François Seneca¹,³, David Davtian¹*, Laurent Boyer²,³, and Dorota Czerucka¹,³

¹Centre Scientifique de Monaco, 8 Quai Antoine 1ᵉʳ, 98000 Monaco.

²Université Côte d'Azur, C3M Inserm, U1065, 06204, Nice Cedex 3, France.

³Laboratoire International Associé, Université Côte d’Azur - Centre Scientifique de Monaco, “Réponse des organismes et populations face au stress environmental”.

*Current address: Division of Population Health & Genetics, Ninewells Hospital and Medical School, Dundee DD19SY, UK.
ABSTRACT

Cnidarians - the most primitive tissue-forming animals - can get infected with pathogens, defend themselves and heal. Recent sequencing projects on cnidarians have unveiled a rich innate immunity gene repertoire but little is known about their involvement in the host’s response against live pathogens over time. *Vibrio parahaemolyticus* (Vp) is a zoonotic threat originating from the ocean, which causes pandemics, and economic struggle to society. Here, we advance our understanding of the innate immunity from the response of a basal marine metazoan to infection with Vp. The dynamic of the response was assessed in a time series experiment following the transcriptomic activity of the anemone *Aiptasia pallida* strain CC7 infected or not with the Vp clinical strain O3:K6 and compared to the gene expression profile under LPS treatment. RNA-sequencing, gene expression and functional analyses detected hundreds of genes responsive to the Vp infection after 1, 3, 6 and 12 hours, including very few shared with the response to LPS. Activated pathways involving TNFRs likely lead to apoptosis which was enzymatically confirmed and suggested as one of the mechanisms in *Aiptasia*’s defense against bacterial infection. A TLR-independent TLR-like pathway represented by MyD88, TRAF6, NF-κB and AP-1 is actively regulated in response to the infection but not by LPS, suggesting an alternative PAMP-PRR trigger to this pathway. In absence of TLRs, recognition of bacterial pathogens was possibly performed instead, by lineage-specific transmembrane immunoglobulin-containing ficolins unknown from vertebrates and/or diverse NOD-like receptor homologs.

**Keywords:** innate immunity, bacteria, infection, transcriptomics, Cnidaria
INTRODUCTION

The immune system is an ancient and complex process that works to detect and defend against pathogens, as well as regulate the interactions between microbes and hosts. In vertebrates, the immune system consists of a two-fold mechanism: the innate immunity which provides protection to the host, and an adaptive immune system that mounts a specific attack against foreign bodies (e.g. bacteria) and display memory. The current consensus is that invertebrates lack the components of the well characterized vertebrate adaptive immune system. However, with the recent increase in sequencing projects on basal metazoans, the ancestral immune repertoire is clearly appearing more complex than previously suspected. As the sister group of Bilateria, Cnidaria together with Ctenophora form the coelenterates - the most basal animals with true tissue layers and a gastro-vascular cavity also known as the coelenteron. This game-changing internal feature is especially relevant to the evolution of innate immunity, as it marks the apparition of an internal location for interactions between host cells and microorganisms. Therefore, questions regarding the evolution of key bilaterian traits such as pathogen and symbiont recognition, can be beneficially explored using cnidarians. Indeed, among cnidarians, anthozoans including corals and anemones have co-evolved since the Paleozoic era, 570-245 million years ago, with a multitude of unicellular algae, bacteria and viruses, and possess an innate immune system with an extensive list of genes homologous to those of the innate immunity of vertebrates (Hemmrich et al. 2007; Brennan and Gilmore 2018; Mansfield and Gilmore 2019).

Although studies of traditional model systems such as the mouse, fruit fly and roundworm have provided a wealth of information on the molecular basis of immunity in humans (Kuo et al. 2018), our knowledge is currently improving dramatically thanks to studies conducted on less conventional species from other taxa such as Amphioxus (Zhang et al. 2018), and Hydra (Bosch 2013). Corals, for example, can have up to 10% of their genes matching human’s but not those of the fruit fly or roundworm models (Kortschak et al. 2003). Moreover, corals show intriguing richness in certain immune gene families such as the TNF receptors (TNFR) - a family counting more members in corals than in humans and other vertebrates (Quistad et al. 2014; Quistad and Traylor-Knowles 2016). Anthozoans forming vital within-cell associations or endosymbiosis with Symbiodinium algae are ‘experts’ in symbiotic relationships and likely hold molecular information on how animal host cells and
microorganisms communicate or fight against each other. Comparison of the genomes of Acropora corals in symbiosis with algae, the aposymbiotic anemone Nematostella vectensis and the freshwater polyp Hydra vulgaris shows increased complexity and varied numbers of Toll/Interleukin-1 Receptor- (TIR), NAIP/C2TA/HET-E/TP1- (NACHT), Tumor Necrosis Factor- (TNF) and Death-domains (DD) containing proteins (Miller et al. 2007; Shinzato et al. 2011; Hamada et al. 2013) across those species. This diversity in the immune repertoire of cnidarian remains unexplained and warrants further work to determine which portion of the cnidarian immunome is functional against various pathogens, and help understand the origin of certain innate immunity processes. Thus, cnidarian models and their transcriptomes/genomes provide the material for testing hypotheses about the nature of ancestral eumetazoan (i.e. Cnidaria and Bilateria) pathways and interactions of innate immune defenses relevant to human health. Of particular interest are the mechanisms of pathogen recognition, containment, and protection among immune cells, as well as the biological processes to protect the rest of the cells against invading pathogens, shared in both cnidarians and bilaterians.

Aiptasia pallida’s transcriptome/genome (Sunagawa et al. 2009; Baumgarten et al. 2015, respectively) show an active repertoire of immune-related genes involved in the establishment of host-algae symbiosis (Poole et al. 2016), and the disruption of this symbiosis also known as bleaching (Detournay et al. 2012), which warrant the question whether the same is true against pathogen invasion. To challenge the innate immune system of Aiptasia, the Gram-negative halophilic bacteria Vibrio parahaemolyticus was chosen for two reasons. Firstly, marine zoonotic pathogens such as Vp strain O3:K6 can have disastrous consequences for human populations. Indeed, Vp is the leading cause of marine-borne illnesses worldwide (Livny et al. 2014) causing gastroenteritis via the consumption of contaminated seafood (Czachor 1992; Lipp and Rose 1997). And secondly, environmental strains naturally occur in the mucus of coral species (Chimetto et al. 2008; Nithyanand and Pandian 2009), which make Vp a member of the rich bacterial community associated with cnidarians. Using Vp to infect Aiptasia makes an ideal - marine zoonotic pathogen versus host - model system that holds great potential for studying ancestral innate immunity, and especially the evolution of those conserved pathways shared with vertebrates including humans.
Brennan and Gilmore (2018) have recently reviewed the information accumulated on the most well-studied pathogen-associated recognition receptors (PRRs) - the Toll-like receptors (TLRs) - in basal phyla including Porifera, Cnidaria, Annelida, Mollusca, and Nematoda. Based on TLR sequence analyses in these phyla, the authors hypothesized that the prototypical TLR originated within the cnidarians. Nevertheless, not all cnidarians possess prototypical TLRs. Studies focusing on the innate immunity of cnidarians have identified various prototypical TLRs as well as TLR-like genes. Surprisingly, the numbers and types of TLR-like genes and TIR-containing proteins vary greatly between Acropora corals, Nematostella vectensis (Nv), Hydra vulgaris (Hv) and Aiptasia pallida (Ap). For example, corals can have one or several multiple cysteine cluster TLRs (mccTLRs), one or no single ccTLR (scC TLR) and many TIR-only proteins (Shinzato et al. 2011; Poole and Weis 2014; Palmer and Traylor-Knowles 2012; Miller et al. 2007). In contrast, Nv only has a single mccTLR and no scC TLR or TIR-only proteins (Brennan ad Gilmore 2018), while Hv possesses separate transmembrane LRR- and TIR-containing proteins (Bosch et al. 2009).

When comparing the conserved components of the TLR to NF-κB pathway, Brennan and Gilmore (2018) show that the pathway’s structure in Ap diverges slightly from that of Nv, Hv, or the three coral species under consideration, as no TLR, NEMO or IκB homologs could be identified. Moreover, recent studies have characterized and provided functional evidences that TLR-like and the key mediator MyD88 proteins can interact to activate the transcription factor NF-κB and certain aspects of the innate immune response in the coral Orbicella faveolata (Williams et al. 2018), Nc (Brennan et al. 2017), and Hv (Bosch et al. 2009). Most recently, a study brought further support for the role of NF-κB in the symbiosis of Ap with Symbiodinium algae and its survival to bacterial infection (Mansfield et al. 2019).

Here, the symbiotic Ap strain CC7 was used to identify the molecular innate immune response of the host to bacterial infection, and compare the responsive genes and represented pathways to the functional data derived from other cnidarian models. This study focuses on the gene expression profiles of immune-related genes in response to infection after 1, 3, 6, and 12 hours with the pathogen, and exposure to the endotoxins lipopolysaccharides (LPS) for 3, 6 and 12 hours. We confirm that no prototypical TLR, nor LRR-containing TLR-like genes are regulated in the innate immune response of Ap, however we identify a TIR- and Immunoglobulin (Ig) -containing transcript responsive to the infection, which we hypothesize
could be the TIR-containing protein interacting with the Ap_MyD88 and inducing signaling down to the activation of Ap_NF-κB. We also discuss the potential roles of homologs belonging to the NOD-like receptor, lectin-complement, and tumor necrosis factor receptor (TNFR) families as well as conserved genes of the extrinsic and intrinsic apoptotic pathways. To our knowledge, this study is pioneering in following the dynamic transcriptomic activity of a basal marine animal under infection with a zoonotic bacterial pathogen in a time series experiment. This work improves our understanding of innate immunity through the measurement of gene activity in response to infection in one of the most primitive tissue-forming animals and highlights several fascinating gene targets for future functional studies.

