-like receptor family are involved in both embryonic development and innate immunity. In *C. elegans*, a clear immune function of the TLR homolog TOL-1 is controversial and central components of vertebrate TLR signaling including the key adapter protein myeloid differentiation primary response gene 88 (MyD88) and the transcription factor NF-κB are not present. In basal metazoans such as the cnidarians *Hydra magnipapillata* and *Nematostella vectensis*, all components of the vertebrate TLR signaling cascade are present, but their role in immunity is unknown. Here, we use a MyD88 loss-of-function approach in *Hydra* to demonstrate that recognition of bacteria is an ancestral function of TLR signaling and that this process contributes to both host-mediated recolonization by commensal bacteria as well as to defense against bacterial pathogens.

Toll-like receptors (TLRs) are conserved throughout animal evolution but appear to serve different functions in different model organisms. TLRs are transmembrane receptors with extracellular leucin-rich repeat (LRR) motifs and an intracellular Toll/interleukin-1 receptor (TIR) domain. Upon stimulation of TLRs, the key adapter protein MyD88 associates with the cytosolic part of the TLR through a homophilic interaction of the TIR domains and then recruits the IL-1R–associated kinase (IRAK), which subsequently associates with the TNFR-associated factor (TRAF). TRAF recruits the TGF-β activated kinase 1 (TAK1). The kinase TAK1 induces a phosphorylation cascade finally leading to the nuclear translocation of the transcription factors NF-κB via the inhibitor of kappa B-kinase (IKK) signalosome or c-Jun via the c-Jun N-terminal kinase (JNK)/p38 branch of TLR signaling (1).

The Toll pathway was initially identified to be essential in early embryonic development in *Drosophila* (2). In addition to its crucial role in the establishment of the dorsal–ventral axis, *Drosophila* Toll was shown to be involved in muscle development (3) and heart formation (4). Later on, it was discovered that Toll signaling in *Drosophila* also contributes to defense reactions against bacteria as well as to antifungal defense by regulating, among others, the expression of the antifungal peptide drosomycin in adult flies (5, 6). Further immunity functions have been identified for Toll-7 (7) and Toll-8 (8). Studies in the mosquito *Aedes aegypti* also identified MyD88-dependent Toll signaling to mediate immune defenses against dengue viruses (9). One other invertebrate model system, the nematode *Caenorhabditis elegans* lacks central proteins of the canonical TLR-signaling cascade (10). Only one Toll homolog, termed TOL-1, was identified in *C. elegans* (10). The fact that TOL-1 mutants show strong developmental defects despite mutants for the putative signaling cascade displaying no developmental abnormalities led to the belief that TOL-1 in *C. elegans* might function as a cell–cell adhesion protein in neurons (10). Other reports state an additional involvement of TOL-1 in pathogen defense (11). In addition, a TIR-domain–containing protein called tir-1 is required for resistance to fungal and bacterial infection in *C. elegans* (12, 13).

Vertebrate homologs of Toll, the TLRs, are receptors of the immune system. Vertebrate TLRs are involved in eliminating pathogens and controlling commensal colonization (1, 14, 15) by recognizing conserved microbe-associated molecular patterns (MAMPs) including lipopolysaccharides, flagellin, and peptidoglycans (1, 16). Therefore, it was proposed that immune function of TLR signaling involving NF-κB and MyD88 has evolved within the bilaterians (17). However, recent genome projects in the nonbilaterians *Hydra magnipapillata* (18) and *Nematostella vectensis* (19) revealed the presence of TLRs, MyD88, and NF-κB (20, 21). Their role in bacterial recognition and innate immunity, however, remains to be shown (22). Cnidaria are a sister group to the Bilateria (19) and one of the earliest branches in the animal tree of life (Fig. 1*A*). The recent genome project of the cnidarian *H. magnipapillata* identified a conserved TLR-signaling cascade (21, 23) (Fig. 1*B* and Table S1), making *Hydra* a suitable model for addressing questions of the ancestral function of TLR signaling. Is the TLR pathway involved in the defense against bacterial pathogens or in maintaining specific host–microbe interactions? Does it affect the mechanisms and routes by which functionally diverse bacteria colonize their host? Is it involved in developmental processes such as axis formation? To gain insight into these questions, we performed MyD88 loss-of-function experiments in *Hydra vulgaris* [AEP strain (24)]. We used a combination of microarray-based gene expression screening and 16S rRNA-gene sequencing to detect changes in both the *Hydra* transcriptome and the associated microbiota. Further, we investigated the role of TLR signaling in pathogen defense against *Pseudomonas aeruginosa*. The patterns of differentially regulated host genes as well as changes in the bacterial colonization process and pathogen susceptibility in MyD88-knockdown polyps point to a role of TLR signaling in sensing of bacteria, be it associated commensals or pathogens. Thus, this functional analysis clearly identifies a role of TLR signaling in innate immunity in an animal at the base of metazoan evolution.

