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Investigating the roles of transforming growth factor-beta in immune response of *Orbicella faveolata*, a scleractinian coral

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ABSTRACT

Symbiotic relationships range from parasitic to mutualistic, yet all endosymbionts face similar challenges, including evasion of host immunity. Many symbiotic organisms have evolved similar mechanisms to face these challenges, including manipulation of the host's transforming growth factor-beta (TGFβ) pathway. Here we investigate the TGFβ pathway in scelaractinian corals which are dependent on symbioses with dinoflagellates from the family Symbiodiniaceae. Using the Caribbean coral, *Orbicella faveolata*, we explore the effects of enhancement and inhibition of the TGFβ pathway on host gene expression. Following transcriptomic analyses, we demonstrated limited effects of pathway manipulation in absence of immune stimulation. However, manipulation of the TGFβ pathway significantly affects the subsequent ability of host corals to mount an immune response. Enhancement of the TGFβ pathway eliminates transcriptomic signatures of host coral immune response, while inhibition of the pathway maintains the response. This is, to our knowledge, the first evidence of an immunomodulatory role for TGFβ in a scelaractinian coral. These findings suggest variation in TGFβ signaling may have implications in the face of increasing disease prevelance. Our results suggest that the TGFβ pathway can modulate tradeoffs between symbiosis and immunity. Further study of links between symbiosis, TGFβ, and immunity is needed to better understand the ecological implications of these findings.

1. Introduction

Symbiotic relationships are present in nearly all of earth's ecosystems and involve a wide diversity of taxa (Brundrett, 2009; Wang and Qiu, 2006; Zeigler, 2014). These relationships range from parasitic, where one partner benefits at the expense of another, to mutualistic, where both partners benefit (Hentschel et al., 2000; Johnson et al., 1997). Additionally these relationships take a variety of forms, ranging from macro relationships, such as those between plants and ants (Palmer and Brody, 2013), to moderate scale relationships such as macro-parasitic infections (de Roij and MacColl, 2012; Koprivnikar et al., 2012), to micro-, endosymbiotic relationships, such as lichens (Perez-Ortega et al., 2010). Particularly in the case of parasitism and endosymbiotic interactions, establishment of these relationships presents challenges for both partners.

One of the largest hurdles to the establishment and maintenance of both parasitic and endosymbiotic relationships is the host's immune response (Anbutsu and Fukatsu, 2010; Chu and Mazmanian, 2013; Molina-Cruz et al., 2013; Toth and Stacey, 2015; Yasuda et al., 2016). Immunity has evolved over millions of years with the specific purpose of eliminating non-host entities. Symbiotic partners, whether mutualistic or parasitic, are by nature non-self and therefore must suppress or evade host immune responses (Anbutsu and Fukatsu, 2010; Molina-Cruz et al., 2013; Yasuda et al., 2016). While some mutualistic symbionts have developed complex recognition pathways to prevent elimination by the immune system and establish symbiosis (Chu and Mazmanian, 2013), many other organisms have developed manipulative techniques for accomplishing similar purposes. Parasites such as *Trypanosoma cruzi* and *Plasmodium falciparum* are known to manipulate the host's transforming growth factor beta (TGFβ) pathway to suppress host immunity to establish symbiosis (Ndungu et al., 2005; Simmons et al., 2006; Waghabi et al., 2005). The TGFβ pathway is found throughout metazoans and is involved in many different processes including development, homeostasis, and immunity (Massague, 1998). Binding of activated TGFβ to receptors activates members of the SMAD transcription factor family, which elicit changes in gene expression, including changes in expression of various immune genes (Detournay et al., 2012; Kulkarni et al., 1993; Letterio and Roberts, 1998;

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Massague, 1998). For example, mice deficient in TGFβ undergo excessive inflammatory responses, suggesting that loss of TGFβ results in impaired immune regulation (Kulkarni et al., 1993). By manipulating and increasing TGFβ activation, parasites and other symbionts may be capable of directly suppressing host immune response to avoid destruction.

