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Review

How *Hydra* senses and destroys microbes

René Augustin, Sebastian Fraune, Thomas C.G. Bosch *

Zoological Institute, Christian-Albrechts-University Kiel, Olshausenstrasse 40, 24098 Kiel, Germany

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ABSTRACT

Molecular genetic evidence has revealed that the basic templates of innate immune sensors were laid down in ancient animals such as the cnidarian *Hydra*. Important functions of *Hydra*'s innate immune sensors and effectors include not only protection against pathogens but also controlling tissue-microbiota homeostasis. The deep evolutionary connections imply that invertebrate and mammalian immune pathways have evolved from a reduced number of common ancestral building blocks to their present configurations.

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1. Introduction

As sister group to the bilateria and one of the earliest branches in the animal tree of life (Fig. 1), Cnidaria are an important phylum potentially providing key insight to the ancestry and evolution of immune reactions. In sharp contrast to the morphological simplicity, cnidarians have attained remarkable diversity through modifications largely in colonial organization and life histories. The diverse phylum includes hydroids (Hydrozoa), sea anemones and corals (Anthozoa), box jellies (Cubozoa), and the true jellyfish (Scyphozoa). Since cnidarians possess most of the gene families found in bilaterians [1,2] and have retained many ancestral genes that have been lost in *Drosophila* and *Caenorhabditis elegans* [2,3], they provide insight into the contents of the "genetic tool kit" present in the cnidarian–bilaterian ancestor. The diversity in cnidarian life histories (solitary vs. colonial, sessile vs. pelagic) and habitats (marine vs. freshwater) raises several important issues relating to immunity [4,5]. First, to maintain tissue integrity, colonial forms have to rely on their capacity of self/non-self discrimination to rapidly detect approaching allogeneic cells as foreign and to eliminate them. Second, since a solid substratum is rare in most of the cnidarians habitats, solitary cnidarians immediately after larval settlement have to defend themselves against other settlers and fight for the substratum. Third, in the absence of protective layers, cnidarians must have effective mechanisms to defend against microbial pathogens. And forth, for some cnidarians, successful growth means to be able to distinguish between friends and foes, i.e., to allow symbionts to live within or on the epithelium and to close the doors for all other intruders.

In this review, we highlight recent advances in our understanding of innate immune recognition of molecular non-self structures in *Hydra* through membrane-bound receptors. Next we describe the host effectors used to destroy pathogens. Finally, we discuss the relation between tissue homeostasis and colonizing microbiota and the implications for evolutionary biology and translational research.

2. How *Hydra* senses microbes

Living in a variety of freshwater habitats, both ectodermal and endodermal epithelial surfaces of *Hydra* are continuously exposed to the environment. One litre of pond water include members from all domains of life (virus, bacteria, archaea, eukarya) and contain up to 2 billion bacteria and 10^{12} virus. Although the majority of microbes appear to exist in peaceful coexistence with *Hydra*, identification of the sensors to detect infection and to trigger an innate immune response is critical in understanding the interaction between hydra tissue and microbes. During the past few years much has been learned about the molecular basis of innate immune perception in *Hydra* [6–11]. In general, the ability of the host to defend against invading pathogens is to a large extent mediated by germ-line encoded receptors known as pattern recognition receptors (PRRs) [12,13]. Current concepts of the hydra innate immune system are largely based on two distinct classes of PRRs [5,14]. One type of receptors comprises Toll-like receptors (TLRs) that are membrane-resident and detect widely conserved microbe-associated molecular patterns on the cell surface such as lipopolysaccharides (LPS), flagellin or not yet defined components of *P. aeruginosa*. The second type of receptors comprises intracellular immune receptors of the nucleotide-binding and oligomerization domain (NOD)-like receptor (NLR) family.
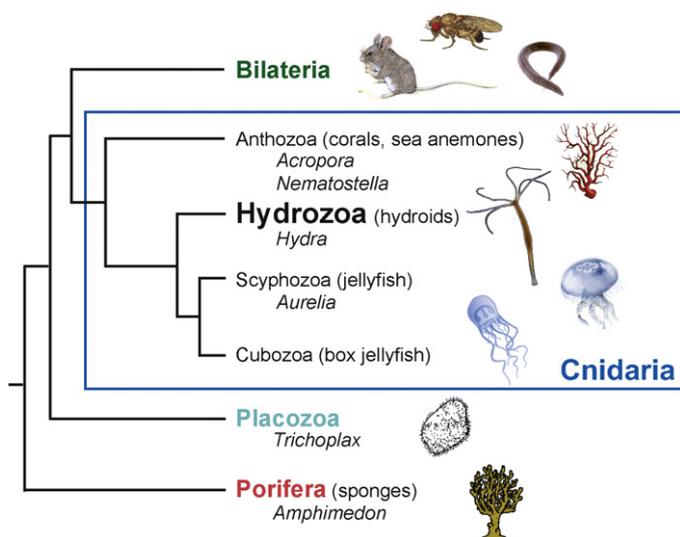
Fig. 1. Phylogeny of the "lower" metazoa. Cnidaria are the sister group to all bilateria. Within the Cnidaria, the Anthozoa (to which the coral *Acropora millepora* and the sea anemone *Nematostella vectensis* belong) are basal.