Results

Generation of MyD88-Knockdown (MyD88$^-$) *H. vulgaris* (AEP). To analyze the function of TLR signaling in the basal metazoan *Hydra*, we generated a stable transgenic *H. vulgaris* (AEP) line with drastically reduced expression levels of the universal adapter

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Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE32383); and the 16S 454 data are deposited at the metagenome analysis server MG-RAST (Project ID 1719).

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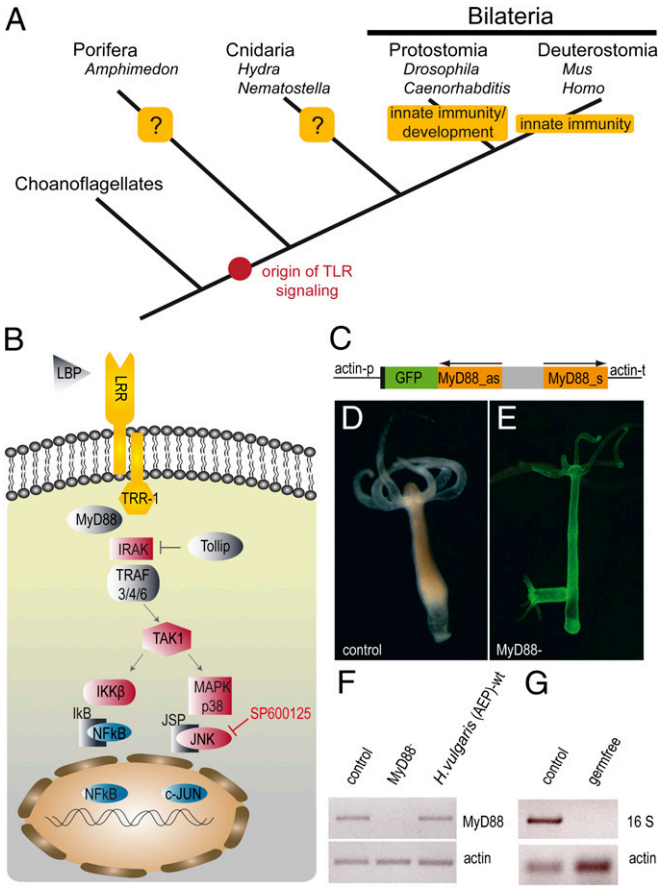

Fig. 1. Interference with the conserved *Hydra* TLR-signaling pathway. (*A*) Function of TLR signaling during metazoan evolution. Cnidaria are the sistergroup to all bilateria and they diverged from the common eumetazoan ancestor ~600–700 million years ago (19). (*B*) Schematic representation of the conserved TLR-signaling pathway in *Hydra*. Note that the functional *Hydra* TLR is assembled by two proteins (HyLRR and HyTRR) (23) and the exogenous JNK inhibitor SP600125 is shown. (*C*) MyD88–Hairpin construct for generation of transgenic *Hydra* (p, promoter; T, terminator, as, antisense; s, sense). (*D*) Live image of a MyD88-control polyp (control). (*E*) Live image of a MyD88-knockdown polyp (MyD88$^-$) showing EGFP expression in the endodermal and the ectodermal cell lineage. (*F*) RT-PCR amplifying *myd88* shows down-regulation in MyD88$^-$ polyps compared with control polyps and wild-type (WT) *H. vulgaris* (AEP). RT-PCR was normalized using the *Hydra actin* gene. (*G*) Absence of bacteria after antibiotic treatment was confirmed by PCR amplification of the bacterial 16S rRNA gene on genomic DNA normalized to *Hydra actin*.

protein MyD88 using a hairpin cassette containing the *myd88* antisense and sense sequences fused to the reporter gene *egfp* (Fig. 1*C*). The transformation of two- to four-cell–stage embryos via microinjection resulted in the founder polyp MyD88-D3. This polyp showed mosaic distribution of GFP$^+$ cells, which could be enriched or depleted in clones generated by budding, depending on where the bud formed. By several rounds of asexual proliferation, two stable lines were established. We termed them the MyD88-control (control) line, which contained no remaining GFP$^+$ cells (Fig. 1*D*), and the MyD88-knockdown (MyD88$^-$) line, which expressed the transgene in the endodermal and ectodermal cell lines (Fig. 1*E*). The resulting double-stranded (ds) RNA triggers the RNAi machinery (25–28), which leads to a decrease in the endogenous MyD88 transcript as shown by RT-PCR (Fig. 1*F*). Because both lines were generated from the same founder polyp by asexual reproduction, we were able to analyze the effects of a drastically decreased MyD88-expression level without the need to account for differences in genomic background. Neither line displayed any obvious developmental or behavioral abnormalities.