RESULTS

Morphological response to infection

In order to study Ap’s innate immune response to Vp infection, we first monitored the response of anemones at the morphological level over 48 hours. Interestingly, animals removed from the treatment medium after 12 or 24 hours, and subsequently left in filtered seawater (FSW) for another 36 or 24 hours, respectively, recovered from such temporary assault suggesting an efficient early innate immune response (Supplementary Figure 1). Morphologically, Ap’s response to infection consists of condensed tentacles, increased mucus production, shriveling and cell detachment (Figure 1). Under the microscope, Vp in contact with the host can be seen swarming on the surface of the anemones including the foot, column and tentacles, which show signs of tissue damage and invasion at 12 and 24 hours post-inoculation. Treatments consisting of 24 hours in the infectious medium started to cause mortality among Ap specimen. To investigate the early innate immune response that allows Ap to defend itself against Vp’s attack, we focused our gene expression analyses on the first 12 hours of infection, when all anemone specimens remain alive.
Figure 1. Photographs of *Aiptasia pallida* under bright light using a stereomicroscope mounted with a digital camera. Shots under 0.8X magnification were taken after 1, 3, 6, 12 hours of exposure to experimental conditions: FSW for the controls and 10⁸ cfu/mL of *Vp* for the treatments.

**General transcriptomic response**

The general trends primarily revealed from the gene expression analyses between control and infected samples over the four time points show that: 1) more genes are up-regulated than down-regulated throughout the host response to infection, 2) the number of regulated genes increases with time, and 3) the regulatory activity in response to the infection over 12 hours ranges from less than 1% to 3% of the whole transcriptome (Table 1).

Table 1. **Proportions of DECs of interest across the response** - Number of DECs (robust Exact test at FDR<0.05) detected at each time point for the holobiont transcriptome, the transcriptome of *Aiptasia* alone, and genes belonging to the four GO classes of interest in this study. The percentage of DECs belonging to the Aiptasia-specific response is given in parentheses next to each DECs number.
The proportion of the *Aiptasia*-specific Differentially Expressed Contiguous sequences (DECs) within the holobiont (i.e. the host and its symbionts) response ranges from 70 to 83% across up-/down- DECs at the four time points of the experiment (Table 1). The numbers of up- and down-regulated DECs are similar after 1 hour post-infection, but the percentage of up-regulated DECs dramatically increased to 81% of the Ap gene activity after 3 hours. This trend will attenuate with time but remains high (73 and 63% of DECs up-regulated) over the next two time points. Among the *Aiptasia*-specific DECs, 2% of up- and < 1% of down-regulated DECs represented the immune related Gene Ontology (GO) terms. To complement the list of DECs potentially involved in the innate immune response of Ap, more permissive lists were created to include genes associated with the recognition of the pathogen, the transduction of a defensive signal and the degradation of the invader. Thus, 2-8% of the *Aiptasia*-specific up-regulated DECs represented the receptor activity, signal transduction and peptidase activity GO classes, while the same GO classes were represented by 2-6% of the down-regulated DECs. The up- and down-regulated genesets were then compared to each other across time points to identify DECs unique or shared between the
different time points via Venn diagrams (Supplementary Figure 2). These analyses show that: 1) there are more genes unique to each time point than genes detected twice or several times, 2) there are very few genes continuously regulated during 12 hours of infection, and 3) the further along the host response, the more genes are shared between time points. These trends suggest a progression in the host response even though anemones were kept in the same infectious medium over 12 hours. As Vp cells settle down and anemones concentrate them from the medium, host-pathogen physical interaction likely diverges with time, while signal transduction from pathogen recognition to effector activation progresses at the same time.

Figure 2. Venn diagrams comparing the up- and down-regulated DECs unique or in common to the Vp infection (in blue circles) and the LPS (in red circles) treatments after 3, 6 and 12 hours.

In order to identify the genes potentially responding to the pathogen-associated molecular pattern molecule (PAMP) LPS, lists of DECs produced as a result of 3, 6 and 12 hours of LPS treatment were compared to the DECs responding to the Vp infection. A few DECs were found in common to both treatments (Figure 2). No TLR homologs or orthologs of the Hydra’s HyLRR-2 and HyTRR-1 genes (Augustin et al. 2010) were identified in the
Ap’s response to LPS (Supplementary Table 1). However, it is very interesting that DECs related to the TLR-like pathway in Aiptasia respond to Vp (Table 2) but are absent among the genes induced by the LPS treatment (Supplementary Table 1). On the other hand, exposure to LPS induces TNF family homologs such as TNFR superfamily member 16 (TNFRSF-mb16) and LPS-induced TNFα factor up-regulation after 6 and 12 hours, respectively. Moreover, searching for potential receptors of PAMPs among the DECs triggered by LPS reveals an active regulation of many genes potentially involved in sensorial pathways including G-protein coupled- and various neuronal receptors (Supplementary Table 1).

Transcriptome assembly and annotation

Transcriptome libraries - made of 6 to 17 million reads across 24 samples - encompassing both control and infected anemones at all four time points, assembled into 321 413 contigs making the holobiont de novo transcriptome (i.e. genome-free assembly). Another assembly was performed as a genome-guided de novo transcriptome using the Aiptasia strain CC7 genome from the Pringle laboratory at Stanford University, but less reads could be later mapped to this reference for gene expression analyses. Thus, in order to derive more accurate information from our sequencing effort, we opted to use our in-house genome-free transcriptome to count reads. The length of contigs ranges from 201 to 49 073 bp with an average length of 950 bp. This de novo transcriptome is still fragmented, i.e. several contigs can belong to the same gene. These contigs represent 198 719 complete and partial gene sequences with 1 to 47 isoforms per gene. On average 74% of reads per sample align to the de novo reference transcriptome.

The alignment of the 321 413 contigs to the 26 042 predicted peptide sequences (from the genome of the Pringle Laboratory) using BLASTx, identified 132 179 contigs (41%) specific to Ap in our de novo transcriptome, which were exclusively used in the remaining of the analyses.

Among these contigs, 130 037 (98%) retrieve a match from the NCBI non-redundant database (e-value < 10⁻³ and % similarity > 35), 84 369 (64%) were annotated with Gene Ontology (GO) terms, 17 192 with KEGG enzyme codes, and 61 980 with InterPro IDs. GO annotations for the whole transcriptome boiled down to 9 240 unique GO terms. Among these, 2 761
belong to 82 different “immune_class” categories of the CateGOrizer tool (Supplementary Figure 3).

Progression of the innate immune response revealed by functional enrichment analyses of DECs

The Fisher’s Exact Test (FET) performed on the different lists of DECs detected many significantly enriched GO classes unrelated to the immune response of *Aiptasia* but rather its maintenance of homeostasis and the energetic cost of its defence against pathogenic invasion. Therefore, for clarity purpose, Figure 3 only shows the results of enrichment analyses performed on up-regulated DECs and GO categories related to different aspects of the immune response. The enrichment analyses’ results on down-regulated DECs are shown in Supplementary Figure 4.

**Figure 3.** Immune response-related GO classes significantly enriched (FET; FDR < 0.05) after 1, 3, 6, and 12 hours post-infection are illustrated by horizontal bars corresponding to the percentage of sequences representing each GO class. The fainted color bars illustrate the
percentage of sequences for each GO class in the reference transcriptome geneset, while the vibrant color bars are the percentage of sequences in the significantly regulated geneset under infection conditions. The evolution of the immune response through time can be followed across the four panels for the three general GO categories: BP: biological processes, MF: molecular functions, and CC: cellular components. A color code is used to help distinguish the categories unique or shared between time points.

The enrichment analyses’ results strongly support an innate immune response activity already detectable after 1 hour and involving up-regulated genes linked to the ‘Toll signaling pathway’ biological process (GO_BP) and ‘tumor necrosis factor receptor binding’ molecular function (GO_MF) represented all along the experiment (Figure 3; FET: FDR < 0.05; Supplementary Table 2 for statistical values). As the immune response progresses, the ‘regulation of apoptotic process’ becomes evident after 3 hours, and will be so through the rest of the experiment. Among the genes indicative of apoptotic activity, some belong to the ‘regulation of: NF-κB transcription factor, interferon-beta production, and MDA-5 signaling pathway’ GO_BP - as well as the GO_MF: ‘caspase binding’ highlighted at 3, 6, and 12 hours post-infection. The role of the TNF pathway in the immune response of Aiptasia gathers even more support at 3 and 6 hours, with the enrichment for ‘TNF-mediated signaling’. In addition, the ‘binding to AP-1 adaptor complex’ is highlighted at these two time points as well. At 6 hours, ‘activity of endopeptidase’ and ‘endopeptidase inhibitor’ was detected for the first time and was supported again at 12 hours. Finally, after 12 hours of infection, while activity within TNF receptor, apoptosis, and proteolysis pathways is still strong, additional peptidase activities join in the host response. In summary, these results reveal a noticeable chronology in the immune activity of Aiptasia against the pathogen, starting with the involvement of TNFRs, then the activation of apoptosis followed by proteolysis. The genes supporting these functional categories, as well as DECs without GO annotations but containing immunity-related conserved domains such as DD, Ig, lectin (Lect), leucine rich-repeat (LRR), NACHT, scavenger receptor (SR), TIR, and TNF represent an interesting source of novel innate immunity candidates, which we partly discussed below.
Apoptosis is a form of cell death regulated by caspase proteases. The caspase-3 (CASP3) like enzymatic activity was measured in Ap during infection using human caspase-3 activity detection kit applied to protein samples. The Ap CASP3-like enzymatic activity was found on average 2.9 fold higher in infected Ap at 12 hours, but showed similar levels to controls at 3 and 6 hours (Figure 4A). In addition, the CASP3 gene expression measured via sequencing detected up-regulation at 3, 6 and 12 hours with a large peak at 6 hours (Supplementary Table 3). This CASP3 gene activity was confirmed by qRT-PCR with specific primers (Figure 4B). These results show that the effector CASP3 gene is positively expressed all along the infection and that CASP3-like enzymatic activity is detectable after 12 hours at the organismal level in response to infection.

