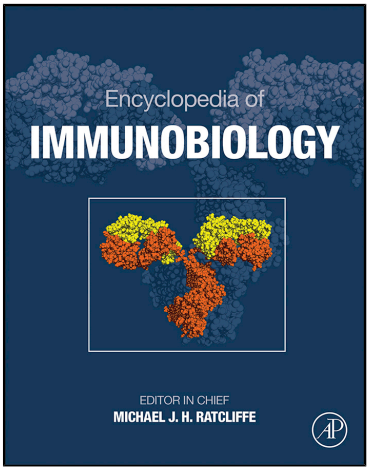

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Emergence of Immune System Components in Cnidarians

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Glossary

Antimicrobial peptides (AMPs) Small molecular weight proteins with broad spectrum antimicrobial activity against bacteria, viruses, and fungi. These evolutionarily conserved peptides are usually positively charged and have both a hydrophobic and hydrophilic side that enables the molecule to be soluble in aqueous environments yet also enter lipid-rich membranes. Once in a target microbial membrane, the peptide kills target cells through diverse mechanisms.

Damage-associated molecular pattern (DAMPs) A host-derived nonmicrobial factor that is released following tissue damage or necrosis and can activate the immune system in a similar way to microorganism-associated molecular patterns.

Microorganism-associated molecular patterns (MAMPs) Molecular motifs that are unique to microorganisms (both pathogens and nonpathogens) and are often recognized by components of the innate immune system.

Nuclear factor-κB pathway An evolutionarily conserved signaling cascade involving multiple protein complexes that control the transcription of immunity genes.

Pattern recognition receptors (PRRs) Eukaryotic proteins that bind to microorganism-associated molecular patterns, activating downstream signaling and, ultimately, innate immunity effector responses. Examples include Toll-like receptors, peptidoglycan recognition proteins, and Gram-negative bacteria-binding proteins.

Symbiont a microorganism that forms a specific, stable, and beneficial association with a particular host. Although this is now a commonly accepted definition, the original definition of symbionts, by Anton de Bary, included pathogenic, commensal, and mutualistic symbionts.

Toll-like receptors (TLRs) A diverse group of evolutionarily conserved pattern recognition receptors that are characterized by extracellular leucine-rich repeats and a cytoplasmic Toll–interleukin-1 receptor motif. Binding of microorganism-associated molecular patterns to TLRs often leads to downstream signaling via the nuclear factor-κB pathway or related pathways and results in the regulation of immune effectors.

Toll–interleukin-1 receptor domain (TIR domain) A cytosolic domain that is common to a diverse group of receptors, including Toll and Toll-like receptors.

Abstract

The phylum Cnidaria, the sister group to the Bilateria that branched off about 600 million years ago is pivotal for understanding the early evolution of immunity. Unlike vertebrates, Cnidaria lack classical antibody-based adaptive immunity, and key molecular and cellular players such as recombination-activating genes, B lymphocytes, and T lymphocytes are absent. Despite this limitation, for hundreds of millions of years, Cnidaria have mounted efficient defense responses to pathogens and are capable of discriminating them from other microorganisms. Cnidarians consist of only two cell layers; all innate immune responses are mediated by epithelial cells. The endodermal epithelium appears as a chemical barrier employing antimicrobial peptides, while the ectodermal epithelium is a physico-chemical barrier. Microbial recognition is mediated by pattern recognition receptors such as Toll- and Nod-like receptors. The aim of this article is to review the experimental evidence for innate immune reactions in Cnidaria and to give an overview of the interactions between the innate immune system of Cnidaria and the symbionts of these organisms. The data available support the hypothesis that the epithelium represents the ancient system of host defense and point to an origin of innate immunity in the Eumetazoan ancestor.

Cnidaria as Model Systems in Comparative Immunology

The phylum Cnidaria is comprised of remarkably diverse and ecologically significant taxa, such as the Anthozoa (reef-forming corals and sea anemones); swimming Scyphozoa (jellyfish); Cubozoa (box jellies); and Hydrozoa, a diverse group that includes all the freshwater cnidarians (such as the freshwater polyp Hydra) as well as many marine forms (Figure 1). Cnidarians originated early in the history of metazoan evolution, as indicated by fossil evidence (Ausich and Babcock, 1998; Cartwright et al., 2007; Chen et al., 2002; Hagadorn and Waggoner, 2000; and Han et al., 2010) and molecular phylogenies (Dunn et al., 2008; Peterson et al., 2004; Peterson et al., 2008; Hejnol et al., 2009; and Park et al., 2012). Their early phylogenetic position as well as the diversity

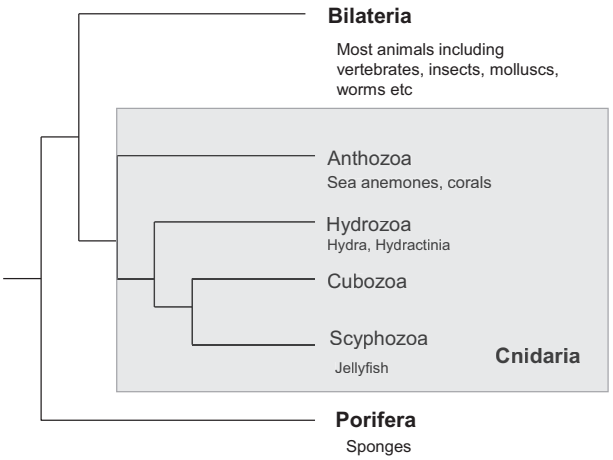

Figure 1 The early occurrence of *Cnidaria* on Earth. Members of different Cnidarian classes serve as models for studying the evolution of innate immunity and host–microbe interactions.