Absence of Bacteria as Well as MyD88 Deficiency Influence Central Parts of the TLR-Signaling Cascade. To assess the transcriptional consequences of a MyD88 knockdown and identify potential downstream effector genes of the TLR-signaling cascade, we performed microarray analyses. Expression levels of both MyD88$^-$ polyps as well as germ-free polyps (Fig. 1*G*) were compared with control polyps. The MyD88$^-$ polyps combined with the germ-free polyps provided unique resources that allowed us to directly investigate the connection between TLR signaling and the regulation of associated bacterial diversity. Statistical analysis was carried out by ANOVA with Student–Newman–Keuls (SNK) post hoc tests and false discovery rate (FDR) correction. The microarray data independently validate the successful MyD88 knockdown. Contig 11552, encoding for MyD88, shows a 4.29-fold down-regulation ($P < 0.001$) in MyD88$^-$ polyps and is not differentially expressed in germ-free polyps (fold change 1.09, N.S.) (Table S1). To check for transcriptional changes of other putative members of the TLR cascade, the *H. vulgaris* (AEP) transcriptome (29) was screened for homologs of previously described members of the pathway. The majority of central cascade members are present in *H. vulgaris* (AEP) (Fig. 1*A* and Table S1). Various central components of the putative TLR cascade including members of the TRAF family of ubiquitin protein ligases, the kinase TAK1, MAP-kinase p38, and the JNK inhibitor JSP-1 show significantly decreased expression in germ-free and/or MyD88-deficient conditions (Table S1). We hypothesize therefore the existence of positive feedback loops of the putative effector transcription factors NF-κB and c-Jun on certain upstream pathway components, pointing toward a functional unity of these proteins in the bacterial sensing process in vivo.

Gene-Expression Profiling of MyD88-Knockdown and Germ-Free Polyps. After identifying the conserved components of the TLR pathway in *Hydra*, we aimed to identify downstream target genes in an analysis not based on gene homologies. Therefore, only contigs exceeding a significant ($P \leq 0.05$) fold-change threshold of 1.5 in at least one of the comparisons were considered for further analyses. This resulted in 183 differentially regulated (122 down-regulated, 61 up-regulated) contigs in MyD88$^-$ polyps (0.5% of total contigs) and in 741 differentially regulated contigs (320 down-regulated, 421 up-regulated) in germ-free polyps (2.0% of total contigs) (Fig. 2*A*). Interestingly, the signature of MyD88$^-$ polyps overlapped substantially (76.5%) with the germ-free signature (Fig. 2*A*). The overlapping signature of 140 contigs included a large proportion (>75%) of taxonomical restricted genes (TRGs), i.e., contigs of unknown function with no homolog detected in other species (Fig. 2*B*). A total of 21% of these TRGs lack a transmembrane domain but have a predicted signal peptide. This is notable because such secreted peptides might directly interact with associated bacteria. Annotated transcripts included metabolic genes such as carbonic anhydrases, protein-modifying enzymes like kinases and ubiquitinases, receptors, chaperones, viral/transposon-related genes such as transposases and reverse transcriptases, as well as transcription factors. Table 1 shows the fold changes of six representative genes. Additionally, we included three significantly ($P < 0.05$) differentially regulated genes of the germ-free vs. control comparison that had a significant fold change below the 1.5× threshold in the MyD88$^-$ samples (Table 1). These genes include previously described TLR-target genes such as a lectin, bcl-2, and alkaline phosphatase (30–32). The differential expression of these representative target genes was validated by qRT-PCR (Fig. S1). All analyzed contigs show a higher fold change in the absence of bacteria than in MyD88-deficient conditions (Table 1). The fact that expression of more than 75% of the MyD88-responsive transcripts is also altered in germ-free polyps (Fig. 2*A*) suggests that these MyD88-downstream genes are bacteria responsive.