2.1. Sensing flagellin via intermolecular interaction of HyTRR-1 and HyLRR-2

Analysis of the *Hydra magnipapillata* genome including about 170,000 ESTs identified two genes whose inferred amino acid sequence contained a Toll/interleukin-1 receptor (TIR) domain, a transmembrane domain, and an extracellular domain lacking any specific domain structure [7,9]. We have termed these *Hydra* genes *Toll-receptor-related* 1 (*HyTRR*-1) and *Toll-receptor-related* 2 (*HyTRR*-2), respectively [7]. Most strikingly, neither HyTRR-1 nor HyTRR-2 contains leucine-rich repeats (LRRs) in its extracellular region. TBLASTN queries of the *Hydra magnipapillata* genome sequence and the corresponding EST datasets did not yield evidence for any additional HyTRR-1 and HyTRR-2 related genes. Thus, *Hydra* has no canonical Toll/TLR protein.

Cell surface molecules containing a leucine-rich-repeat (LRR) domain composed of 19–25 tandem copies of LRR motifs, 20–30 amino acids in length, are good candidates for immune receptors for two reasons. First, the LRR domain is present in the major classes of pathogen receptors in both plants and animals. And second, LRR domains are thought to be major determinants of effector recognition. This is supported by studies on several LRR-domain-containing protein families such as the human Toll-like receptor TLR4 [13]. Therefore, to study how HyTRR-1 and HyTRR-2 transmembrane receptors can function in the absence of extracellular LRRs, we screened the *Hydra* EST dataset as well as the *Hydra magnipapillata* genome for LRR-containing genes by using LRR sequences from human TLR4. We identified two full length transcripts that encode putative transmembrane proteins carrying TLR-related LRRs on the extracellular part [9]. We termed these genes HyLRR-1 and HyLRR-2, respectively. Since HyLRR-2 is structurally more similar to vertebrate TLRs than HyLRR-1, we next examined whether this transmembrane protein can recognize PAMPs such as flagellin.

To functionally assess the capacity of HyLRR-2 to elicit intracellular signals induced by PAMP recognition, and to test our hypothesis that in *Hydra* recognition of flagellin is mediated by an intermolecular interaction of HyLRR-2 as receptor and HyTRR-1 as signal transducer, we expressed the combination of *Hydra* HyLRR-2 lacking the TIR domain together with HyTRR-1 in human HEK293 cells. Since only transfected cells expressing both transmembrane proteins showed an increased NF-κB activation induced by flag-