in cnidarian life histories (solitary vs colonial, sessile vs pelagic) and habitats (marine vs freshwater) raises several important issues relating to the origin of immunity. In the absence of protective layers such as cuticulae, Cnidarians must have effective mechanisms to defend against invading microbial pathogens. Moreover, successful growth means for many Cnidarian species to be able to distinguish between friends and foes, that is, to allow symbiotic algae to live within the endodermal epithelial cells and to close the doors for all other intruders. In addition, Cnidarians such as sea anemones and corals have extremely long life spans, were discovered to be more than 4200-years old (Roark et al., 2009) – and, therefore, must have some very effective immune systems in order to assure longevity. How do Cnidarians interact so successfully with their environment? How did the cross-kingdom communication systems between Cnidaria and microbes evolve?

Although cnidarians have a long history as model systems in comparative immunology (Campbell and Bibb, 1970; DuPasquier, 1974, 2001), the underlying molecular mechanisms only recently have been uncovered. Results from Cnidaria genome projects in *Nematostella* (Putnam et al., 2007), *Hydra* (Chapman et al., 2010), and *Acropora* (Shinzato et al., 2011) have revealed that the genomes of these early emerging animals with their seemingly simple body plans are unexpectedly complex. They possess most of the gene families found in bilaterians and have retained many ancestral genes that have been lost in *Drosophila* and *Caenorhabditis elegans* (Kusserow et al., 2005; Miller et al., 2005; Technau et al., 2005; Schwaiger et al., 2014). In addition, Cnidarians have evolved rich epithelial defense mechanisms to cope with the variety of immunological challenges they encounter. Characterization of the innate immune repertoire of Cnidarians is, therefore, of both fundamental and applied interest – it not only provides insights into the basic immunological 'tool kit' of the common ancestor of all animals, but is also likely to be important in understanding human barrier disorders by describing ancient mechanisms of host–microbial interactions and the resulting evolutionary selection processes.

Cnidaria Use a Variety of Molecular Pathways to Illicit Complex Immune Responses

Cnidarians are constantly exposed to microbes. Molecular analyses have revealed that the Cnidarian host uses a variety of molecular pathways to elicit complex immune responses (Miller et al., 2007; Hemmrich et al., 2007). When microbes are approaching the epithelium of a Cnidarian, they encounter extracellular germ line–encoded surface receptors that recognize microbe-associated molecular patterns (MAMPs) (Figure 2). Examples of MAMPs include lipopolysaccharides, peptidoglycan, flagellin, and microbial nucleic acids. Perception of a microorganism at the cell surface by Toll-like receptors (TLRs) initiates MAMP-triggered immunity.

Toll-Like Receptors

Our most complete understanding of the Cnidarian response to MAMPs relates to perception via the Toll/TLR pathway. TLRs are conserved throughout animal evolution (Pasare and Medzhitov,

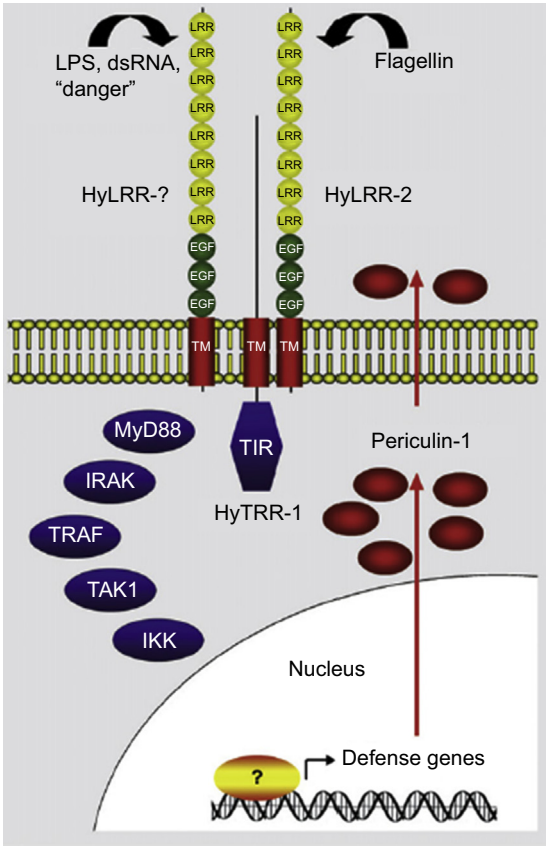

Figure 2 Molecular components of the pathways used by *Hydra* epithelial cells to elicit complex immune responses. LRR, leucine-rich repeat domain; HyTRR, Hydra Toll-receptor-related; TIR, Toll/IL-1R; TIR, Toll/IL-1R domain; MyD88, Myeloid differentiation primary response gene; IRAK, IL-1R-associated kinase family; TRAF, TNF receptor-associated factor protein family; TAK1, Transforming growth factor β (TGF-β)-activated kinase 1; IKK, inhibitor of nuclear factor kappa-B kinase.