Figure 4. A) Normalized fluorescence of AMC cleaved by caspase-3-like activity measured in anemones after 3, 6 and 12 hours of exposure to control conditions (Ap control), *V. parahaemolyticus* (Ap + Vp), or 1 μM staurosporine (Ap + staurosporine) are shown relative to control levels. To confirm that the fluorescent signal corresponds to caspase-3-like proteolytic activity, the inhibitor Ac-DEVD-CHO was used in infected Aiptasia samples (Ap + Vp + inhibitor). B) In the vignette, the caspase-3 gene regulation detected by RNA sequencing is validated by quantitative real-time PCR.
Genes pointing to active innate immunity pathways and apoptosis

The results shown in Table 2 follow the organization chosen by Zhang and colleagues (2018) in a study extremely relevant to this one, analyzing the transcriptomic response of the amphioxus - Branchiostoma belcheri - to Vp. Here, a majority of the DECs reported in the tables 2 and 3 also represent the significantly enriched GO classes mentioned above. The tables of DECs shown below are subsets of exhaustive lists given in Supplementary Tables 3 and 4. For clarity purpose, Table 2 and 3 show DECs with fold changes > 5 or < -5, and homolog peptide sequence similarity values > 70%.

Table 2. Innate immunity-associated DECs - Immune response related DECs are organized as in Zhang et al. (2018) into the following four broad categories: complement and coagulation cascades, PRRs, cytokines and regulators, and adaptors and signal transducers. Vertically, DECs appear: 1) in chronological order of their time of detection, 2) from highly to lowly regulated, and 3) per gene family. While horizontally, the time of detection, the fold change in expression level and the false discovery rate (FDR) corrected p-value from the Robust Exact Test (edgeR package) represent gene expression analysis results. In addition, the contig sequence length in base pairs (bp), the score and similarity percentage of the alignment (e-value and similarity %, respectively) represent the level of confidence in the best gene identification (i.e. gene name) derived from the BLASTx to nr (from NCBI database) alignment analysis. The DECs highlighted here were filtered for a fold change greater or equal to 5 and lower or equal to -5, as well as a percentage of similarity greater or equal to 70%. The entire list of DECs is provided in Supplemental Table 3.
Among the most regulated and well supported (i.e. showing high similarity) DECs (Table 2), several gene families including coagulation factor, lectin, NOD-like receptor...
(NLR), SR, interferon regulatory factor (IRF), and TNF were highly represented. The most highly regulated immune related DEC families at 1 hour post-infection are the IRF1-like sequences (FC = 795.7, Robust Exact Test - FDR = 6.52E⁻³; and FC = -98.1, Robust Exact Test - FDR = 4.09E⁻²), which are accompanied by isoforms up-regulated at all three other time points (Supplementary Table 3). Another DEC annotated IRF2-like isoform X1 is also the most up-regulated sequence at 3 hours post-infection (FC = 220.6, FDR = 3.62E⁻²), and isoforms were also up-regulated at 3, 6 and 12 hours (Supplementary Table 3). At the same time point, the TNF receptor-associated factor 5 (TRAF5) -like DEC is down-regulated 230.6 times (Robust Exact Test, FDR = 1.79E⁻²), but isoforms are up-regulated at 3, 6 and 12 hours (Supplementary Table 3). From the same gene family, but after 6 hours post-infection, the TRAF4 DEC shows the highest up-regulation by a fold change of 488.2 (Robust Exact Test, FDR = 1.04E⁻³). After 12 hours post-infection, the stimulator of interferon genes protein (STING) DEC is the most up-regulated with a fold change of 171.5 (Robust Exact Test, FDR = 4.12E⁻³). Moreover, several isoforms of this gene are also up-regulated at 3, 6, and 12 hours post-infection (Supplementary Table 3). Based on comparisons between the Vp and LPS results (Supplementary Table 3 versus Table 1, respectively), a TLR-independent TLR-like pathway possibly represented by homologs of Myeloid differentiation primary response 88 (MyD88), TRAFs, TANK-binding kinase 1 (TBK1), NF-κ-B-inducing kinase (NIK), IRFs, NF-κB and activator protein 1 (AP-1) but no TLR-like genes, is activated under Vp infection but not LPS exposure. In absence of TLRs in Ap’s innate immunity, genes containing SR, Lect, or LRR domains belonging to the macrophage scavenger receptor (MSR), lectin or NLR families potentially represent the pathogen recognition capacity in Ap, discussed further below. Furthermore, ligands and receptors of the TNF family suggest the induction of potential cytokine dependent pathways possibly connecting to an apoptosis-like pathway reported below.

Similarly, to the innate immunity genes illustrated in Table 2, highly regulated genes associated with apoptosis are shown in Table 3. DECs likely involved in the apoptotic process were already detected after 1 hour post-infection and further progression of the apoptotic signal was observed at each following time point. The DECs annotated ‘acidic leucine-rich nuclear phosphoprotein 32 family member A’ are the most regulated sequences at 1 hour post-infection (Table 3, FC = 635.0, Robust Exact Test - FDR = 3.03E⁻⁹; and FC = -256.4,
Robust Exact Test - FDR = 6.78E-5). At the 3 hours time point, a homolog of ‘v-ets erythroleukemia virus E26 oncogene’ is up-regulated 249.4 times (FRD = 3.68E-5) and was still up-regulated at 6 and 12 hours (Supplementary Table 4). A homolog of the key apoptotic executor – CASP3 – is detected as the overall most up-regulated DEC of this analysis, with a fold change of 157.2 in (FRD = 4.56E-5), while the DEC described as T-box transcription factor (TBX1) is down-regulated 11.9 times (FDR = 1.05E-3). Briefly, the genes in Table 3 suggest that both the extrinsic and intrinsic apoptotic pathways are playing a role in the anemone’s response. The former likely represented by caspase 8 (CASP8), Ced3 (CASP9) and CASP3, and the latter by mitochondria-associated genes such as B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), and Bcl-2 antagonist/killer (Bak), among others.

Table 3. Apoptosis-associated DECs  - Apoptotic response related DECs. The DECs represented here were filtered and organized as in Table 2. The entire list of DECs is provided in Supplemental Table 4.
DISCUSSION

Overall, studies of innate immunity in cnidarians have focused on its evolutionary history (Hemmrich et al. 2007; Miller et al. 2007), its role in bacteria sensing and aging (Franzenburg et al. 2012; Bosch 2013), self-/non-self-recognition (Dishaw and Litman 2009; Puill-Stephan et al. 2012), or within ecological contexts such as host-algae symbiosis (Davy et al. 2012), bleaching (Weis 2019), wound healing (van de Water et al. 2015) and diseases (Mydlarz et al. 2006; Palmer and Traylor-Knowles 2012; Mydlarz et al. 2016; van de Water et al. 2018). Although important knowledge has been gained about the immune capacity of cnidarians during the past 15 years, their innate immune response to pathogens remains poorly understood. Genome sequencing of several cnidarian species revealed that the representation of innate immunity gene families and pathways is surprisingly divergent across cnidarian classes, families and species (Mansfield and Gilmore 2018). This provides a set of models to study the evolution of innate immunity as it is known in vertebrates. In particular, studies focusing on the characterization of the TLR to NF-κB like pathway and protein activity of its components in Hydra (Bosch et al. 2009; Franzenburg et al. 2012), Nematostella (Wolenski et al. 2011; Brennan et al. 2017) and Orbicella faveolata (Williams et al. 2018), reveal some functional conservation in response to immune challenges (discussed below). With this in mind, this study aimed to contribute information about the active molecular component of innate immunity towards bacterial pathogens in Aiptasia, in order to identify genes and pathways relevant to vertebrate and human health. To do so, the transcriptomic activity of the cnidarian model - Aiptasia pallida strain CC7 - was analyzed while challenged with the human pathogen Vp strain O3:K6 over 12 hours.

Anemones are suspension-feeders and therefore concentrate particles from the seawater within their coelenteron over time, via the mechanical action of cilia covering their surfaces. This feeding mechanism is relevant to the pathway from which humans get affected by Vp. Indeed, a majority of the vector species of vibrios are filter-feeders and constitute “seafood” which can accumulate plankton-associated pathogens if present in their environment (Baffone et al. 2006). When in contact with the high concentration of Vp used here, anemones can die in 24 hours, however if relieved from bathing too long in the highly infectious medium, anemones are capable of recovery, denoting an effective immune system.
Several relevant studies have looked at the physiological and gene expression response of the coral *Pocillopora damicornis* under temperature-dependent infection with *Vibrio corallililyticus* (Vc) - a known coral pathogen (Vidal-Dupiol *et al.* 2011a, b; Vidal-Dupiol *et al.* 2014). Although several key components of the recognition and signaling immune pathways detected by Vidal-Dupiol and colleagues (2014) are also underlined by the present study, discrepancies in the regulation of those genes is striking. However, we have to keep in mind that, on one hand, Vc’s virulence is triggered by increased temperature over several days, i.e. 25 + 7.5 °C, which caused coral bleaching, and on the other hand, bleaching caused by increased temperature alone involves coral innate immune genes activation such as NF-κB and TNFRs (DeSalvo *et al.* 2010; Barshis *et al.* 2013). Therefore, the confounding effects of temperature-induced bleaching and Vc’s invasion, the timeline difference (i.e. days versus hours), as well as the various virulence mechanisms used by the different pathogens (Vc versus Vp), can explain the discrepancies between Vidal-Dupiol’s and our experiment.