ellin [9], in *Hydra* only the functional interaction of HyLRR-2 and HyTRR-1 mediates recognition of the bacterial PAMP flagellin (see Fig. 2). This receptor complex activation then triggers the innate immune response which involves the production of antimicrobial peptides. Most interestingly and supporting this view, in HyTRR-1 silenced polyps we observed a loss of antimicrobial activity in tissue extracts prepared from these animals which is partly due to a significant reduction of the expression of the antimicrobial peptide Periculin-1 [9]. As noted earlier [7,8], using HMM-based search methods in the anthozoan Cnidaria *Nematostella vectensis* uncovered a protein (NvTLR-1) clearly related to members of the Toll/TLR family containing LRRs proximal to the membrane in the extracellular part of the protein (see Fig. 2). Since within the phylum Cnidaria anthozoans are considered the ancestral group [15,16], the unusual situation in *Hydra* of sensing microbial patterns by functional interaction of a LRR-domain-containing transmembrane receptor (HyLRR-2) and a TIR-domain containing protein (HyTRR-1) appears not to reflect evolutionary ancestry but rather a *Hydra* specific development.

2.2. Sensing viruses

In addition to bacteria, *Hydra* is constantly exposed to myriads of viruses. Viruses enter cells either directly into the cytoplasm or through a pH-dependent process involving the endocytotic pathway [17]. How do *Hydra* polyps detect them? As described above, the innate immune system senses and destroys pathogens by recognizing families of molecules, PAMPs, that are conserved across broad classes of pathogens [13]. In the case of viruses, they can be detected if they produce dsRNA. DsRNA can be used to distinguish self from non-self because eukaryotes typically do not produce long stretches of dsRNA. However, many RNA viruses have double-stranded genomes, and even viruses with single-stranded genomes can produce dsRNA during replication, when viral RNA forms secondary structures, or when there are complementary sense and antisense replicative intermediates [18]. To test whether *Hydra* can sense the presence of viruses and to mimic the induction of an immune response by viral infection, we transferred dsRNA into intact polyps by electroporation followed by monitoring the expression of innate immunity genes [9]. Strikingly, the introduction of dsRNA caused a strong upregulation of expression of some but not all of the known antimicrobial peptide encoding genes in a dosage dependent manner [9]. While the strong response to the presence of "foreign" RNA may reflect an antiviral response, the pathway involved in hydra's defence against viruses remains to be identified. Since, however, *Hydra* encodes homologous to Dicer and Argonaute-containing effector genes [19], our observations [9] call for functional studies to assess whether the antiviral viRNA pathway, an important component of innate antiviral immunity in plants, fungi, invertebrates and vertebrates [18], is involved.