2005; Leulier and Lemaitre, 2008). A canonical Toll/TLR pathway is present in representatives of the basal Cnidarian class Anthozoa, but neither a classic Toll/TLR receptor nor a conventional nuclear factor (NF)-κB could be identified in the hydrozoan *Hydra* (Hemmrich et al., 2007). There, two genes could be identified 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 (Bosch et al., 2009). We have termed these *Hydra* genes Toll-receptor-related 1 (HyTRR-1) and Toll-receptor-related 2 (HyTRR-2) respectively (Bosch et al., 2009). Most strikingly, neither HyTRR-1 nor HyTRR-2 contains leucine-rich repeats (LRRs) in its extracellular region. TLR function in *Hydra* is realized by the interaction of a LRR-domain-containing protein with a TIR-domain-containing protein lacking LRRs (Bosch et al., 2009). This receptor complex activation then triggers the innate immune response which involves the production of species-specific antimicrobial peptides (AMPs) such as periculin (Bosch et al., 2009).

NOD-Like Receptors

Microbes evading membrane-bound TLR receptors or specifically invading epithelial cells encounter another line of defense inside the host cell, the NOD-like receptors (NLRs) (Rosenstiel et al., 2009). Homologs of the NLRs (e.g., R genes) have been discovered throughout the plant and animal kingdoms, including cnidarians (Lange et al., 2011), the sea urchin (Sodergren et al., 2006), and the zebrafish (Laing et al., 2008). The high evolutionary conservation of the NLRs underlines their significance in host defense. Similar to sea urchin which has at least 203 identified putative NLRs (Sodergren et al., 2006), cnidarians such as *Nematostella* and *Hydra* have hundreds of NLRs. In a systematic survey of the NACHT (NAIP (neuronal apoptosis inhibitor protein), C2TA (MHC class 2 transcription activator), HET-E (incompatibility locus protein from Podospora anserina) and TP1 (telomerase-associated protein)) and NB-ARC (Nucleotide-binding fold with APAF-1 (apoptotic protease-activating factor-1), R proteins, and CED-4 (Caenorhabditis elegans death-4 protein)) domain genes in existing expressed sequence tag and genome data sets, we observed that the *Hydra* genome has 290 putative nucleotide binding domain (NBD) loci falling into two large groups: 130 of the NACHT domain type and 160 of the NB-ARC domain type (Lange et al., 2011). The *Hydra* and Cnidaria NACHT/NB-ARC complements include novel combinations of domains. The number of one specific type (NB-ARC and tetratricopeptide repeat containing) in *Hydra* is particularly large (Lange et al., 2011). Thus, as in vertebrates, a broad repertoire of NLRs seems to be involved in the recognition of conserved microbial components in *Hydra*. While these observations clearly show that NLRs are ancient genes, their immune function and significance in host–microbe interactions remains to be determined. The main localization of NLR expression in *Hydra* is confined to the endodermal layer, where NLRs could play a crucial role in maintaining a balance with the commensals/symbionts of the gastric cavity. The advent of novel sequencing techniques will allow for the first time the analysis of the diversification of this class of genes in organisms at the base of animal evolution and will contribute to understand evolutionary pressures that have shaped genetic diversity profiles of immune genes.

MyD88-Mediated Signal Transduction

Innate immune reactions in cnidarians are initiated upon recognition of conserved microbial features (MAMPs) by cell surface receptors and can originate from the cytoplasmic TIR domains of TLRs (Hemmrich et al., 2007; Bosch et al., 2009). For further signal transduction, a conserved adaptor, MyD88, has been identified in *Nematostella* as well as in *Hydra* (Hemmrich et al., 2007), which appears as an essential component for the activation of innate immunity. MyD88 possesses the TIR domain in the C-terminal portion, and a death domain in the N-terminal portion. MyD88 associates with the TIR domain of TLRs. 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 signaling pathways, and finally to the activation of c-Jun N-terminal kinase (JNK) and NF-κB (Hemmrich et al., 2007).

To address the function of MyD88 directly, a MyD88 loss-of-function study in *Hydra vulgaris* (AEP) using a stable transgenic *Hydra* line with reduced expression level of MyD88 was combined with microarray analyses to identify effector genes downstream of the TLR-signaling cascade (Franzenburg et al., 2012). In parallel, the gene expression profile of germfree animals was examined to directly investigate the connection between TLR-signaling and bacterial recognition. More than 75% of the MyD88-responsive transcripts appeared to be also altered in germfree polyps, indicating that expression of these genes is responsive to bacterial recognition (Franzenburg et al., 2012). These observations show that recognition of bacteria is an ancestral function of TLR-signaling, which is important for the selection and maintenance of resident microbiota, and contributes to defense against bacterial pathogens.

Cnidarian Defense Molecules

Antimicrobial Peptides

Following microbe recognition and invasion, there is an activation of an inducible defense system marked by an increased expression of highly active AMPs. AMPs are known as prominent effector molecules of the innate immune system in vertebrates and invertebrates, where they act by disrupting the structure or function of the microbial cell membranes (Zasloff, 2002). They are gene-encoded small peptides, most consisting of less than 50 amino acids, are amphipathic, and typically carry an overall positive charge. They have been found in every species examined to date and over 900 have been identified across species. The peptides show a broad spectrum of antimicrobial activity. The primary sources of Cnidarian AMPs are epithelial cells. In *Hydra magnipapillata*, a multitude of AMPs have been found including Hydramacin, Periculin (Bosch et al., 2009; Jung et al., 2009), and Arminin (Augustin et al., 2009a; Franzenburg et al., 2013). Which role do they play in the *Hydra* host?