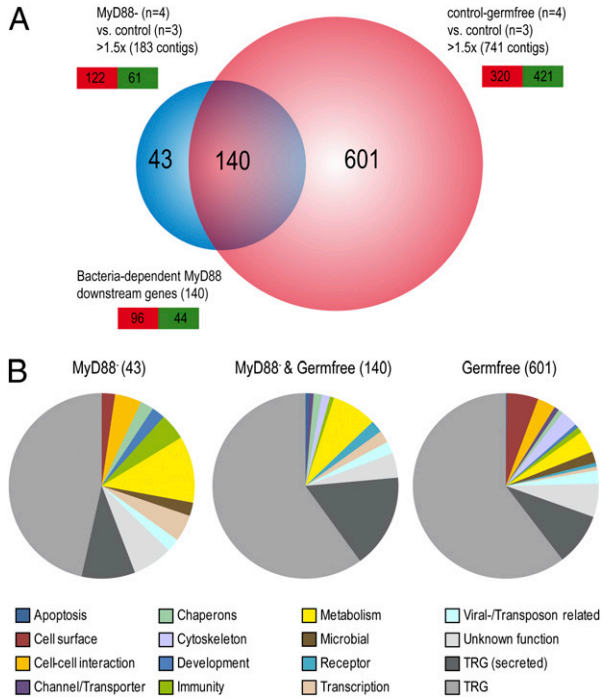

Fig. 2. Microarray analysis reveals differential gene expression due to MyD88 down-regulation and the absence of the associated microbiota. (*A*) Graphic representation of differentially regulated (≥1.5-fold change, $P \leq 0.05$) contigs in MyD88$^-$ and germ-free compared with control polyps. Note the overlap between both experiments. Down-regulated contigs are highlighted in red, up-regulated contigs in green. (*B*) Categorization of differential contigs. Pie charts were separated in MyD88- but not bacterial-regulated contigs (*Left*), MyD88- as well as bacterial-regulated contigs (*Center*), and MyD88-independent bacterial-regulated contigs (*Right*). Contigs were assigned into self-chosen categories.

Notably, MyD88-dependent transcripts were not enriched for genes with known developmental functions (Fig. 2*B*). However, a developmental role of certain TRGs cannot be excluded. In comparison, the 601 transcripts that are regulated by the presence of the bacterial microbiota in a MyD88-independent way include a noticeable proportion of genes involved in cell-surface components and cell–cell interaction like mucins, lectins, and cadherins. Because the glycocalyx of epithelial cells is a habitat for *Hydra*-associated bacteria, it is possible that these colonizers induce changes in their own *Hydra*-cell–associated environment.

Table 1. Differential expression of representative genes in MyD88$^-$ and germ-free polyps

		MyD88$^-$		Germ-free	
Annotation	Contig*	Fold change	*P* value	Fold change	*P* value
Secreted peptide	732	0.66	0.002	0.44	0.001
Secreted EGF	12837	0.57	0.006	0.50	0.005
	24241	0.57	0.004	0.49	0.007
Secreted protein	16151	0.63	0.000	0.54	0.004
T-box	19777	0.64	0.000	0.45	0.001
Cadherin	34924	0.54	0.001	0.30	0.001
	14903	0.59	0.009	0.33	0.002
Secreted protein	43476	0.62	0.026	0.40	0.002
Gal-lectin	1372	0.73	0.005	0.39	0.004
bcl-2	7659	0.72	0.015	0.56	0.005
Alkaline phosphatase	45829	0.70	0.000	0.49	0.002

*Contigs are available at http://compagen.zoologie.uni-kiel.de/.

Subset of MyD88-Downstream Genes Is Regulated by JNK. Upon stimulation by MAMPs, the TLR-signaling cascade in vertebrates can regulate the expression of target genes via two major downstream branches, leading to the nuclear translocation of the transcription factor NF-κB or c-Jun (Fig. 1*A*) (1). In *Hydra*, the expression levels of putative members of the JNK/p38 branch of TLR signaling leading to c-Jun translocation (TAK1, p38α, p38β, and JSP-1) are significantly affected by both the absence of bacteria as well as by silencing the MyD88 expression (Table S1). To directly analyze the role of JNK in TLR signaling, we examined the expression of the nine representative contigs (Table 1) in the presence of the JNK-specific inhibitor SP600125 (33, 34), which was shown to act specifically in the cnidarian *Hydra* (34). As shown in Fig. 3, four of nine candidate genes were negatively regulated by SP600125 in a concentration-dependent manner. This includes bcl-2 (contig 7659), a known NF-κB target gene in vertebrates (31). Thus, bcl-2 might be transcriptionally regulated by both NF-κB and c-Jun transcription factors. To exclude an unspecific, broad effect of the inhibitor SP600125, we conducted qRT-PCRs with a set of nine random genes. None of these genes were affected by SP600125 treatment (Fig. S2). Our results point toward a key role of MAP-kinase signaling in the MyD88-mediated perception of bacterial signals via TLRs in *Hydra*.

MyD88-Deficient *Hydra* Display Delayed Bacterial Recolonization upon Antibiotic Treatment. Next we examined whether a MyD88 knockdown affects the colonizing microbiota. We performed 454 pyrosequencing of the variable region 2 (V2) of the bacterial 16S rRNA gene, amplified from total DNA of control and MyD88$^-$ polyps after a 4-wk period of cocultivation. We found the microbiota of both control and MyD88$^-$ polyps to be dominated by β-proteobacteria (Fig. 4*A* and Fig. S3), mainly of the genus *Curvibacter*. On average, *Curvibacter* sp. accounts for 94% of the microbiota in control polyps and 92% in MyD88$^-$ polyps (Fig. 4*A*). This bacterium was previously cosequenced with the genome of *H. magnipapillata* (18). Rare fractions of the *Hydra*-associated bacterial community include α-proteobacteria, γ-proteobacteria, and flavobacteria (Fig. 4*A*).