2.3. Hydra's innate-immune-sensing pathway is a shared character of most metazoans

Orthologs of key components of the signal transduction pathway downstream of Toll/TLR known from flies and vertebrates are present in *Hydra* indicating that the pathway described below, was part of the basic eumetazoan gene repertoire [7,8]. Upon activation by ligands such as flagellin [9], the cytoplasmic Toll-IL-1 receptor (TIR) motif of HyTRR-1 and HyTRR-2 recruits specific adaptor molecules such as myeloid differentiation factor 88 (MyD88) to propagate the signals received [7]. Upon stimulation, MyD88 recruits IL-1 receptor-associated serine/threonine kinase (IRAK) to TLRs through interaction of the death domains of both molecules. IRAK is activated by phosphorylation and then associates with TRAF6, leading to the activation of two distinct signalling path-
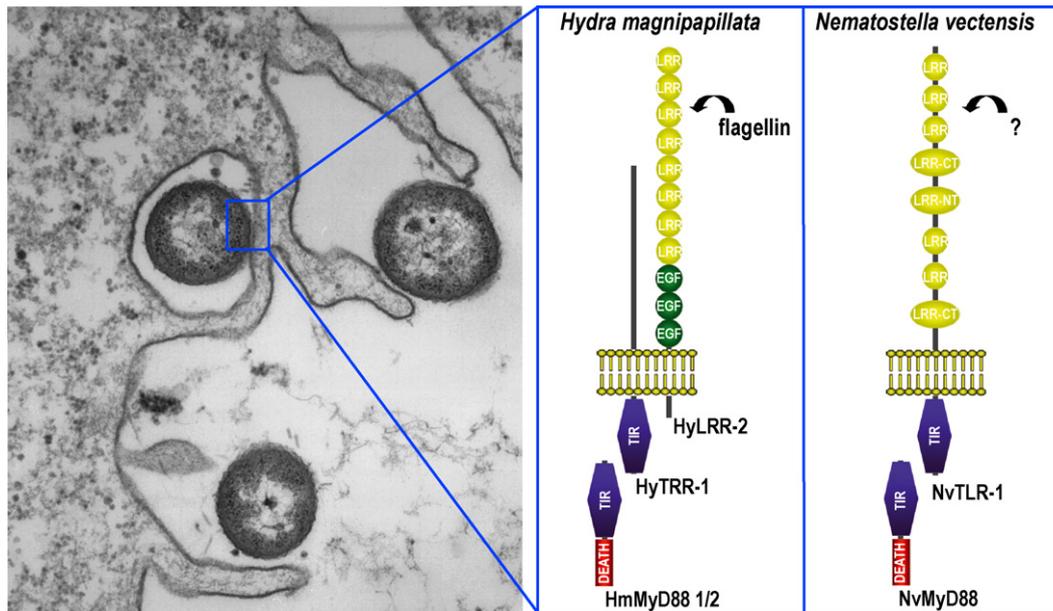
Fig. 2. Epithelial cells of the freshwater polyp *Hydra* sense bacterial pathogen associated molecular patterns (AMPs) via activation of pattern recognition receptors (PRRs) such as HyTRR-1 and HyLRR-2. While in *Hydra* leucine-rich-repeats (LRRs) and the canonical TIR domain are located on two separate proteins, the anthozoan *Nematostella vectensis* encodes a gene, NvTLR-1, which contains both the LRRs and the TIR domain within one protein.

ways, and finally to the activation of JNK [7] and NF-κB transcription factors which in turn induce the expression of hundreds if not thousands of genes. The presence of only two transmembrane receptors with a TIR domain together with the limited number of adaptor molecules raises the obvious question, how in *Hydra* signalling diversity can be obtained. For answering this question and uncovering the controlling components responsible for signal diversification downstream of the TLR response, next generation sequencing may be of help. Another puzzling fact is that there is not yet a single innate immunity receptor identified with an inhibitory role. All PRR receptors known so far in *Hydra* and other invertebrates are sensors which induce an innate immune response. Since mechanisms preventing unwanted responses (e.g. to commensals) and ensuring that the immune response is terminated in due course are critical in any attempt towards understanding how innate immune reactions are controlled in *Hydra*. Future studies should be focused on identifying negative control mechanisms.

3. How *Hydra* disarms microbial attackers

3.1. Antimicrobial peptides

Since the late 1960s and the work of H. Boman in Sweden [20] it has been known that invertebrates are highly resistant to microbial infections and that the molecules accounting for inducible antimicrobial activity are cationic, membrane-active peptides.

Due to a large number of antimicrobial peptides, the hydra epithelium is remarkably well equipped to prevent infectious agents from entering the body. Following pathogen invasion, there is an activation of an inducible defence system marked by an increased expression of genes encoding antimicrobial peptides. One of them is Hydramacin-1, a basic, 8 cysteines containing cationic 60 aa peptide with a calculated molecular mass of 6994 Da [9]. *Hydramacin*-1 mRNA is expressed exclusively in endodermal epithelium. LPS at low concentrations upregulates *Hydramacin*-1 expression, indicating that *Hydramacin*-1 is inducible by microbial products. Determination of the solution structure [10] revealed that the molecule possesses two short α-helices (10–14 and 27–33) at the N-terminus which are separated by a long flexible loop. The C-terminal region contains two β-strands (38–42 and 56–60) in an antiparallel arrangement separated by a long flexible loop. When used in liquid growth inhibition assays, recombinant Hydramacin-1 was capable of killing a large number of human gram negative pathogens, including the extended spectrum beta-lactamase (esbl) strains of *Escherichia coli*, *Klebsiella oxytoca* and *Klebsiella pneumoniae*, which are resistant to penicillin derivates.