In most Cnidarians, embryos develop outside the mother and are directly exposed to environment which is full of potential pathogens. Surprisingly, nothing is known so far how cnidarian embryos are protected when released in an environment loaded with potential pathogens. This ignorance seems to apply not only to cnidarians but to all animals

including man: "Considering the importance of the fetus to our survival as a species, it is surprising that we know so little about what protects it from microbial assault" (Zasloff, 2003). It was, therefore, interesting to discover an AMP in *Hydra* which is expressed exclusively in females to provide protection of the early embryo (Fraune et al., 2010, 2011). The protein, termed Periculin-1, is maternally expressed in female germ line cells. After midblastula transition, the expression of Periculin-1 in the nurse cells diminishes. In contrast to Periculin-1, other members of the peptide family such as Periculin-2 are not expressed during oogenesis and early stages of embryogenesis. After midblastula transition, the blastomeres of the outer epithelial layer start to express Periculin-2. Immune staining reveals that after fertilization the Periculin peptides are secreted to the surface of the embryo. During embryogenesis, all translated Periculin peptides seem to be secreted directly to the epithelium and not stored in vesicles anymore. Thus, the *Hydra* embryo appears to be encoated with a thick layer of highly active antibacterial peptides.

While nothing is known yet from coral embryos, there is some evidence that adult corals depend on their antimicrobial mucus to remove and lyse bacterial invaders (Phillips, 1963; Bigger and Hildemann, 1982). In the jellyfish, *Aurelia aurita*, one of the most common and widely recognized types of jellyfish found near the coasts in the Atlantic, Arctic, and Pacific Oceans, a novel AMP, Aurelin, was purified from the mesoglea (Ovchinnikova et al., 2006). Aurelin, a 40-residue AMP with a molecular mass of 4.3 kDa was shown to exhibit activity against Gram-positive (*Listeria monocytogenes*, strain EGD) and Gram-negative (*Escherichia coli*, strain ML-35p) bacteria. Its primary structure, including six cysteines forming three disulfide bonds, as well as the primary structure of its molecular precursor, consisting of a canonical signal peptide, anionic propiece and a mature cationic part, resembles the common structural features of animal defensins (Ovchinnikova et al., 2006). However, the distribution of cysteine residues makes it also similar to K^+ channel-blocking toxins of sea anemones. Although aligning the aurelin and sea anemone toxin sequences shows rather moderate homology, Ovchinnikova et al. (2006) suggest that aurelin could be functionally related to the Stichodactyla toxin (ShK)-like toxins known to block voltage-gated potassium channels. Both the expression patterns and the *in vivo* function of Aurelin remain to be clarified.

In light of the risk that antimicrobial resistance poses to public health, it is interesting to note that recombinant Hydramacin-1 is capable of killing a large number of human Gram-negative pathogens, including the extended spectrum beta-lactamase (esbl) strains of *E. coli*, *Klebsiella oxytoca*, and *Klebsiella pneumoniae*, which are resistant to penicillin derivates, but also highly active against some Gram-positive strains such as *Bacillus megaterium* ATCC14581 (Bosch et al., 2009; Jung et al., 2009). Cnidarian AMPs such as Hydramacin-1 may, therefore, make an interesting leading structure for design of a novel generation of antibiotics.

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 defenses, acquisition of nutrients for growth and proliferation, facilitation of dissemination, or tissue damage during infection. Inhibiting one or more of these described processes will lead to growth inhibition or reduced pathogenesis, if not to death of the bacteria. It is not too surprising, therefore, that the innate immune system in cnidarians involves not only AMPs but also serine protease inhibitors. When biochemically analyzing *H. magnipapillata* tissue for antistaphylococcal activity we discovered, unexpectedly, that not only epithelial cells but also gland cells are critically involved in *Hydra*'s innate host defense by producing a kazal-type serine protease inhibitor, kazal-2 (Augustin et al., 2009b). In liquid growth inhibition assays, native Kazal-2 protein has a minimal inhibitory concentration of 0.7–0.8 μM against *Staphylococcus aureus*, indicating potent antistaphylococcal activity. Furthermore, we could demonstrate that the separated kazal-2 domains exhibit an inhibitory activity for the serine proteases trypsin and subtilisin. The significantly stronger inhibitory activity against subtilisin, a bacterial protease, may indicate that kazal-2's activity directed against *S. aureus* is up to the inhibition of a bacterial-specific serine protease (Augustin et al., 2009b). Recombinant kazal-2 domains not only inhibit growth of *S. aureus* but also kill the microbes. The discovery of an antimicrobial serine protease inhibitor in *Hydra* not only sheds new light on the mechanisms of host defense early in metazoan evolution. In light of the already mentioned increasing prevalence of antibiotic-resistant microbes and the undisputed need for new antibiotics, kazal-2-type protease inhibitors – similar to novel AMPs such as Arminin – might also constitute a new class of highly effective antibiotics, suitable for optimization into new antistaphylococcal compounds.