Similarly, to the cross-pathogenicity observed here, another study using the gram-negative human pathogen - *Serratia marcescens* - shows that Ap exposed to $10^8$ cfu/mL has a survival rate of 70% after 24 hours (Krediet *et al.* 2014), which supports the present observations using Vp at the same concentration. *S. marcescens* does not originate from the marine environment unlike Vp, but a couple of strains (PLD100 and PDR60) were isolated from lesions on the Elkhorn coral *Acropora palmata*, and are therefore also relevant to cnidarian etiology. Here, we show that the clinical Vp strain O3:K6 responsible for gastroenteritis in humans remains pathogenic in saltwater at 27 degrees celsius and can kill *Aiptasia* anemones over 24 hours.

**Potential cytoplasmic PRRs**

One of the essential aspects of innate immunity this study sheds light on, is the identification of active genes with PAMPs recognition potential in *Aiptasia*. Within the cells, intracellular PRRs are critical to respond to microbial components in the cytosol and detect pathogens. The NLRs are cytoplasmic proteins, which recognize bacterial peptidoglycans and trigger proinflammatory and antimicrobial immune response. Among the NLR family, the NLR family CARD domain containing protein 4 (NLRC4) is the best-known mammalian member capable of triggering the inflammasome upon detection of microbial ligands (Duncan
and Canna 2018). Moreover, inherited NLRC4 mutations are responsible for autoinflammatory diseases in humans (Kitamura et al. 2014). Van der Burg and colleagues (2016) hypothesized that the diverse repertoire of NLR-like genes in cnidarians may perform the work done by TLRs in vertebrates. Inspection of the innate immunome of several anemone species including Ap, identified 35 actinarian NLRs including eight with transmembrane domains (TMDs; van der Burg et al. 2016). Here, homologs of NLRs, NLRC-like genes were significantly regulated at each time point (Table 2 and Supplementary Table 1) and include up-regulated sequences with NACHT-like domains and LRR as well as a down-regulated sequence with TMDs (Figure 5). This is in contrast with human’s cytoplasmic NLRs lacking TMDs. However, these infection-induced NLRC contigs in Aiptasia may encode for intracellular proteins playing a role in the recognition of Vp molecules within the cells via LRRs.
Figure 5. Aiptasia’s NLR family candidates regulated in response to infection and their hypothetical cellular localization. Depictions (not to scale) of domain architecture are based on conserved Domain Architecture Retrieval Tool (NCBI) results. Transmembrane domains are identified via the TMHMM (v.2.0) of the DTU Bioinformatics server. GenBank code followed by the gene name of the closest vertebrate homolog (when applicable) is given for each candidate. The domain abbreviations correspond to: transmembrane domain (TMD), domain of unknown function (DUF), P-loop containing Nucleoside Triphosphate Hydrolases
(P-loop_NT), Predicted NTPase - NACHT family domain (COG5635), Leucine-rich repeats - ribonuclease inhibitor (LRR_RI).

Additional genes were identified as potential cytoplasmic PRRs, interferon-induced helicase C domain-containing protein 1 (IFIH1) and RIG-I-like receptor 3 (RLR-3), which contains the RIG domain of the retinoic acid-inducible gene-I-like receptors (RLRs), play a role in the intracellular recognition of viruses. In total eight IFIH1 sequences and three RLR-3 sequences were detected as up-regulated at 3, 6 and 12 hours (Supplementary Table 1). As the pathogen Vp acquired several new genomic islands through horizontal transfer, which contain phage-encoded virulence factors (Ceccarelli et al. 2013), it is possible that the Ap genes mentioned above are induced by viral-like genetic material brought into the host cells by invading Vp.

**Lineage-specific hypothetical PRRs and their putative pathways**

In search for potential genes involved in *Symbiodinium* recognition, Baumgarten and colleagues (2015) suggested that Cnidarian ficolin-like genes (CniFLs) containing a TMD and several Ig domains (unlike bilaterian ficolins) may contribute to microbe recognition and activation of the lectin-complement pathway (van der Burg et al. 2016). Here, TMD/Ig-containing Ficolin-1 homologs were up-regulated at 3, 6, 12 hours. In addition, several components of the lectin-complement pathway were also up-regulated during the infection. For example, complement component C3 was up-regulated as early as 1 hour after exposure to Vp and continued after that as well. Members of the lectin pathway were up-regulated at 3, 6 and 12 hours post-infection, including complement C2 and C4, but no Mannan-binding lectin serine protease (MASP) homologs. Finally, Ap’s factor B (Ap_Bf-1) of the alternative pathway was up-regulated in the infected anemones at 3, 6 and 12 hours (Supplementary Table 1). These results support the potential role of TMD/Ig-containing CniFLs in the recognition of pathogens and strongly suggest that both the lectin and alternative pathways play an active role in the defense of *Aiptasia* against Vp. However, it remains to be demonstrated whether the CniFLs bind to certain PAMPs and can trigger the lectin-complement pathway in *Aiptasia*.
The scavenger receptor lectin-like family is rich and diverse in Anthozoa (Neubauer et al. 2016) and plays an important role in host-symbiont interactions during onset of symbiosis (Davy et al. 2012). Moreover, a mannose-binding lectin is capable of binding pathogens in the coral Acropora millepora (Kvennefors et al. 2008). In Aiptasia, several lectins were detected during the response to Vp infection. For example, the L-rhamnose-binding lectins (RBLs) interacts with various types of bacteria in fish and invertebrates to induce proinflammatory cytokines such as IL-1β, TNF-α and IL-8, and enhanced macrophage’s phagocytosis (Watanabe et al. 2009). Here, Ap_RBL contigs are up-regulated at 3, 6 and 12 hours post-infection (Table 2 and Supplementary Table 1). In addition, homologs of collectin-12 (colec12) - a scavenger receptor that promotes binding and phagocytosis of Gram-positive, Gram-negative bacteria and yeast in humans, are up-regulated after 3 hours post-infection. Colec12 possesses a C-type lectin domain, and this domain was also found in several other DECs such as lectin C-type domain protein, L-rhamnose-binding lectin and galectin. A single C-type lectin LRR-containing sequence was up-regulated 4.9 and 22.0 in fold change at 3 and 6 hours respectively during the infection. In addition, Galectin-3–binding protein (gal3bp) homologs were up-regulated after 12 hours. In invertebrates, Gal3bp and its receptor/ligand, galectin-3 (gal3) can interact with each other to promote cell-to-cell adhesion and initiate pathologic proinflammatory signaling cascades. These results highlight several interesting lectins candidates for the recognition, potential activation of phagocytosis, and proinflammatory signaling upon contact with a pathogen in Aiptasia.

**Active components of TLR-like pathway**

TIR domain containing genes including TLR and IL-1R-like homologs have been identified in several anthozoans (Poole and Weis 2014), however many of those genes lack the ectodomain LRR and only possess TM- and TIR-domains (Baumgarten et al. 2015). As mentioned above, Ap’s genome possesses homologs of the major PRR types including NLRs, RLRs, and C-type lectin receptors (CLR) but lack TLRs. However, TLR to NF-κB pathway components are present in Aiptasia as well as in A. digitifera and O. faveolata corals, Hydra and Nematostella (reviewed in Brennan and Gilmore 2018). For example, in Nematostella, one multiple cysteine cluster TLR (Nv_TLR) is required for proper embryonic development and can also interact with MyD88 to induce NF-κB signaling in human cells after stimulation
with the heat-inactivated Vc or the *Salmonella typhimurium* flagellin. This multifunctional capacity from a single mccTLR is unknown from more complex protostomes and deuterostomes (Brennan et al. 2017). In Hydra, the closest matches to TLRs are separate transmembrane proteins containing extracellular LRRs (HyLRRs) or an intracellular TIR domain (HyTRRs) (Augustin et al. 2010). Similarly to the results with Nv_TLR, the HyLRR-2 fused to a human TIR domain, activates NF-κB signaling in human cells when stimulated with flagellin. But in *Hydra*, LPS among many other PAMPs failed to activate NF-κB signaling. Moreover, gene knocked down experiments by RNAi of either HyTRR-1 or HyLRR-2 reciprocally reduced the expression of the other gene, and the production of antimicrobial peptides (Bosch et al. 2009). In the coral *O. faveolata* too, the mccTLR protein can interact with human MyD88 (Williams et al. 2018), while MyD88-deficient *Hydra* were more susceptible to infection with the human pathogen *Pseudomonas aeruginosa* (Franzenburg et al. 2012). Taken together, these reports support an ancestral role of TLRs and TLR-like to NF-κB signaling in response to bacterial stimuli in cnidarians.