To investigate whether TLR signaling plays a role in the microbiota assembly process, we generated germ-free control and germ-free MyD88$^-$ polyps by antibiotic treatment and reinfected them with a complex pool of potential colonizing bacteria retrieved from the supernatant of a nongerm-free culture, pond water, and *Hydra* tissue homogenates (Fig. 4*B*). Individual polyps were examined for their microbiota by 454 pyrosequencing 2 wk and 19 wk postbacterial inoculation. Principle coordinate analysis (PCoA) of the weighted UniFrac metric revealed that after 2 wk,

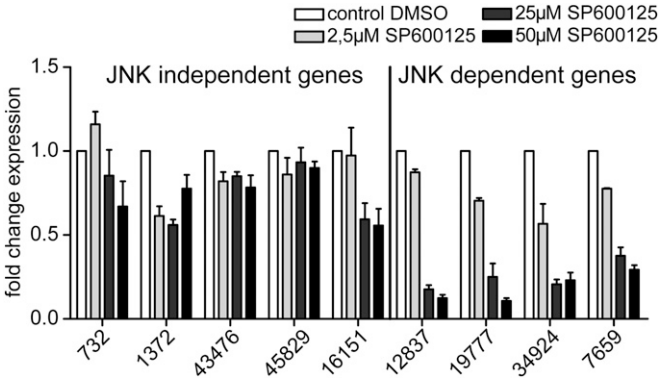

Fig. 3. JNK phosphorylation is mediating the expression of several MyD88-downstream genes. Relative expression level of the candidate genes upon administration of the JNK inhibitor SP600125 (34), determined by qRT-PCR. Note that the expression of 12837, 19777, 34924, and 7659 is influenced by SP600125 in a concentration-dependent manner. cDNA amounts were equilibrated by elongation factor 1 α. The graphic shows means + SD ($n = 3$).