The Apical Epithelial Cell Surface

A characteristic feature of most animal epithelial cells is a dense carbohydrate-rich layer protruding up to 500 nm from the apical cell surface, referred to as the glycocalyx. The dense layer of transmembrane glycoproteins, glycolipids, and proteoglycans excludes large molecules and organisms from having direct access to the cell surface by steric hindrance, whereas smaller molecules might pass through. Thus, similar to the mucus layer in vertebrates (Johansson et al., 2008), the *Hydra* glycocalyx appears to represent the first line of contact between the cell and other cells or bacteria and viruses providing a physical and chemical barrier against the external environment and also representing the main site of interaction between the host and its associated commensal microbial community.

Visualization of the glycocalyx in Cnidarians is not a trivial task. Since conventional chemical fixation procedures fail to preserve this extracellular structure, it has been ignored and overlooked for a long time. Pilot electron microscopic analysis of *Hydra*'s ectodermal epithelium revealed a smooth uninterrupted layer covering the whole body with exception of the lowest part of the foot (Bosch et al., 2009). Using high-pressure freezing/freeze substitution, Holstein et al. (2010) resolved the ultrastructure of *Hydra*'s glycocalyx, which appears to consist of five distinct layers (Holstein et al., 2010). Although the overall structure was termed glycocalyx, at least the outer layer appears to be not membrane bound, but resembling a mucuslike gel. First insights into the molecular

composition of *Hydra*'s glycocalyx come from work of Böttger et al. (2012). They report that the glycocalyx, here termed 'cuticle layer,' contains glycosaminoglycans such as chondroitin and chondroitin-6-sulfate. While Böttger et al. did not identify high-molecular-weight glycoproteins such as mucins capable of forming the complex meshlike structure of *Hydra*'s glycocalyx, in the cnidarian *Nematostella vectensis* at least five gel-forming mucins were shown to be present indicating an early origin of mucins in metazoan evolution (Lang et al., 2007). Taken together, there is convincing evidence that the glycocalyx in *Hydra* is at least partly composed of species-specific proteins. In addition, the strong Periodic acid–Schiff reactivity of all glycocalyx layers as well as the large secretory vesicles under the apical surface indicates a high content of not yet identified carbohydrate moieties (Böttger et al., 2012). It is possible that the glycans provide attachment sides and nutrients for bacteria; and that the glycocalyx is an important interface for mediating host–microbe interactions in Cnidarians. Preliminary studies in support of this view indicate that *Hydra*'s ectodermal surface is densely colonized by a distinct bacterial community (Fraune et al., 2014). Interestingly, only the outer mucus layer of *Hydra*'s glycocalyx is colonized by bacteria, whereas the dense inner layers seem to remain sterile providing a barrier to both the commensal microorganisms and potential pathogens. This principle of separation into a habitat for symbiotic bacteria and a physical barrier preventing excessive immune activation was previously described for the mucosal surface of the mammalian colon (Johansson et al., 2008) and apparently is a conserved feature which can be traced back to the ancestral metazoan *Hydra*.

Dynamic Relationships between Cnidarians and Microorganisms

Cnidarian-associated microbial communities, including protists, bacteria, archaea, and viruses, are important components of the Cnidarian holobiont that influence the health of, for example, corals and coral reef ecosystems (Bosch and McFall-Ngai, 2011; Kimes et al., 2013). Evidence suggests that the composition of these microbial communities is affected by numerous parameters; however, little is known about the confluence of these ecological and temporal effects. Increasing evidence suggests that the innate immune system in Cnidarians not only is used to destroy harmful microorganisms but also has a role in structuring tissue-associated microbial communities which may contribute to the animal's health (Franzenburg et al., 2013; Bosch, 2013). Exploring the structure and function of the microbiome is thus a major objective of current research in Cnidaria. The coral probiotic hypothesis (Reshef et al., 2006; Rosenberg et al., 2007) proposes that a dynamic relationship exists between symbiotic microorganisms and corals at different environmental conditions that selects for the most advantageous coral holobiont in the context of the prevailing conditions.

Coral-Associated Microbial Communities

Coral tissue and coral mucus support a diverse community of microbes (Nissimov et al., 2009; Ainsworth et al., 2010; Porporato et al., 2013; Kimes et al., 2013; Bourne et al., 2013; Roder et al., 2014). Phylogenetic analyses as well as molecular techniques including metagenomic approaches have demonstrated that coral microbial communities are highly complex, spatially and temporally stable, and most likely have multiple roles in the physiological function of the coral host (Rohwer et al., 2001; Ainsworth et al., 2006; Breitbart et al., 2005; Yokouchi et al., 2006; Dinsdale et al., 2008a,b; Littman et al., 2009). Observations in *Oculina patagonica* indicate (Koren and Rosenberg, 2006) that microbial communities associated with tissue and mucus of corals can differ between seasons. Disturbances in the balance between corals and colonizing microbiota appear to facilitate emergence of coral disease (Ritchie, 2006; Sharp and Ritchie, 2012). Metagenomic approaches underline this view by showing that, for example, in *Porites compressa*, the contribution of a just a few members of a microbial community can profoundly shift the health status of the coral holobiont (Thurber et al., 2009). Relative changes in taxonomy demonstrated that coral-associated microbiota (archaea, bacteria, protists) shifted from a healthy-associated coral community (e.g., Cyanobacteria, Proteobacteria, and the zooxanthellae Symbiodinium) to a community (e.g., Bacteroidetes, Fusobacteria, and Fungi) of microbes often found on diseased corals (Thurber et al., 2009). Thus, a homeostatic balance of cnidarians with their colonizing microbiota may well explain the evolutionary success of corals. Environmental stressors such as increased sea surface temperatures and subsequent bleaching are accompanied by drastic shifts in the microbial community factors including changes in the production of antimicrobial compounds (Ritchie, 2006; Bourne et al., 2008; La Rivière et al., 2013; Tout et al., 2014). These results contribute to the idea that coral bleaching is caused by the disruption of the coral-microbiota balance, that is, 'the microbial hypothesis of coral bleaching' (Rosenberg et al., 2009).