Here, with the exception of two DECs with low similarity to TLR-6 and -13, neither homologs of vertebrate TLRs, nor orthologs of the *Hydra*’s HyTRR or HyLRR genes were identified in *Ap*’s response. On the other hand, similarly to the results in *Nematostella* and *Hydra*, *Ap* genes possibly constituting a TLR-like pathway include homologs of MyD88, TRAF6, IRFs, AP-1, and NF-κB, possess several conserved domains (Figure 6) and are responsive to the infection but remain uninduced during LPS exposure (Supplementary Table 1). The TIR-domain containing MyD88 homolog in *Aiptasia* (Ap_MyD88), is 254 times more regulated in infected anemones at 3 hours, and remains up-regulated by a fold change of 2 after 12 hours. Ap_TRAF6, an important signal mediator likely acting downstream of Ap_MyD88 is up-regulated 254.7 times at 6 hours and continues at a lesser degree after 12 hours. This suggests that in *Ap*, the recruitment of MyD88 and downstream signaling to activation of NF-κB is induced by a PAMP other than LPS and must be triggered by a PRR other than a prototypical TLR or HyLRR-2 homolog.
Figure 6. Aiptasia’s TLR-like pathway candidates regulated in response to infection and their hypothetical cellular localization. Depictions (not to scale) of domain architecture are based on conserved Domain Architecture Retrieval Tool (NCBI) results. GenBank code followed by the gene name of the closest vertebrate homolog (when applicable) is given for each candidate. The domain abbreviations correspond to: Toll-Interleukin receptor domain (TIR), death domain (DD), leucine-rich repeat (LRR), P-loop containing Nucleoside Triphosphate Hydrolases (P-loop_NT), C-terminal of Roc domain (COR), caspase domain (CASc), Protein Kinases catalytic domain (PKC), N-terminal sub-domain of the Rel homology domain (RHD-n), Immunoglobulin-like fold - Plexins - Transcription factor (IPT), Jun-like
transcription factor (Jun), basic leucine zipper (bZIP), Immunoglobulin domain (Ig), Interferon-Regulatory Factor (IRF).

When searching further for genes potentially interacting with MyD88 via their TIR or Death domains, a DEC up-regulated at 6 and 12 hours annotated substance-P receptor-like (SPR) containing a TIR-2 and Ig domains actually matches a TLR-2 type-1-like gene (GenBank: KXJ09394) in the Ap genome (Figure 6). Here, we hypothesize that this Ap TLR-2 type-1-like gene may be the cytosolic TIR-containing protein interacting with the Ap_MyD88. We suggest that similarly to the results in Hydra, Ap_MyD88 could be the central mediator of the pathogen-recognition signal to transcription factors or regulators such as NF-κB, AP-1, and IRFs, which are all up-regulated during the infection and likely control the production of cytokines.

**Involvement of TNFR pathways**

The next important aspect revealed by this study is the activation of cytokine-dependent signaling pathways taking part in the innate immune response to Vp. Two of these pathways could lead to apoptosis and an inflammatory response. The conservation of the TNF-induced apoptotic response was demonstrated in the coral *Acropora digitifera* (Quistad et al. 2014). Here, the regulation of the ‘TNF-mediated signaling pathway’ was enriched at 3, 6 and 12 h post infection. When focusing on the genes representing this GO_BP, key players of two pathways stand out as highly regulated and contain some of the conserved domains in support of functional conservation in *Aiptasia*’s innate immunity (Figure 7).
Figure 7. Aiptasia’s TNF family candidates regulated in response to infection and their hypothetical cellular localization. Depictions (not to scale) of domain architecture are based on conserved Domain Architecture Retrieval Tool (NCBI) results. Transmembrane domains are identified via the TMHMM (v.2.0) of the DTU Bioinformatics server. GenBank code followed by the gene name of the closest vertebrate homolog (when applicable) is given for each candidate. The domain abbreviations correspond to: tumor necrosis factor (TNF) receptor superfamily (TNFRSF), transmembrane domain (TMD), death domain (DD), really interesting new gene finger and U-box domain (RING_Ubox), TNF receptor-associated...
factor-type zinc finger (zf-TRAF), A20-like zinc finger (zf-A20), LPS-induced TNFα-like zinc ribon domain (zf-LIT), structural maintenance of chromosomes (SMC_prok_B), meprin and TRAF-C homology domain (MATH), ovarian tumor gene-like cysteine protease (OTU), PRLI-interacting factor K (PLNO3086). The length in amino acids is given at the C-terminus.

One of these pathways likely starts with the activation of the TNFR superfamily member 1A (TNFRSF-mb1A/TNFR1), which is the receptor for the proinflammatory cytokine TNFα and the cytotoxic protein lymphotixin-alpha in humans. The Ap_TNFR1 DEC (GenBank: KXJ17455) is up-regulated 223.9 times after 1 hour, then again at a lower level after 3, 6 and 12 hours. Several TRAFs including TRAF2, -5 and the mitogen-activated protein kinase kinase kinase 14 (MAP3K14 or NIK) among other MAPK3 homologs, are potentially acting downstream of Ap_TNFR1 and are up-regulated at 6 and 12 hours. Interestingly, more support for the TNFR1 pathway is shown with the TNFα-induced protein 3 (TNFAIP3 or A20) and the TNFAIP3-interacting protein 1 (TNIP1) homologs that are both up-regulated at 3, 6, 12 hours. TNFAIP3 is an essential component of the ubiquitin-editing protein complex that ensures the transient nature of inflammatory signaling pathways. While TNIP1 inhibits the NF-κB activation and TNF-induced NF-κB-dependent gene expression by regulating the activity of TNFAIP3. Finally, the transcription factor AP-1 is up-regulated at each time point and may influence the immune response via activation of Ap_TNFR1.

The other cytokine-dependent pathway involves the TNFR superfamily member 16-like (TNFRSF-mb16; GenBank: KXJ29589) homologs, which contain the Death domain and are up-regulated at 3, 6 and 12 hours. This Ap_TNFRSF-mb16 homolog resembles the Fas receptor (FasR or TNFRSF6 gene) which plays a central role upon Fas ligand (FasL) binding in the physiological regulation of programmed cell death in humans via its interaction with FADD and CASP8. In the coral A. digitifera, thirteen TNFR-like sequences containing the DD were identified and proposed as mediators of apoptosis through caspase activation (Quistad and Traylor-Knowles 2016). Interestingly, we also identified candidates potentially acting up- and down-stream of FasR. Extracellularly, the soluble FasL is generated by cleaving membrane-bound FasL by the external matrix metalloproteinase MMP-7 (Mitsiades et al. 2001). Here, two Ap matrix metalloproteinases are up-regulated along the infection and
might interact with the TNF ligand superfamily member 6 (FasLG) homolog which is up-regulated at 12 hours. The Ap_CASP8 is up-regulated at 3 and 6 hours and likely functions within the cells. If interacting with the Ap_TNFRSF proteins, Ap_CASP8 would represent the extrinsic (receptor-mediated) pathway to apoptosis. On the other hand, the intrinsic (mitochondria-mediated) pathway is strongly supported by the up-regulation of ATF4, Bcl-2, Bcl-W, Bax, Bak, Apaf-1 as well as CASP9 homologs in Ap (Supplementary Table 4). Both pathways could engage the apoptotic executor - CASP3, which is up-regulated all along the infection, with a peak of 1 250 in fold change at 6 hours and enzymatically active at 12 hours.

Apoptosis as defense mechanism

An essential immune defense mechanism in eukaryotes is to sacrifice infected cells in order to protect healthy cells. The major cell death pathways, including apoptotic cell death, is a crucial barrier against microbial infection (Bergsbaken et al. 2009; Lamkanfi and Dixit 2010; Zitvogel et al. 2010). For instance, in response to bacterial infection, apoptosis or programmed cell death is used in the host innate immune response to: 1) eliminate pathogens at the early stage of infection without emitting alarm signals, and 2) induce dendritic cells (DCs) to engulf apoptotic bodies containing infected microbes (Elliott and Ravichandran 2010). In Aiptasia's response to infection, a functional enrichment for ‘regulation of apoptotic process’ could be detected at 3 hours. Among the DECs supporting the enrichment, many key players of apoptosis as well as genes with potential connections to the apoptotic pathway were identified at each time point, while an increased CASP3-like enzymatic activity was observed at 12 hours. More specifically, 216 contigs annotated with a potential role in apoptosis took part in Ap’s response to infection. Besides the hallmarks of apoptosis mentioned above, other genes connected to apoptosis were highly regulated. For example, isoforms of the acidic leucine-rich nuclear phosphoprotein 32 (ANP32) gene were 635 and 265 times up- and down-regulated, respectively at 1 hour, and up-regulated 174 times at 3 hours. Several studies showed that ANP32 proteins allowed apoptosome activation at physiological levels of dATP and also promoted CASP3 activation directly (Reilly et al. 2014). The transcription factor v-ets erythroblastosis virus E26 oncogene homolog 1 (Ets-1) is involved in the regulation of apoptosis (Teruyama et al. 2001), by regulating genes encoding Bax, Bcl-2, Caspase-1 and Fas ligand (Nagarajan et al. 2009). Here, the Aiptasia’s Ets-1 homolog is up-regulated at 3, 6,
and 12 hours post infection including a peak expression of 249 fold change at 3 hours. It is noteworthy that the FasLG, Bax and Bcl-2 homologs are among the DECs detected in this study. These results strongly suggest that apoptosis plays an important role in the innate immune response of Aiptasia against bacterial pathogens. As Aiptasia is capable of recovering from a punctual exposure to *Vp*, it is probable that apoptosis plays a part in the removal of invading bacteria and the survival of the host. Further work is envisaged to study if and how Aiptasia is capable of containing bacterial invasion via programmed cell death.