When screening hydra tissue more systematically for the presence of novel antibiotics against particularly pernicious strains of bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), we recently discovered a novel antimicrobial peptide, arminin 1a. This peptide shows no sequence homology to any known AMP. Following proteolytic processing, the 31 amino acid residue long positively charged C-terminal part exhibits a potent and broad-spectrum activity against bacteria including multi-resistant human pathogenic bacteria such as methicillin-resistant *Staphylcoccus aureus* strains and vancomycin-resistant strains of *Enterococcus faecalis* und *Enterococcus faecium*. Ultrastructural observations indicate that this peptide kills bacteria by disruption of the bacterial cell wall. Because of the high activity against multi-resistant human pathogenic bacterial strains this peptide appears as promising template for a new class of antibiotics [21].

A third group of antimicrobial peptides includes Periculin-1, termed due to its rapid response to a wide variety of bacterial and tissue "danger" signals [9]. Analysis of the deduced amino acid sequence of Periculin-1 and the charge distribution within the molecule reveals an anionic N-terminal region and an 8 cysteine residues containing cationic C-terminal region. No identifiable orthologues were found in any database. Periculin-1 is localized in the endodermal epithelium as well as in some interstitial cells in the ectoderm. The cationic C-terminal region of Periculin-1 has strong bactericidal activity against *Bacillus megaterium* indicating that this peptide also is involved in the hydra host defence.

3.2. Kazal-type serine protease inhibitors

Serine-proteases are widely spread in many pathogenic bacteria, where they have critical functions related to colonization of

host tissue and evasion of host immune defences. Inhibiting one or more of these described processes will lead to growth inhibition or reduced pathogenesis, if not to death of the bacteria. It was not too surprising, therefore, to discover that the innate immune system in *Hydra* involves not only antimicrobial peptides but also serine protease inhibitors [11]. Gland cells located between endodermal epithelial cells produce a kazal-type serine protease inhibitor, Kazal-2 [11]. In liquid growth inhibition assays, native Kazal-2 protein has a MIC of 0.7–0.8 μM against *S. aureus*, indicating potent anti-staphylococcal activity.

3.3. Taxonomically restricted effectors

The discovery of an antimicrobial serine protease inhibitor which is used in addition and in combination to the numerous antibacterial peptides produced by epithelial cells demonstrates that early in metazoan evolution *Hydra* has developed an incredible effective and versatile chemical warfare system for host defence. This view captures an important feature: the *Hydra* epithelial defence system appears to employ both, evolutionary conserved signalling pathways such as the Toll/TLR pathway as well as novel, taxon-specific host defence-associated molecules including Hydramacin-1 and Periculin. Thus, although the common ancestor of bilaterians appeared to sense non-self by using receptors and signal transduction pathways that have been remarkably conserved all the way up to man, the precise nature of the pathogen-recognition system as well as details of the signalling pathways may been modified early during animal evolution and branched off into a variety of unique components in present day organisms.

4. Sensing microbes to maintain tissue homeostasis

Similar to the human intestine, the *Hydra* epithelium is colonized by a complex and dynamic community of microbes. Individuals from different *Hydra* species differ greatly in their microfauna. When analyzing individuals of, for example, *Hydra oligactis* and *Hydra vulgaris* from laboratory cultures, we discovered that individuals from both species have drastically different bacterial microbiota although so they were cultured under identical conditions [22]. Comparing the cultures maintained in the laboratory for >30 years and polyps directly isolated from the wild revealed surprising similarity in the microfauna [22]. The differences in the microbial communities between the species and the maintenance of specific microbial communities over long periods of time strongly indicate distinct selective pressures imposed on and within the hydra epithelium, and suggest that the *Hydra* genotype may structure the colonizing microbial communities. This compelling evidence for a complex dialog between an epithelial barrier and the residing microbiota at a basal level of evolution raises fundamental questions: what is the genetic basis of maintaining distinct community profiles in different *Hydra* species? Which functions do the resident bacteria have on hydra's physiology? Is the taxonomic diversity of microbiota related to functional diversity? How is diversity maintained? What are the host effectors that control colonizing bacteria? What are the host sensors which distinguish between pathogenic and beneficial (commensal/symbiotic) microbes? What are the microbial effectors that mediate this regulation?