In sum, there is general agreement that the coral holobiont – the polyp which coexists in a mutualistic relationship with unicellular algae, zooxanthellae, and a complex microbial community – is of complexity which just now is getting to be appreciated (Ellner et al., 2007; Harvell et al., 2007). Up to now, however, the many roles of coral-associated microbial communities are not uncovered; and there is little if any understanding about the molecular mechanisms underlying pathogen defense and host–microbe interactions in corals (Kvennefors et al., 2008; Ainsworth et al., 2010).

Tissue-Associated Microbial Communities in *Hydra*

Observations in the experimentally facile 'model' cnidarian *Hydra* may serve as experimental substitute for studies on innate immunity in corals. As a project along these lines, to test theories regarding the assembly of tissue-associated microbial communities and to gain insight into the function of the resident microbiome of cnidarian epithelia, we have started to characterize the microbiome of different species of *Hydra* (Fraune and Bosch, 2007). When analyzing individuals of *Hydra oligactis* and *H. vulgaris* from long-term laboratory cultures, we discovered that individuals from both species differ greatly in their bacterial microbiota although they were cultured under identical conditions. Comparing the cultures

maintained in the laboratory for >30 years and polyps directly isolated from the wild revealed surprising similarity in the associated microfauna. The significant differences in the microbial communities between the two species and the maintenance of specific microbial communities over long periods of time strongly indicate distinct selective pressures imposed on and within the epithelium. Our analysis suggests that the *Hydra* epithelium actively selects and shapes its own microbial community (Fraune and Bosch, 2007). The fact that individuals from different species differ greatly in their microbiota and that individuals living in the wild are colonized by microbiota similar to that in individuals grown in the lab, point to the maintenance of specific microbial communities over long periods of time (Fraune and Bosch, 2007). Since it is evident that there are pressures exerted both by the Cnidarian host and the external environment to mold these ecosystems, the question arises as to which forces are shaping the colonizing microbial composition.

Rethinking the Role of AMPs as Host-Derived Regulators of the Microbiota

AMPs are known as prominent effector molecules of the innate immune system. Recent studies in *Hydra* provide compelling evidence that AMPs are not only involved in pathogen defense, but also affect the composition or behavior of the commensal microbiota. To date, three families of potent AMPs have been identified in *Hydra*: the hydramacin, periculin, and arminin families of peptides (Bosch et al., 2009; Augustin et al., 2009a). To investigate whether the ectotopic expression of a single AMP may affect the number and composition of the colonizing microbiota at the ectodermal epithelial surface, we generated transgenic *Hydra* expressing the AMP periculin1a in ectoderm epithelial cells (Fraune et al., 2010). Comparing the bacterial load of these transgenic polyps with that of wild-type control polyps revealed not only a significantly lower bacterial load in transgenic polyps overexpressing Periculin1a but also drastic changes in the bacterial community structure. Analyzing the identity of the colonizing bacteria showed that the dominant β-Proteobacteria decreased in number, whereas α-Proteobacteria were more prevalent cells (Fraune et al., 2010). Thus, overexpression of Periculin causes not only a decrease in the number of associated bacteria but also a changed bacterial composition. With the transgenic polyps overexpressing periculin, we apparently have created a new holobiont that is different from all investigated *Hydra* species (Fraune et al., 2010). From these results, we assume that specific associations between hosts and bacteria are a result of bacterial adaptation to different repertoires on AMPs in different host species.

Species-specific variability and constitutive high-level expression made in particular the arminin peptide family, an excellent candidate for investigating the role of AMPs in shaping the host-specific microbiota of *Hydra*. To examine the impact of arminin function on the resident microbial population in *Hydra*, and to broadly interfere with the host's arminin expression, we generated transgenic *H. vulgaris* (*AEP*) polyps expressing a hairpin cassette containing *arminin* antisense and sense sequences fused to a reporter gene (Franzenburg et al., 2013). The resulting dsRNA triggers the RNAi machinery, which led to a 97% decrease in the endogenous arminin transcript. Tissue extract of arminin knockdown polyps showed a 50% decrease in bacteriocidal activity. For functional analysis, germfree control and arminin knockdown polyps were generated and subsequently recolonized by foreign bacterial consortia provided by cocultivation with other *Hydra* species, that is, *H. oligactis* or *Hydra viridissima*. 454 pyrosequencing revealed (Franzenburg et al., 2013) that arminin-deficient *H. vulgaris* (*AEP*) polyps have a decreased ability to select suitable bacterial partners from a pool of foreign potential colonizers as they are colonized differently than control polyps, which select for bacterial types partially resembling their native microbiota. These findings suggest that AMPs shape the stable associated microbiota, acting as host-derived regulators of microbial diversity rather than nonspecific bacteriocides. Evolutionary changes in the AMP repertoire of host species, therefore, are expected to lead to changes in the composition of the associated bacterial community. Future efforts will be directed toward analyzing the fitness-related performance of this new phenotype under different environmental conditions.