Coral reefs, some of the most biodiverse ecosystems on the planet (Odum and Odum, 1955; Roberts, 1995; Sebens, 1994), are comprised predominately of scelaractinian corals (Odum and Odum, 1955). Cnidarians are largely dependent on endosymbiotic dinoflagellates from the family Symbiodiniaceae (specifically the genera *Symbiodinium, Breviolum*, *Cladocopium*, and *Durusidinium*) to meet their nutritional needs (Muscatine, 1984, 1990; Muscatine and Porter, 1977); however thermal stress in particular can cause a breakdown of this relationship, resulting in eventual starvation and death of the host coral (Glynn, 1993). Consequently, these ecosystems have experienced unprecedented declines due to rising sea surface temperatures, coupled with increases in the severity and prevalence of marine disease. New findings in cnidarian models have implicated the TGFβ pathway as a link between symbiosis and immunity in cnidarians (Berthelier et al., 2017; Detournay et al., 2012). However the mechanisms and consequences of TGFβ manipulation by symbionts are poorly understood.

To investigate potential roles of the TGFβ pathway in coral immunity, we conducted a controlled multi-factorial experiment. To address the hypothesis that TGFβ signaling in cnidarians affects the immune response, we leveraged existing methodologies which have successfully been used to investigate function of TGFβ in cnidarian species (Berthelier et al., 2017; Detournay et al., 2012) as well as other non-model species (Berthelier et al., 2017; Detournay et al., 2012; Padgett et al., 1993; Sampath et al., 1993; Zoccola et al., 2009). Specificially, we used common pharmaceutical compounds to enhance or inhibit the TGFβ pathway and measured subsequent effects on host gene expression under controlled and immune stimulation conditions. Our findings here provide evidence for the immune-regulatory role of the TGFβ pathway in corals.

2. Methods

2.1. Sample collection

Colonies of *Orbicella faveolata* were collected from Brewer's Bay (GPS coordinates: 18° 20′ 38.9, −64° 58′ 56.6), a fringing reef located in a shallow embayment on the south side of St. Thomas Island, U.S. Virgin Islands. Each of the five colonies was collected using a hammer and chisel and transported in ambient seawater back to the University of the Virgin Islands Marine Laboratory facility. There, colonies were labeled and fragmented into eight pieces of approximately 5 cm in size. Fragmented corals were then placed in large water tables with flow through filtered seawater maintained at 27 °C for the duration of the experiment. Tanks were covered with shade cloth to prevent stress due to ultraviolet radiation or excessive sunlight and samples were maintained in these conditions for approximately four days prior to experimentation.

2.2. Experimental design

Prior to the experiment, the eight fragments per colony were randomly assigned to a treatment group (Table 1). Colonies were then placed in individual, sterilized 4.7 L buckets filled with ambient seawater and supplied with continuous individual aeration though out the duration of the experiment. Individual buckets were than randomly distributed between water tables filled with flow through seawater to maintain temperature. Samples were allowed to acclimate to new conditions for approximately 2 h prior to experimentation.

The experiment employed a full factorial design, combining one of four TGFβ treatments with one of two immune stimulation treatments, for a single replicate per colony (n = 5 unique genets). First, samples were inoculated with one of four TGFβ pre-treatments to manipulate activity of the TGFβ pathway. Samples were injected with 100 μL of either 0.125 μg/mL recombinant TGFβ-3 expressed in *Escherichia coli* prepared in filtered seawater (TGFβ, Sigma-Aldrich, SRP3171), 10 μg/mL anti-TGFβ, pan antibody produced in rabbit and prepared in filtered seawater (anti-TGFβ, Sigma-Aldrich, T9429), or an appropriate vehicle control. Two controls were used for this portion of the experiment: a filtered seawater vehicle served as a control for TGFβ inoculations while 10 μg/mL normal rabbit IgG antibody in filtered seawater (Sigma-Aldrich, NIO1) was used as a control for the anti-TGFβ treatment. Inoculants were injected in equal amounts in five random locations across the coral fragment. Samples were then placed back into their individual containers and incubated for approximately 2 h to allow the compounds to diffuse around the entire fragment, including the connective tissue. These methods were developed based on published studies documenting the functionaliy of TGFβ signaling in cnidarian symbioses and follow similar design to these studies (concentration and product type), adapted for coral systems (Berthelier et al., 2017; Detournay et al., 2012). To our knowledge these are the best existing methods to study the functionality of the TGFβ pathway in a non-model cnidarian species for which limited genomic and proteomic resources exist.