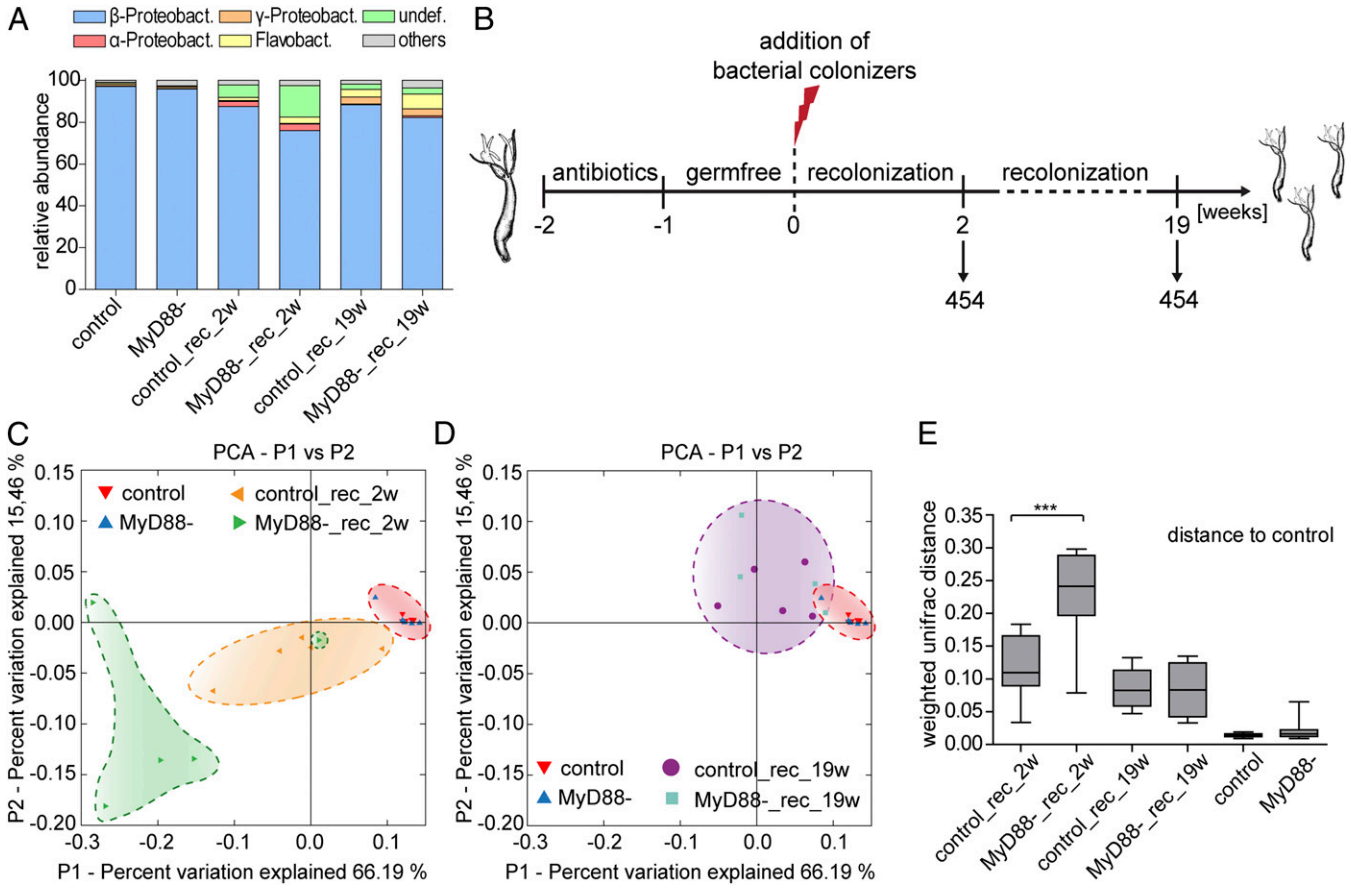

Fig. 4. 454 sequencing of bacterial 16S rRNA reveals minor impact of MyD88 in bacterial colonization of *Hydra* polyps. (*A*) Bar charts representing bacterial communities of *Hydra* polyps on class level (means of five replicates). Rare bacterial taxa (<1% relative abundance) were grouped to the fraction "others." (*B*) Experimental design. Germ-free MyD88$^-$ and control polyps were inoculated with potential bacterial colonizers derived from pond water, *Hydra*-culture supernatant, and *Hydra*-tissue homogenates. Single polyps were removed from clonally growing cultures 2 wk and 19 wk postinoculation and subjected to 454 sequencing of the microbiota. (*C* and *D*) Bacterial communities clustered using principle coordinate analysis of the weighted UniFrac distance matrix. Percent variation explained by the principle coordinates is indicated in the axes. (*E*) Weighted UniFrac differences calculated by pairwise comparisons of the bacterial profile to control polyps. Statistical analysis was carried out using two-tailed *t* test (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

bacterial communities of MyD88$^-$ polyps (MyD88$^-$_rec_2w) display a relatively high separation from the preantibiotic control state, whereas control samples (control_rec_2w) cluster in close proximity to the preantibiotic state (red cloud) (Fig. 4 *C* and *E*). After 19 wk of recolonization (Fig. 4 *D* and *E*), this initial difference between the bacterial communities of control and MyD88$^-$ polyps disappeared. Taken together, these results demonstrate a role of MyD88-mediated TLR signaling in the reestablishment of *Hydra*-associated bacterial communities by promoting bacterial recolonization. However, MyD88 does not appear to be essential for maintaining bacterial homeostasis in adult polyps of *H. vulgaris* (AEP).

TLR Signaling in Pathogen Defense in *Hydra*. *P. aeruginosa* is a pathogenic bacteria that can infect a broad range of host organisms including plants, nematodes, insects, and mice (35). To examine whether silencing of MyD88 affects pathogen defense in *Hydra*, we used the isolate of *P. aeruginosa*, strain UCBPP-PA14 (PA14; http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/www.tax.cgi?id=208963) (36) to infect MyD88$^-$ and control polyps. Single polyps (n = 24 each) were cultured in medium containing 1.8×10^8 PA14 cells per milliliter. Polyps were screened daily and the impact of the pathogenic bacterium was scored according to the disease phenotypes presented in Fig. 5*A*. Twenty-four hours postinfection, MyD88$^-$ as well as control polyps suffered from severe tentacle shortening (mean score ~2) (Fig. 5*B*). Forty-eight hours postinfection, the disease state was characterized by a complete loss of tentacles in a subset of polyps both in control (7/24 polyps with score 3) and MyD88$^-$ (10/24). Whereas the control polyps started to recover, disease severity increased in MyD88$^-$ polyps in the following 2 d (Fig. 5*B*), culminating in the complete lysis of 3 MyD88$^-$ polyps after 96 h (Fig. 5*C*). Taken together, the PA14 infection assay shows a significantly higher pathogen susceptibility of MyD88$^-$ polyps compared with control polyps.

Discussion

Bacterial Sensing Is an Ancient Function of TLR Signaling. In vertebrates, most TLRs act as pattern recognition receptors (PRRs) and are involved in the elimination of pathogens and controlling commensal colonization (1, 14, 15). In contrast, the role of *Drosophila* Toll receptors is less uniform. Their temporal and spatial expression patterns during embryonic development point toward roles in developmental processes by either inducing signaling cascades or acting as cell-adhesion molecules (37). However, a role in development is clearly proven only for Toll (2). In addition, Toll (5, 6) as well as Toll-7 (7) and Toll-8 (8) act in immune defense against fungi, bacteria, or viruses. This functional duality prompted us to search for the ancestral role of TLR signaling in a more basal metazoan (22).