We conclude from these results that AMPs are host-derived regulators of microbial diversity rather than nonspecific bacteriocides. Because the genes of the arminin family have no identifiable orthologs in other species outside the genus *Hydra*, they represent taxonomically restricted genes. As discussed elsewhere, each animal species contains a significant number of such 'orphan' genes encoding potent AMPs, which are involved in defense of taxon-specific microbial pathogens as well as in shaping the species-specific commensal microbiota (Khalturin et al., 2009). Evolutionary changes in the AMP repertoire of host species, therefore, are expected to lead to changes in the composition of the associated bacterial community. From these results, we assume that specific associations between hosts and bacteria are a result of bacterial adaptation to different repertoires on AMPs in different host species.

How do Cnidarians Distinguish between Friends and Foes?

Cnidarians lack conventional antibody-based immunity but harbor large numbers of beneficial microorganisms. How are pathogens eliminated and long-lived and specific symbiotic associations established and maintained?

Insights from Corals

There is increasing evidence that the inter-partner signaling pathways involved during the onset of symbiosis are homologous to those driving animal host–pathogenic microbe interactions. In both types of relationships, there are common principles and beside released signals, pathogen/symbiont surface molecules most likely are major determinants for an interaction with the host. The cellular and molecular interactions underlying this interaction with particular emphasis on the establishment, maintenance, and breakdown of these cooperative partnerships are currently investigated in several coral species, including the Hawaiian stony coral *Fungia scutaria*, the tropical sea anemone *Aiptasia pallida*, a temperate sea

anemone found on the Oregon coast, *Anthopleura elegantissima*, and a Red Sea soft coral, *Heteroxenia fuscescens*. Most species must acquire symbionts anew with each generation and therefore must engage in a complex recognition and specificity process that results in the establishment of a stable symbiosis. To identify genes that initiate, regulate, and maintain, this host–symbiont interaction, the Weis lab at Oregon State University has conducted a comparative transcriptome analysis in the host sea anemone *A. elegantissima* using a cDNA microarray platform (Rodriguez-Lanetty et al., 2006). Although statistically significant differences in host gene expression profiles were detected between *A. elegantissima* in a symbiotic and nonsymbiotic state, the group of genes whose expression is altered, is diverse, suggesting that the molecular regulation of the symbiosis is governed by changes in multiple cellular processes (Rodriguez-Lanetty et al., 2006). To search for symbiosis-specific proteins during the natural onset of symbiosis in early host ontogeny, Barneah et al. (2006) used two-dimensional polyacrylamide gel electrophoresis and compared patterns of proteins synthesized in symbiotic and aposymbiotic primary polyps of the Red Sea soft coral *H. fuscescens* in the initiation phase, in which the partners interact for the first time. Surprisingly, there were no changes detectable in the host proteome as a function of symbiotic state (Barneah et al., 2006). To examine molecular mechanisms during initiation and establishment of coral–algae symbioses, Medina and colleagues (Voolstra et al., 2009) followed gene expression profiles of coral larvae of *Acropora palmata* and *Montastraea faveolata* after exposure to *Symbiodinium* strains that differed in their ability to establish symbioses. Most interestingly, the coral host transcriptome remained almost unchanged during infection by symbionts that were able to establish symbioses. It was, however, massively altered during infection by symbionts that were not able to establish symbioses. Taken together, both the transcriptome (Rodriguez-Lanetty et al., 2006; Voolstra et al., 2009) and proteomics study (Barneah et al., 2006) do not support the existence of symbiosis-specific host genes involved in controlling and regulating the symbiosis. Instead, it appears that regulation of the immune system, apoptosis, and proteolysis may be the key players in the regulation of early coral–algae symbioses. "Successful coral–algal symbioses depend mainly on the symbionts' ability to enter the host in a stealth manner" (cited from Voolstra et al., 2009). If this scenario turns out to be true, then several important questions await to be answered: How does the symbiont manage to survive within the host cell? Which molecules are released by the symbiont that participate in host–symbiont recognition and modification of the immune response? Which host pathways are responding to the molecules released by the symbiont? How is the delicate balance between successful metabolism and replication of the symbiont and survival of the host maintained on a long-term basis?