**Novel genes responsive to infection**

Several of the genes discussed above as potential actors in the innate immune response of Ap appear to possess cnidarian specific domain architectures and await functional characterization (van der Burg *et al.* 2016). For example, novel domain architectures and/or innovations, such as double TMD-containing TNFRs and TMD/Ig-containing CniFLs may confer these genes new roles in innate immunity. In addition, genes similar to the potential novel actinarian immune genes (NG1, NG2, and NG3) proposed by van der Burg (2016) based on domain architectures, are here annotated F-box only protein 11, 4 and F-box/LRR-repeat protein 14, and found up-regulated at all four time points. Finally, this study also reveals for the first time in a cnidarian that diverse G protein-coupled receptor (GPCRs) homologs with seven-transmembrane domains are highly regulated all along the infection and in response to LPS exposure. A variety of GPCRs are expressed in T cells and were recently linked to an important role in the mediation of immunity in humans (Mashaghi *et al.* 2016; Wang 2018; Lu and Cyster 2019). Thus Ap_GPCRs constitute fascinating molecular targets towards the identification of ancestors of immune cells in basal metazoans and/or the activation of innate immunity via sensory pathways.

**CONCLUSION**

To our knowledge, this is the first transcriptomic time series study of the innate immune response to bacterial infection in a cnidarian model. The main goal of this study was to identify and follow the active immunome of *Aiptasia pallida*’s (strain CC7) in response to the O3:K6 pathogenic strain of *Vibrio parahaemolyticus*. The transcriptomic analyses in the host over 12 hours detected thousands of differentially expressed sequences responsive to the
infection. Among the most interesting discoveries were indications that several innovations consisting of: possible neofunctionalization, conservation of signaling pathways over conservation of particular key genes, and involvement of lineage-specific immunity genes, denote the innate immune response of *Aiptasia*. Indeed, recognition of bacterial pathogens is most likely performed by PRRs others than the canonical TLRs, and probably assured instead by putative novel immune receptors, such as TMD/Ig-containing CnIFLs and/or cytoplasmic LRR-containing NLRs, which would represent novel bacterial pathogen recognition resources in eukaryotes. Transduction of immune signals through molecular pathways, in part resembling the TLR pathway, is supported by active homologs of MyD88, TRAF6, IRFs, NF-κB and AP-1, but not TLRs. Interestingly, two TNFR pathways could possibly lead to apoptosis and an inflammatory response, and in turn, apoptosis is likely one of the mechanisms taking place to fend off bacterial infection in *Aiptasia*. Finally, previously identified but uncharacterized actinarian-specific genes, with novel immune-domain architectures are responsive to bacterial infection. Taken together these results reveal the anemone’s innate immunity genes responsive to bacterial infection as well as potential novel players offering many new exciting research subjects to advance innate immune knowledge.

**MATERIAL AND METHOD**

**Experimental design**

Individual clones of the sea anemone Ap strain CC7 were acquired from the Pringle laboratory at Stanford University and kept at the Scientific Center of Monaco under controlled conditions in the Ecosystems and Immunity laboratory of the Biomedical department. Anemones are maintained in three liters tanks inside a culture chamber (Percival Scientific, USA) at constant 27°C with 20 μmol.m⁻².s⁻¹ of light (12:12h). Filtered seawater (FSW) at 0.45 μm is renewed every week and anemones are fed twice a week with artemias except on the week of the experiment during which they fast.

For the infection experiment, anemones of approximately half a centimeter in diameter were placed in 6-well plates filled with 10 mL of 0.22 μm FSW. Anemones were kept still overnight until fixed to the bottom.
Vp strain RIMD 2210633 serotype O3:K6 came from the laboratory of Prof. Kodama (Osaka University, Japan). Routinely, frozen Vp are cultured at 37°C in Luria-Bertani (LB) supplemented up to a final concentration of 1.5 g/L NaCl. For the infection treatment, Vp was then grown again at 27°C in Luria-Bertani (LB) supplemented with NaCl to a final of 3.5 g/L and without agitation for 24 hours. The Vp cultures were centrifuged at 2,500 rpm for 10 minutes, the bacterial pellet resuspended in 0.2 μm FSW to be concentrated 10 times, and 500 μL were dispensed into the wells containing the anemones in 9.5 ml of FSW. The final concentration of Vp was 10^8 cfu/mL. Preliminary kinetics experiments using this concentration of pathogen were performed to choose relevant time points at which samples would be fixed for further molecular analyses. For the LPS treatment, ultrapure lipopolysaccharide (LPS) from E. coli K12 (Invivogen cat. ttrl-pekLps) was added to reach a final concentration of 10 μg/mL in the medium containing the anemones.

After Vp or LPS added to treatment wells, anemones were placed in the Percival chamber at 27 °C under gentle orbital agitation of 500 rpm for 1, 3, 6 and 12 hours. At each time point, whole anemones were detached, transferred in 1.5 mL tubes and frozen in liquid nitrogen for further molecular analyses. The LPS treated samples were collected after 3, 6 and 12 hours.

**Monitoring of morphological appearance**

Before collecting the anemones for molecular analyses, photographs were taken using a stereomicroscope Stemi 305 trino (Zeiss) mounted with a digital camera 5MP Toupcam (Touptek). Shots were taken at each time point under bright light and the 0.8X magnification to monitor the overall appearance of the animals. Further observations were recorded at 5X magnification to assess the progression of the infection and the integrity of the animal tissue. Animals were considered dead when not contracting after being touched.

**RNAs isolations**

Frozen anemone’s tissues were disrupted and cells lysed in Trizol reagent (Ambion life technologies) to isolate RNAs. To do so, approximately 100 uL of 1mm diameter glass beads in 1.5 mL tubes filled with 1 mL of lysis buffer were used for anemones weighing approximately 100 mg. Tissue was completely disrupted using a precellys 24 tissue lyser.
(Bertin Technologies) at 500 bpm for 2x 5 seconds. Samples were then immediately placed on ice and total RNA extraction protocol followed.

For RNA extraction, two chloroform phase separations were performed in order to precipitate the polysaccharides from the aqueous phase as much as possible. 300 uL of chloroform was added to the tissue lysate, hand shaken for 15 seconds and settled at room temperature for 15 minutes. The aqueous phase was then added to 400 uL of 75% ethanol and transferred onto a column for purification. The purification followed the Direct-zol RNA MiniPrep Plus protocol (cat# R2072) from Zymoresearch. RNA samples were then sent to Eurofins (Europe) for cDNA library preparation using polyA selection (96 RNA-seq library from total RNA protocol, Illumina) and sequencing. cDNA libraries were sequenced as 2x 100 bp paired-end reads on the Illumina HiSeq2500 with chemistry v4 and high-output run mode.

**Sequencing data processing**

Raw sequences in fastq files were processed to remove Illumina adapters, low quality bases and short reads using the Trimmomatic-0.36 tool (Bolger *et al.* 2014). New reference transcriptomes were assembled *de novo* using all of the *Aptasia pallida* libraries produced here and either guided by the Ap genome produced by the Pringle laboratory (version 1.1) or genome-free using Trinity v2.6.5 release (Grabherr *et al.* 2011). Contiguous sequences or contigs were used to retrieve gene descriptions using blatTx against the non-redundant NCBI database with the following filter: E-value < $10^{-3}$, and only Blast description annotation excluding the terms ‘unknown’, ‘hypothetical’, or ‘uncharacterized’. In addition, coding regions were predicted using TransDecoder and the predicted peptide sequences were used to retrieve further gene annotations from several different databases using blastp against Uniprot, Pfam, Swissprot and UniRef90. HMMER and the Pfam database were also used to identify conserved protein domains present in our predicted peptide database. Depictions of domain architecture were based on conserved Domain Architecture Retrieval Tool (NCBI) results. Transmembrane domains were identified via the TMHMM (v.2.0) of the DTU Bioinformatics server. Contigs belonging to the anemone were isolated from the rest of sequences using blastx against the Ap predicted protein database produced by the Pringle laboratory (Baumgarten *et al.* 2015). Transcript-level quantification was estimated for gene and isoform expression by allocating multi-mapping reads among all transcripts of the
reference transcriptome using the RSEM (Li and Dewey 2011) and Bowtie-2 (Langmead and Salzberg 2012) packages. The pairwise differential expression analysis comparing the transcript abundance in control anemones to anemones in contact of the pathogen was performed using edgeR (Robinson et al. 2010) on the Blast2GO platform (Götz et al. 2008). The exact test based on the quantile-adjusted conditional maximum likelihood method for single-factor experimental design was applied here to detect differentially expressed contigs (DECs) between control and infected anemones at different time points. All parameters, command lines and scripts used for the transcriptomics and bioinformatics are provided in Supplementary File 1.

**Functional analyses**

**Immune class GO categorization**

To visualize the progress of Ap’s immune response to the infection, GO terms for differentially expressed contigs at each time point were categorized according to the ‘immune-class’ categorization method by Zhi-Liang Hu (2008). Briefly, the CateGOrizer tool allows simplifying the visualization of functional annotations from gene lists, by counting all paths between child and parent GO terms. Here, all paths between child and parent GO terms (using the cumulative count method: multiple paths possible between a single child and a single parent) were counted for the whole transcriptome as well as for the lists of DECs corresponding to each time point. The percentage of ‘immune_class’ GO term paths was then calculated as follows: GO path % = nbr. of GO paths in DECs list for GO:000xyz / nbr. of GO paths in transcriptome for GO:000xyz.

**Targeted Gene Ontology**

The GO codes corresponding to the parent terms ‘immune response’, ‘receptor activity’, ‘signal transduction’, and ‘peptidase activity’ were used to count all corresponding child terms within the DECs list of each time point. These DECs were then compared between each time point and visualized via a Venn diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/ ; Supplementary Figure 2). The largest fold changes in expression level for the immune response related DECs analyzed in Supplementary Figure 2 are shown in Table 2. The complete list of DECs containing sequence
IDs, hits information, folds changes and statistical results is accessible in the Supplementary Table 3.