Following incubation with a given TGFβ treatment, fragments were again removed and injected with one of two immune stimulation treatments. Samples received either a cocktail of bacteria lipopolysaccharides from *Escherichia coli* O127:B8 (LPS, Sigma-Aldrich, L3129) and peptidoglycan from *Staphylococcus aureus* (PGN, Sigma-Aldrich, 77,140) prepared in filtered seawater (final concentration: 0.15 μg/μL LPS and 0.05 μg/μL PGN) or a vehicle (filtered seawater) control. Corals were injected with 100 μL of inoculant in equal parts across five random locations on the coral fragment and then were placed back into their individual containers for an additional 2-h incubation.

At the conclusion of the experiment, samples were removed from their individual containers and processed for later analysis. Two small (~10 μg) portions of tissue were removed and placed in microcentrifuge tubes containing 1 mL RNA-later (ThermoFisher, AM7021). RNA samples were then preserved according to manufactures instructions for later RNA extraction. The remaining core was then flash frozen in a liquid nitrogen dry shipper and stored at −80C.

2.3. RNA extraction and sequencing

RNA was extracted from a small fragment of each sample using an RNAaqueous with DNAse step kit (Life Technologies AM 1914) using a modified version of the manufacturer's instructions. Fragments with first homogenized with 800 μL of Lysis Buffer in a 2 mL microcentrifuge tube for approximately 1 min. The tube was then centrifuged on an AccuSpin Micro (Fisher Scientific) and 700 μL of the supernatant was removed and processed using the manufacturer's instructions for RNA extraction. Final RNA was eluted twice with 50 μL elution solution for a total of 100 μL eluted RNA. In order to remove DNA contamination, eluted RNA was treated with an additional DNAse step. A 25 μL aliquot of final extract was combined with 1.5 μL DNase solution and 2.95 μL of Master Mix and incubated at 37 °C for 1 h. This solution was then incubated at room temperature for 2 min with 2.95 μL inactivation reagent. The DNase step was repeated in triplicate to increase RNA yield. The final extracts (~30 μL per reaction) were combined and transferred to a new 1.5 mL tube, stored at −80 °C.

Prior to sequencing, all samples were quality assessed using an Agilent BioAnalyzer 2100 at the University of Texas at Arlington Genomics Core facility. Samples with quality values (RIN number) greater than 5 were selected for library preparation and sequencing. Seven samples did not pass quality requirements and were therefore not sequenced (see Table 1 for final n per treatment). Samples were then processed by the NovoGene Corporation for library construction and

Table 1
List of relevant contrasts analyzed to determine the effects of manipulation of the TGFβ pathway, immune stimulation, and the combination of both treatments. Included are the specific effects examined in each contrast as well as the comparisons which were made between groups of differentially expressed transcripts/ significantly enriched GO terms affected by each transcripts.

Abbreviation	1st Treatment		2nd Treatment		Effect Examined	Compared To
	TGFβ	Immune	TGFβ	Immune		
SCvRC	**S**eawater	**C**ontrol	**R**abbit Antibody	**C**ontrol	Effects of rabbit antibody	NA
SCvTC	**S**eawater	**C**ontrol	**T**GFβ	**C**ontrol	Effects of TGFβ pathway enhancement	NA
RCvAC	**R**abbit Antibody	**C**ontrol	**A**nti-TGFβ	**C**ontrol	Effects of TGFβ pathway inhibition	SCvRC
SCvSI	**S**eawater	**C**ontrol	**S**eawater	**I**mmune	Baseline immune response	RCvRI TCvTI ACvAI
RCvRI	**R**abbit Antibody	**C**ontrol	**R**abbit Antibody	**I**mmune	Effects of rabbit antibody on immune response	SCvSI ACvAI
TCvTI	**T**GFβ	**C**ontrol	**T**GFβ	**I**mmune	Effects of TGFβ enhancement on immune response	SCvSI ACvAI
ACvAI	**A**nti-TGFβ	**C**ontrol	**A**nti-TGFβ	**I**mmune	Effects of TGFβ inhibition on immune response	SCvSI RCvRI TCvTI

sequencing. A eukaryotic RNA-Seq library of total RNA extracts was constructed for each sample and libraries were sequenced on an Illumina Hiseq X with 150 base pair, paired end reads.