The cnidarian *Hydra* possesses a bona fide TLR-signaling cascade (Fig. 1*A*) (21), making it a suitable model for investigating the ancestral function of the TLR pathway. Together with previous results showing that the HyLRR-2 receptor in *Hydra* is able to bind flagellin in vitro (23), the in vivo results of this study

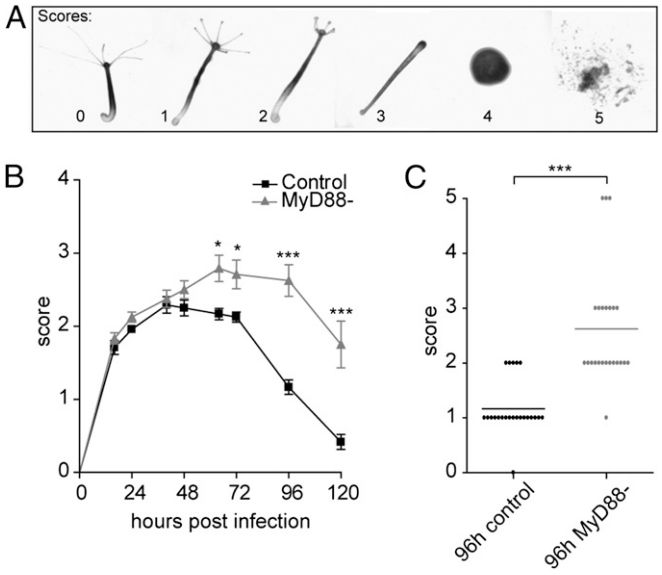

Fig. 5. MyD88$^-$ and control polyps show differential susceptibility to infection by *P. aeruginosa* (*P.a.*) (*A*) Phenotypic scores of the *Hydra* infection model. Disease always starts with swelling of the tentacle tips (score 1), followed by subsequent shortening (score 2), and loss (score 3) of tentacles. Score 4 indicates the loss of body shape with maintenance of an intact epithelium. Score 5 is characterized by tissue lysis. (*B*) Temporal profile of *P.a.*14 infection in *Hydra*. Polyps were incubated in 1 mL *Hydra* medium containing 1.8×10^8 cfu *P.a.*14. Values are plotted as mean + SEM ($n = 24$). (*C*) Detailed representation of the time point 96 h postinfection from *B*. Each dot represents one polyp; horizontal line shows the mean. Note that three polyps died in the MyD88-knockdown group, whereas the maximum score observed in the control group was 2 (tentacle shortening). Statistical significance was tested by two-tailed Mann–Whitney test (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

suggest a role for TLR signaling in bacterial recognition in the cnidarian *Hydra.* Notably, MyD88 loss of function, as well as the absence of commensal bacteria in germ-free polyps, causes significant and overlapping changes in the transcriptome (Fig. 2). A subset of differential genes is regulated by the JNK/p38 branch of TLR signaling (Fig. 3). This shows that TLR signaling in *Hydra* does not act unidirectionally via the transcription factor NF-κB but is also linked to MAP kinases. Notably, a p38 MAPK cascade was identified as a key component of the *C. elegans* immune response and is also present in *Monosiga brevicollis*, a single-cell eukaryote that is most closely related to metazoans (22). Because JNK and p38 in *Hydra* are also activated by many other stimuli, including G-protein–coupled receptors (38) and the wnt pathway (34), innate immune reactions appear to be controlled by a complex signal transduction network.

MyD88 Promotes Reestablishment of Bacterial Homeostasis After Disturbance. Many studies show that microbes have direct beneficial effects on the host (39–42). This is supported by the fact that a dysregulation of host–bacteria homeostasis seems to be involved in the occurrence of disorders, like chronic inflammatory bowel diseases (43, 44). *Hydra* polyps are colonized by species-specific bacterial communities (45), which are largely determined by the host rather than by the environment (45, 46). After verifying that TLR signaling in *Hydra* is clearly involved in the recognition of bacteria, we therefore asked whether this active cross-talk is involved in the maintenance of host–bacterial homeostasis. Our results show no impact of impaired TLR signaling on the composition of the bacterial microbiota in healthy animals. However, after active disturbance of the bacterial community by antibiotic treatment or upon bacterial infection with *P. aeruginosa*, MyD88-mediated TLR signaling promotes a reestablishment of bacterial homeostasis. This is concordant with several mechanisms to restrict active immune signaling to disturbance, such as pathogen defense while being hyporesponsive to the healthy commensal microbiota (47). These mechanisms include (*i*) a decreased apical surface expression of TLRs, (*ii*) spatial segregation of host cells and commensal bacteria by mucus layers to limit detection of invasive bacteria that crossed the epithelial barrier, and (*iii*) active bacterial suppression of host immunity, e.g., via the induction of the TLR-signaling suppressor Tollip (Fig. 1*B*) by commensal bacteria (47, 48).