**Enrichment analyses**

To detect a significant enrichment for the immunity associated GO terms within the lists of DECs, two tests were applied: one quite permissive – the Fisher’s exact test and the other more stringent – the gene set enrichment analysis method. Both tests were run using the Blast2GO platform (Götz et al. 2008) and the whole transcriptome annotated with GO terms as the reference gene set. For both tests, only contigs with false discovery rate corrected p-value lower than 0.05 were considered. The enrichment analysis using the Fisher’s exact test was performed on the up- and down-regulated genesets produced via the gene expression analyses for the 1h, 3h, 6h and 12h time points. Results were then reduced to the most specific GO categories applying a lower than 0.05 filter on FDR values to produce Figure 3. Some limitations of the functional analysis (FET) need to be kept in mind and concerns the level of fragmentation of our transcriptome, meaning that several contigs can belong to the same transcript. This can introduce a bias when running enrichment analysis which compares the proportion of genes representing a gene ontology category in a test geneset, with the proportion of genes for that same GO category in a reference geneset. In a case where, a test geneset made of a large number of DECs representing a certain number of transcripts is compared to a different geneset representing the same number of transcripts but by less DECs. The Fisher’s exact test is more likely to find GO categories significantly represented in the former geneset than the latter. Therefore the enrichment is somewhat dependent on the level of fragmentation of the genes represented in the geneset. The enrichment for the GO categories discussed here were manually validated and are truly represented by many relevant genes provided in Supplementary Table 3 and 4.

**Caspase-3-like activity assay**

Caspase-mediated apoptosis in control and infected anemones was assessed by increased activity of caspase-3-like and other DEVD-specific proteases using an enzymatic assay (EnzCheck Caspase-3 assay kit, Molecular Probes: Cat.# E-13183) on freshly isolated protein extracts. Frozen anemone’s tissues were disrupted and cells lysed using the cell lysis buffer of the kit (EnzChek Caspase-3 kit #1, Molecular Probes) and the precellys as for RNA
extractions above. The fluorescence of the benzylloxycarbonyl group (Z-DEVD-AMC) upon proteolytic cleavage was continuously measured in triplicate samples using the microplate reader Synergy H1M (BioTek) at 441 nm for 1 hour. Confirmation of the caspase-3-like activity was obtained by using the Ac-DEVD-CHO inhibitor (negative control), successfully suppressing the signal detected in this experiment almost entirely. The positive control consisted of inducing apoptosis in anemones exposed to 1 μM staurosporine (Cat.# 9953S, Cell Signaling) and triggering a caspase-3-like activity up to 9.2 times higher than in controls. The fluorescence signal was normalized to total protein quantity measured with the DC protein assay (Cat.# 5000116, Bio-Rad).

Quantitative real-time PCR

RNAs were extracted as explained for the sequencing analyses. Complementary DNAs were then synthesized from 100ng of purified total RNAs using oligodT primers following the RevertAid first strand cDNA protocol (cat. K1622; ThermoScientific). Specific primers designed from the DEC sequences identified as caspase-3 in Ap’s transcriptome were used in quantitative real-time PCR (qRT-PCR) reactions run on the StepOnePlus machine (Applied Biosystems). The GoTaq qPCR master mix (cat. A6002; Promega) was used in reactions containing: 1μL of each primer at 10pmol/μL, 10uL of SYBR, 0.1uL of CXR, 5.9uL of DEPC treated water, and 2uL of 10X diluted cDNA. Normalized expression levels for the gene of interest (GOI) were calculated using normalization factors (geometric mean) based on the stable expression levels across time points and treatments of three reference genes: 60S ribosomal protein L11, F-actin and adenosylhomocysteinase 2.

Ethics approval and consent to participate

Not applicable

Availability of data and materials

The datasets generated during and/or analysed during the current study WILL BE available in the NCBI BIOPROJECT repository

Consent for publication
Competing interests

The authors declare that they have no competing interests

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Authors' contributions

F.S. designed and ran the experiment, performed the bulk of the computational analyses, interpreted the results and wrote the manuscript. D.D. performed data mining for novel domain architectures. D.C. and L.B. supervised all aspects of the project. All authors have read, improved and approved the manuscript.

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REFERENCES

Augustin R, Fraune S, Bosch TCG. 2010. How Hydra senses and destroys microbes. Seminars in Immunology 22:54–58.

Baffone W, Tarsi R, Pane L, Campana R, Repetto B, Mariottini GL, Pruzzo C. 2006. Detection of free-living and plankton-bound vibrios in coastal waters of the Adriatic Sea (Italy) and study of their pathogenicity-associated properties. Environ Microbiol 8:1299–1305.

Barshis DJ, Ladner JT, Oliver TA, Seneca FO, Traylor-Knowles N, Palumbi SR. 2013. Genomic basis for coral resilience to climate change. Proc Natl Acad Sci USA 110:1387–1392.

Baumgarten S, Simakov O, Esherick LY, Liew YJ, Lehnert EM, Michell CT, Li Y, Hambleton EA, Guse A, Oates ME, et al. 2015. The genome of Aiptasia, a sea anemone
model for coral symbiosis. Proc Natl Acad Sci USA 112:11893–11898.

Bergsbaken T, Fink SL, Cookson BT. 2009. Pyroptosis: host cell death and inflammation. Nat Rev Microbiol 7:99–109.

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–2120.

Bosch TCG. 2013. Cnidarian-Microbe Interactions and the Origin of Innate Immunity in Metazoans. Annu. Rev. Microbiol. 67:499–518.

Bosch TC, Augustin R, Anton-Erxleben F, Fraune S, Hemmrich G, Zill H, Rosenstiel P, Jacobs G, Schreiber S, Leippe M and Stanisak M. 2009. Uncovering the evolutionary history of innate immunity: the simple metazoan Hydra uses epithelial cells for host defence. Developmental & Comparative Immunology. 33(4):559–569.

Brennan JJ, Gilmore TD. 2018. Evolutionary origins of toll-like receptor signaling. Molecular biology and evolution. 35(7):1576–1587.

Brennan JJ, Messerschmidt JL, Williams LM, Matthews BJ, Reynoso M, Gilmore TD. 2017. Sea anemone model has a single Toll-like receptor that can function in pathogen detection, NF-κB signal transduction, and development. Proc Natl Acad Sci USA 114:E10122–E10131.

van der Burg CA, Prentis PJ, Surm JM, Pavasovic A. 2016. Insights into the innate immunome of actiniarians using a comparative genomic approach. BMC Genomics 17:850.

Ceccarelli D, Hasan NA, Huq A, Colwell RR. 2013. Distribution and dynamics of epidemic and pandemic Vibrio parahaemolyticus virulence factors. Frontiers in cellular and infection microbiology. 3:97.

Chimetto LA, Brocchi M, Thompson CC, Martins RCR, Ramos HR, Thompson FL. 2008. Vibrios dominate as culturable nitrogen-fixing bacteria of the Brazilian coral Mussismilia hispida. Systematic and Applied Microbiology 31:312–319.

Czachor JS. 1992. Unusual aspects of bacterial water-borne illnesses. American family physician 46:797–804.

Davy SK, Allemand D, Weis VM. 2012. Cell Biology of Cnidarian-Dinoflagellate Symbiosis. Microbiology and Molecular Biology Reviews 76:229–261.

DeSalvo M, Sunagawa S, Voolstra C, Medina M. 2010. Transcriptomic responses to heat stress and bleaching in the elkhorn coral Acropora palmata. Mar. Ecol. Prog. Ser. 402:97–113.

Detournay O, Schnitzler CE, Poole A, Weis VM. 2012. Regulation of cnidarian–dinoflagellate mutualisms: Evidence that activation of a host TGFβ innate immune pathway promotes tolerance of the symbiont. Developmental & Comparative Immunology 38:525–537.

Dishaw LJ, Litman GW. 2009. Invertebrate Allorecognition: The Origins of Histocompatibility. Current Biology 19:R286–R288.
Duncan JA, Canna SW. 2018. The NLRC4 Inflammasome. Immunol Rev 281:115–123.

Elliott MR, Ravichandran KS. 2010. Clearance of apoptotic cells: implications in health and disease. The Journal of Cell Biology 189:1059–1070.

Franzenburg S, Fraune S, Künzel S, Baines JF, Domazet-Lošo T, Bosch TC. 2012. MyD88-deficient Hydra reveal an ancient function of TLR signaling in sensing bacterial colonizers. PNAS. 109(47):19374–19379.

Götz S, García-Gómez JM, Terol J, Williams TD, Nagaraj SH, Nueda MJ, Robles M, Talón M, Dopazo J, Conesa A. 2008. High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic Acids Res 36:3420–3435.

Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q, et al. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol 29:644–652.

Hamada M, Shoguchi E, Shinzato C, Kawashima T, Miller DJ, Satoh N. 2013. The Complex NOD-Like Receptor Repertoire of the Coral Acropora digitifera Includes Novel Domain Combinations. Molecular Biology and Evolution 30:167–176.

Hemmrich G, Miller DJ, Bosch TC. 2007. The evolution of immunity: a low-life perspective. Trends in Immunology. 28(10):449–454.

Hu ZL, Bao J, Reecy JM. 2008. CateGOrizer: a web-based program to batch analyze gene ontology classification categories. Online J Bioinform. 9:108–112.

Kitamura A, Sasaki Y, Abe T, Kano H, Yasutomo K. 2014. An inherited mutation in NLRC4 causes autoinflammation in human and mice. The Journal of Experimental Medicine 211:2385–2396.