Insights from *Hydra*

Observations in *Hydra* may teach us another lesson. *Hydra* can mount a powerful immune response in the absence of motile phagocytic cells (Bosch et al., 2009). This response is contingent on two independent components: the presence of bacterial flagellin (a classic example of a MAMP) and the secretion of a tissue damage or danger signal, known as a damage-associated molecular pattern (DAMP). As outlined above, in *Hydra*, two proteins are required for MAMP recognition and signal transduction: a *Hydra* TIR domain-containing protein (HyTRR) and *Hydra* LRR protein 2 (HyLRR-2), containing a transmembrane domain. HyLRR-2 interacts with the HyTRR protein in response to flagellin. When exposed to a DAMP, such as monosodium urate (which is released from injured cells), an even stronger induction of defense genes is observed. For example, there is increased production of AMPs such as periculin-1 (Bosch et al., 2009). Nyholm and Graf propose (2012) that the requirement for both a MAMP and a DAMP to launch an effective immune response constitutes a mechanism that allows *Hydra* to distinguish pathogens from symbionts. Further studies will show whether in *Hydra*, this ability to integrate the two signals (MAMPs and DAMPs) may contribute to shaping of the microbiota. In line with the conclusions drawn from coral studies, and based on the fact that AMPs such as periculins (Fraune et al., 2010) and arminins (Franzenburg et al., 2013) are instrumental in establishing the symbiotic bacterial community in *Hydra*, the innate immune system seems crucial for both the establishment and the long-term maintenance of host-associated bacterial communities in Cnidarians.

Concluding Remarks

Cnidaria share deep evolutionary connections with all animals including humans. The combined evidence suggests that, in addition to the classical mechanisms of responding to pathogens, Cnidaria are capable of using the innate immune system to interact with symbionts in order to promote the establishment and maintenance of symbiotic associations. Although the mechanisms behind this ability are not fully elucidated, the use of Cnidarian models gives researchers the opportunity to discover additional regulatory pathways that are likely to be ancient and might reveal novel immunological mechanisms that confer high microbial specificity. Microbial recognition by Cnidarian hosts is at least partially mediated by PRRs such as TLRs and NLRs that are conserved among metazoans. A likely crucial role for the ancient MyD88/NF-κB pathway has been implied. Thus, these apparently simple animals will have important roles in understanding the evolutionary foundations of epithelial-based innate immunity.

Future Prospects

Thinking about future approaches and trends crucial to advance this exciting field I discuss here four aspects.

First, since many human diseases have ancient genetic origins, Cnidaria in the future may emerge as attractive and simple model systems to understand human barrier disorders by describing ancient mechanisms of host–microbial interactions and the resulting evolutionary selection processes or advantages. The general importance of these principles also for human health has become increasingly clear, as it was demonstrated that genetic variants in phylogenetically ancient innate immune genes are involved in the etiology of

emerging chronic inflammatory diseases of epithelial barrier organs (Schreiber et al., 2005). Moreover, searching for the evolutionary origin of human disease-causing genes and characterizing the variation in such genes under known evolutionary pressures has demonstrated that almost none of the disease-associated genes emerged after the origin of mammals, although many of the diseases do only occur in humans (Domazet-Loso et al., 2007; Domazet-Loso and Tautz, 2008).

Second, to unravel the complex interplay of host–microbiota signaling cascades that are also relevant to human barrier organs and its microbiota, future research in Cnidarian immunity should focus on examining an even more diverse array of Cnidaria species for the identification of evolutionarily conserved mechanisms.

Third, bioinformatic analyses are challenging, especially when the identified sequences are not functionally characterized or when the characterized gene comes from a distantly related organism. Thus, the use of omics in parallel with a model systems approach which allows functional studies and detailed study of the cellular and molecular processes underlying host–pathogen/symbiont interaction will be crucial to advance this exciting field. The freshwater polyp (member of the Cnidarian class Hydrozoa) has been developed in that direction and offers many interesting insights. This situation is different in different phylogenetic lineages such as the reef-forming corals (Anthozoa), the swimming Scyphozoa (jellyfish), and the Cubozoa (box jellies) for which experimental models revealing clues about how the innate immune system functions are missing (Weis et al., 2008). To overcome these inherent difficulties at least in the Anthozoan class, the small sea anemone Aiptasia recently has received much attention as a model system for making effective progress in understanding coral cell biology (Lehnert et al., 2012). Aiptasia is normally symbiotic with dinoflagellates closely related to those found in corals (Lajeunesse et al., 2012; Lehnert et al., 2012) and offers many experimental advantages (Weis et al., 2008). First observations are promising and indicate that Aiptasia indeed may provide us with novel insights in the mechanisms controlling coral–symbiont interaction (Hambleton et al., 2014; Lehnert et al., 2014).

Fourth, more effort should also be put into understanding how interactions with the microbiota influence not only the development of the immune system but also affect the fitness of the corresponding Cnidarian host.

In sum, a comparative approach in various species of Cnidaria will reveal a much more complex picture of the role of innate immune system in fostering symbiotic relationships with microorganisms than currently available.

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See also: **Anatomy and Microanatomy of the Immune System:** Anatomy and Function of the Gut Immune System; Roles of Chemokines in Immune Cell Trafficking to Lymphoid Tissues; Skin Immune System: Microanatomy. **Cytokines and Their Receptors:** Inflammasomes. **Phylogeny of the Immune System:** Defense Mechanisms in Plants; Immunity in Insects; Immunity in Molluscs; Molecular Evolution of Defense Pathways in Sponges: Self–Self-recognition and Fight against the Nonself; The Immune System of Crustaceans; The Immune System of Echinoderms. **Signal Transduction:** Adapter Molecules in Immune Receptor Signaling.

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