MyD88-Dependent Target Genes Include Taxonomically Restricted and Conserved Genes. Interestingly, around 75% of differential contigs could not be assigned to functional categories due to the lack of BLAST hits (Fig. 2). This fraction of TRGs (49) is largely overrepresented compared with the overall fraction of TRGs in the whole transcriptome. This large number might indicate that the TLR-dependent response toward commensal bacteria is by and large taxon specific. Furthermore, 21% of these TRGs possess a predicted signal peptide. Because these secreted peptides may contribute to the properties of *Hydra*'s epithelial environment, they might also affect the colonizing microbiota. We have previously shown (50) that antimicrobial peptides, apart from their role in defense against pathogenic bacteria, also have regulatory functions in host–bacterial homeostasis. This adds support to our previously proposed hypothesis (49) that taxonomically restricted host defense molecules facilitate the disarming of taxon-specific microbial attackers, and at the same time shape the colonizing microbiota. Nevertheless, a small proportion of MyD88-target genes is conserved throughout the animal kingdom. These genes include previously described vertebrate MyD88-dependent NFκB-target genes such as lectins or bcl-2 (30, 31). In addition, alkaline phosphatase in zebrafish also responds to LPS through a mechanism that involves MyD88. It is required to detoxify LPS and prevent intestinal inflammation in response to the resident microbiota (32). We propose that the differentially expressed alkaline phosphatase in *Hydra* (Table 1) plays a similar role.

Conclusion

Based on our results we propose that the TLR/MyD88 pathway is an ancestral immune signaling pathway predating the evolution of TLR-dependent immune signaling pathways at the origin of metazoan evolution. Recognizing and managing the bacterial communities typically present at epithelial surfaces throughout the animal kingdom likely contributed to its evolution and maintenance.

Materials and Methods

Experimental details are provided in *SI Materials and Methods*.

Animal Culture. Experiments were carried out using *H. vulgaris* (AEP). All animals were cultured under constant, identical environmental conditions including culture medium, food, and temperature according to standard procedures. All animal experiments were conducted in accordance with the German law concerning animal experimentation.

Generation of Transgenic *H. vulgaris* (AEP) Expressing an EGFP–MyD88–Hairpin Fusion Construct in Endodermal and Ectodermal Cells. For generation of *H. vulgaris* (AEP) egfp:myd88–hairpin transgenics, a hairpin construct was designed. By selecting for EGFP expression, polyps with full endodermal and ectodermal expression of EGFP (MyD88 knockdown) were generated.

Generation of Germ-Free *Hydra*. Polyps were incubated for 1 wk in an antibiotic solution containing 50 μg/mL each of ampicillin, rifampicin, streptomycin, and neomycin with daily exchange of the solution.

Custom-Made *H. vulgaris* (AEP) Microarray. The microarray is based on a full transcriptome of *H. vulgaris* (AEP) sequenced by 454 technology (29). This results in a microarray platform having 45,220 oligos of 60 nucleotides in length, resembling 37,063 unique contigs.

RNA Isolation and Microarray Gene Expression Experiments. Total RNA was isolated from 15 polyps using the TRIzol Plus protocol (Invitrogen). Labeled

cDNAs were hybridized to custom-made Agilent *Hydra* (AEP) gene expression microarray slides (4 × 44K).

Microarray Data Extraction, Filtering, and Analysis. Raw microarray image files were processed and quality checked by Agilent's Feature Extraction 10.7 Image Analysis software. Statistical analysis was conducted by ANOVA with SNK post hoc test and FDR correction for multiple comparisons.

qRT-PCR. qRT-PCR was conducted in biological triplicates (n = 3), using the GoTaq qPCR Master Mix (Promega) and a 7300 Real-Time PCR system (ABI).

SP600125 JNK Inhibitor Treatment. For the treatment with SP600125 (A.G. Scientific), polyps (25 each) were incubated at a density of 1 polyp per milliliter in SP600125 diluted in 0.1% DMSO/*Hydra* medium in the dark for 24 h at 18 °C (34).

DNA Extraction and Sequencing of 16S rRNA Genes. For total DNA extraction, single polyps were subjected to the DNeasy Blood and Tissue kit (Qiagen). For sequencing of the bacterial 16s rRNA genes, the variable region 2 (V2) was amplified using the universal forward primer V2_B_Pyro_27F and the barcoded reverse primer V2_A_338R. A sample of each library was run on an Agilent Bioanalyzer before emulsion PCR and sequencing as recommended by Roche.

16S rRNA 454 Analysis. 16S rRNA amplicon sequence analysis was conducted using the Qiime 1.3 package (51). Fig. S4 shows the alpha-diversity curves for all samples ensuring sufficient sequencing depth.

***P. aeruginosa* Infection.** Experiments were carried out using the *P. aeruginosa* strain PA14. Single MyD88-knockdown and -control polyps (n = 24 each) were incubated in single wells of 24-well plates and incubated in 1 mL of the PA14 solution with an optical density (OD_{600}) of 0.1 containing 1.8×10^8 cells per milliliter. Polyps were screened daily and scored by following the criteria shown in Fig. 5.

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