Kortschak RD, Samuel G, Saint R, Miller DJ. 2003. EST Analysis of the Cnidarian Acropora millepora Reveals Extensive Gene Loss and Rapid Sequence Divergence in the Model Invertebrates. Current Biology 13:2190–2195.

Krediet CJ, Meyer JL, Gimbrone N, Yanong R, Berzins I, Alagely A, Castro H, Ritchie KB, Paul VJ, Teplitzki M. 2014. Interactions between the tropical sea anemone Aiptasia pallida and Serratia marcescens, an opportunistic pathogen of corals: Aiptasia pallida and a coral pathogen. Environmental Microbiology Reports 6:287–292.

Kuo C-J, Hansen M, Troemel E. 2018. Autophagy and innate immunity: Insights from invertebrate model organisms. Autophagy 14:233–242.

Kvennefors ECE, Leggat W, Hoegh-Guldberg O, Degnan BM, Barnes AC. 2008. An ancient and variable mannose-binding lectin from the coral Acropora millepora binds both pathogens and symbionts. Developmental & Comparative Immunology 32:1582–1592.

Lamkanfi M, Dixit VM. 2010. Manipulation of Host Cell Death Pathways during Microbial Infections. Cell Host & Microbe 8:44–54.

Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. Nat Methods
9:357–359.

Li B, Dewey CN. 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. :16.

Lipp EK, Rose JB. 1997. The role of seafood in foodborne diseases in the United States of America. Revue Scientifique et Technique-Office International des Epizooties 16:620–640.

Livny J, Zhou X, Mandlik A, Hubbard T, Davis BM, Waldor MK. 2014. Comparative RNA-Seq based dissection of the regulatory networks and environmental stimuli underlying Vibrio parahaemolyticus gene expression during infection. Nucleic Acids Research 42:12212–12223.

Lu E, Cyster JG. 2019. G-protein coupled receptors and ligands that organize humoral immune responses. Immunol Rev 289:158–172.

Mansfield KM, Gilmore TD. 2019. Innate immunity and cnidarian-Symbiodiniaceae mutualism. Developmental & Comparative Immunology 90:199–209.

Mansfield KM, Cleves PA, Van Vlack E, Kriefall NG, Benson BE, Camacho DJ, Hammond O, Pedroza M, Siggers T, Pringle JR, Davies SW. 2019. Varied effects of algal symbionts on transcription factor NF-κB in a sea anemone and a coral: possible roles in symbiosis and thermotolerance. BioRxiv:640177.

Mashaghi A, Marmalidou A, Tehrani M, Grace PM, Pothoulakis C, Dana R. 2016. Neuropeptide substance P and the immune response. Cell. Mol. Life Sci. 73:4249–4264.

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

Mitsiades N, Yu W, Poulaki V, Tsokos M, Stamenkovic I. 2001. Matrix Metalloproteinase-7-mediated Cleavage of Fas Ligand Protects Tumor Cells from Chemotherapeutic Drug Cytotoxicity. :6.

Mydlarz LD, Fuess L, Mann W, Pinzón JH, Gochfeld DJ. 2016. Cnidarian immunity: from genomes to phenomes. In: The Cnidaria, Past, Present and Future. Springer. p. 441–466.

Mydlarz LD, Jones LE, Harvell CD. 2006. Innate Immunity, Environmental Drivers, and Disease Ecology of Marine and Freshwater Invertebrates. Annu. Rev. Ecol. Evol. Syst. 37:251–288.

Nagarajan P, Parikh N, Garrett-Sinha LA, Sinha S. 2009. Ets1 induces dysplastic changes when expressed in terminally-differentiating squamous epidermal cells. PloS one. 4(1).

Neubauer EF, Poole AZ, Weis VM, Davy SK. 2016. The scavenger receptor repertoire in six cnidarian species and its putative role in cnidarian-dinoflagellate symbiosis. PeerJ 4:e2692.

Nithyanand P, Pandian SK. 2009. Phylogenetic characterization of culturable bacterial diversity associated with the mucus and tissue of the coral Acropora digitifera from the Gulf of Mannar: Culturable bacterial diversity of A. digitifera. FEMS Microbiology Ecology
Palmer CV, Traylor-Knowles N. 2012. Towards an integrated network of coral immune mechanisms. Proceedings of the Royal Society B: Biological Sciences 279:4106–4114.

Poole AZ, Kitchen SA, Weis VM. 2016. The role of complement in cnidarian-dinoflagellate symbiosis and immune challenge in the sea anemone Aiptasia pallida. Frontiers in microbiology. 7:519.

Poole AZ, Weis VM. 2014. TIR-domain-containing protein repertoire of nine anthozoan species reveals coral–specific expansions and uncharacterized proteins. Developmental & Comparative Immunology 46:480–488.

Puill-Stephan E, Seneca FO, Miller DJ, van Oppen MJ and Willis BL. 2012. Expression of putative immune response genes during early ontogeny in the coral Acropora millepora. PLoS One:7(7).

Quistad SD, Stotland A, Barott KL, Smurthwaite CA, Hilton BJ, Grasis JA, Wolkowicz R, Rohwer FL. 2014. Evolution of TNF-induced apoptosis reveals 550 My of functional conservation. Proc Natl Acad Sci USA 111:9567–9572.

Quistad SD, Traylor-Knowles N. 2016. Precambrian origins of the TNFR superfamily. Cell Death Discovery 2:16058.

Reilly PT, Yu Y, Hamiche A, Wang L. 2014. Cracking the ANP32 whips: Important functions, unequal requirement, and hints at disease implications: Prospects & Overviews. BioEssays 36:1062–1071.

Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26:139–140.

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

Sunagawa S, Wilson EC, Thaler M, Smith ML, Caruso C, Pringle JR, Weis VM, Medina M, Schwarz JA. 2009. Generation and analysis of transcriptomic resources for a model system on the rise: the sea anemone Aiptasia pallida and its dinoflagellate endosymbiont. BMC Genomics 10:258.

Teruyama K, Abe M, Nakano T, Iwasaka-Yagi C, Takahashi S, Yamada S, Sato Y. 2001. Role of transcription factor Ets-1 in the apoptosis of human vascular endothelial cells. Journal of cellular physiology 188:243–252.

Vidal-Dupiol J, Dheilly NM, Rondon R, Grunau C, Cosseau C, Smith KM, Freitag M, Adjeroud M, Mitta G. 2014. Thermal stress triggers broad Pocillopora damicornis transcriptomic remodeling, while Vibrio coralliilyticus infection induces a more targeted immuno-suppression response. PloS one: 9(9).

Vidal-Dupiol Jeremie, Ladrière O, Destoumieux-Garzón D, Sautière P-E, Meistertzheim A-L,
Tambutté E, Tambutté S, Duval D, Fouré L, Adjeroud M, et al. 2011. Innate Immune Responses of a Scleractinian Coral to Vibriosis. J. Biol. Chem. 286:22688–22698.

Vidal-Dupiol J., Ladriere O, Meistertzheim A-L, Foure L, Adjeroud M, Mitta G. 2011. Physiological responses of the scleractinian coral Pocillopora damicornis to bacterial stress from Vibrio coralliilyticus. Journal of Experimental Biology 214:1533–1545.

Wang D. 2018. The essential role of G protein-coupled receptor (GPCR) signaling in regulating T cell immunity. Immunopharmacology and Immunotoxicology 40:187–192.

Watanabe Y, Tateno H, Nakamura-Tsuruta S, Kominami J, Hirabayashi J, Nakamura O, Watanabe T, Kamiya H, Naganuma T, Ogawa T, et al. 2009. The function of rhamnose-binding lectin in innate immunity by restricted binding to Gb3. Developmental & Comparative Immunology 33:187–197.

van de Water JAJM, Ainsworth TD, Leggat W, Bourne DG, Willis BL, van Oppen MJH. 2015. The coral immune response facilitates protection against microbes during tissue regeneration. Mol Ecol 24:3390–3404.

van de Water JAJM, Chaib De Mares M, Dixon GB, Raina J-B, Willis BL, Bourne DG, van Oppen MJH. 2018. Antimicrobial and stress responses to increased temperature and bacterial pathogen challenge in the holobiont of a reef-building coral. Mol Ecol 27:1065–1080.

Weis VM. 2019. Cell Biology of Coral Symbiosis: Foundational Study Can Inform Solutions to the Coral Reef Crisis. Integr Comp Biol 59:845–855.

Williams LM, Fuess LE, Brennan JJ, Mansfield KM, Salas-Rodriguez E, Welsh J, Awtry J, Banic S, Chacko C, Chezian A, Dowers D. 2018. A conserved Toll-like receptor-to-NF-κB signaling pathway in the endangered coral Orbicella faveolata. Developmental & Comparative Immunology. 79:128–136.

Wolenski FS, Garbati MR, Lubinski TJ, Traylor-Knowles N, Dresselhaus E, Stefanik DJ, Goucher H, Finnerty JR, Gilmore TD. 2011. Characterization of the core elements of the NF-κB signaling pathway of the sea anemone Nematostella vectensis. Molecular and cellular biology. 31(5):1076–1087.

Zhang Q-L, Zhu Q-H, Liang M-Z, Wang F, Guo J, Deng X-Y, Chen J-Y, Wang Y-J, Lin L-B. 2018. Comparative transcriptomic analysis provides insights into antibacterial mechanisms of Branchiostoma belcheri under Vibrio parahaemolyticus infection. Fish & Shellfish Immunology 76:196–205.

Zitvogel L, Kepp O, Kroemer G. 2010. Decoding Cell Death Signals in Inflammation and Immunity. Cell 140:798–804.