There are no answers yet to all these questions. However, one old and some rather recent observations may provide informative insight. In experimental studies in axenic *Hydra* [23], these animals were shown to have severe developmental defects. Culturing them under sterile conditions resulted in reduced budding rate; after inoculation with bacteria from *Hydra* stock-culture normal budding was resumed. While there are several explanations for this effect, one attractive hypothesis is that *Hydra* needs bacte-

ria for normal tissue homeostasis. More recently, to test theories regarding the nature of host effectors mediating the host-microbe dialog and to gain insight into how epithelial homeostasis affects microbial community structure, we eliminated distinct cell types from the *Hydra magnipapillata* (strain sf-1) epithelium and asked whether this affects the composition of the colonizing microbiota [24]. Intriguingly, when the tissue was lacking neurons and gland cells, the bacterial composition changed drastically compared to normal tissue. Bacteria of the Bacteriodetes group showed a drastic increase in abundance while bacteria of the β-proteobacteria decreased in abundance. These data show that changes in hydra's epithelial homeostasis causes significant changes in the microbial community, implying direct interaction between epithelia and microbiota.

5. Conclusion—why *Hydra* matters

What general conclusions can be drawn from the molecular analysis of immune sensing pathways in *Hydra*? First, they are conserved to a remarkable degree. By demonstrating that TLR-related sensors can mediate a flagellin triggered immune response, the *Hydra* data provide a conceptual framework for the control of innate immune defences dependent on genome-encoded receptors of microbial ligands at the base of animal evolution. Thus, the basic templates of innate immunity were laid down in ancient simple animals such as cnidarians. Important areas that require further studies include the characterization of the signalling networks that influence hydra's defence response. Is, for example, a β-catenin-dependent defence pathway part of hydra's immune response as it is in *C. elegans* (F. Ausubel, pers. communication) and humans [25,26]? If so, how is β-catenin regulated during bacterial stimulation? Second, immune responses obviously are highly tuned by evolution. Since in *C. elegans* the only TLR present, TOL-1, is not a central component of the innate immune response, and since there is no evidence for MyD88 and NF-κB in *C. elegans*, it was proposed that TLR's role in immune signalling evolved recently [27]. However, the genomes of *N. vectensis* and *Hydra magnipapillata* as well as EST data from various other basal metazoan taxa clearly revealed the existence of TLRs, MyD88, and NF-kB in the eumetazoan ancestor before the cnidarian–bilaterian split [7,8]. The obvious conclusion, therefore, is that MyD88 and NF-κB genes have been lost during evolution in the *C. elegans* lineage. Third, the available data point to a level of complexity in hydra's immune response that has ecological implications. Our studies [7–9,11] identified not only conserved components of various signalling pathways, but it also showed that taxonomically related genes showing no homology to sequences in other species play important roles as antimicrobial effector molecules. Screening other animal taxa indicates that each animal species contains a significant number of such "orphan" genes encoding potent antimicrobial peptides [28]. We, therefore, suggested elsewhere [28] that taxonomically restricted host defence molecules contribute to disarm taxon-specific microbial attackers and to cope with specific environmental challenges. Last, the findings have medical implications. Analyzing the innate immune system in *Hydra* has uncovered some of the most powerful antimicrobial peptides we know so far from metazoans [9–11]. These highly active antimicrobial peptides now can be used in translational research to design novel antimicrobial compounds with broad-spectrum antimicrobial activity.

Acknowledgement

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