2.4. Transcriptome analysis

Raw reads were obtained from NovoGene and filtered using the software package Trimmomatic, (v. 0.32; (Bolger et al., 2014). Adaptors and low quality reads were removed, and resulting sequences less than 36 base pairs in length were discarded. Sequences were then aligned to the reference *Orbicella faveolata* transcriptome (Pinzon et al., 2015) using the Tophat software package (v. 2.1.1 (Trapnell et al., 2009); with default parameters. Read counts were obtained using the Cufflinks package (v 2.2.1 (Trapnell et al., 2010); cufflinks and cuffmerge) and a read count matrix was generated from results using the htseq software package (v 0.6.1; (Anders et al., 2015).

Differential expression was conducted using the R package DESeq2 (R v. 3.4.3; DESeq2 v. 1.18.1; (Love et al., 2014). Outliers were assessed using PCA analyses, and none wdetected. Prior to analysis, reads were variance stabilizing transformed to generate normalized read counts. Differential expression was modeled using treatment combination as a single effect. Average $\log_2$fold change per transcript was then generated for all relevant contrasts between treatment combinations (Table 1). Significantly differentially expressed transcripts were identified as those where $padj < 0.05$ with Bonferroni corrections made for multiple comparisons (padj values from each contrast from DESeq2 were multiplied by the number of contrasts (7); resulting values less than 0.05 were considered significant).

2.5. Transcriptome annotation and gene ontology analyses

The reference host transcriptome was re-annotated against the UniProtKB/Swiss-Prot database downloaded in January of 2018 prior to analysis of differentially expressed transcripts. A blastx algorithm with a minimum e-value threshold of 1.0E-5 was used for this analysis. The resulting annotations were used to conduct gene ontology analysis of the effects of each treatment using the R script GOMWU (Wright et al., 2015) with default parameters. The script, which has been used in numerous similar transcriptomics studies (Kenkel and Bay, 2017; Wright et al., 2015), uses any continuous measure of significance (here $\log_2$fold change) and associated GO terms (if annotated) for every transcript in the dataset to generate a rank-based estimate of enriched GO terms. This rank-based approach increases statistical power and generates a statistically meaningful picture of the biological processes that are changing under a given treatment (as opposed to single genes). GO terms were considered significant if their adjusted *p*-value was less than 0.05.

3. Results

3.1. Differential gene expression analysis

We conducted differential expression analyses to identify genes which were significantly effected by each of our treatments. Full transcriptome sequencing resulted in a total of 833,787,572 combined reads for all 30 samples following initial quality control and prior to further trimming (average of 25,266,290 reads per sample; minimum 20,000,000 reads per sample). Raw sequencing reads are available for download via NCBI Genbank (PRJNA579770). Seven contrasts were conducted to test for the effects of manipulation of the TGFβ pathway under control and immune stimulation condition (Table 1). In total, 68 unique transcripts were affected by one of these teatments (Supplementary File 1). Manipulation of TGFβ pathway in the absence of immune stimulation had a limited effect on gene expression. Addition of exogenous TGFβ resulted in differential expression of five transcripts, of which one was annotated (histamine H2 receptor). Addition of anti-TGFβ resulted in the differential expression of six transcripts when compared to rabbit antibody controls, of which one was annotated (kinesin-1 heavy chain).

In contrast, immune stimulation affected host gene expression to a greater extent. Immune stimulation alone (contrast between **S**eawater control and immune buffer **C**ontrol (SC) and **S**eawater control and **I**mmune stimulation (SI)) resulted in the differential expression of 40 transcripts, of which 11 were annotated. This included a transcript annotated as a putative stress-response protein, protein dual specificity protein phosphatase 1B, and one annotated as a putative immune protein, serine/threonine-protein phosphatase 5 (PP5). The group of transcripts differentially expressed as a result of immune stimulation in specimens pre-treated with the rabbit antibody control (contrast between **R**abbit antibody control and immune buffer **C**ontrol (RC) and **R**abbit antibody control and **I**mmune stimulation (RI)) largely xoverlap with those transcripts differentially expressed as a result of immune stimulation alone (Fig. 1). Addition of immune stimulant to corals treated with rabbit antibody controls (RCvRI) resulted in differential expression of 47 transcripts, of which 27 were also differentially expressed as a result of immune treatment alone (SCvSI). 14 transcripts in this group were annotated, including PP5 and putative defense transcript probable serine/threonine-protein kinase PBL18.

A single transcript was differentially expressed following immune stimulation by samples pretreated with TGFβ (contrast between **T**GFβ pretreatment and **C**ontrol immune buffer (TC) and **T**GFβ pretreatment and **I**mmune stimulation (TI)). This transcript was annotated as tolloid-like protein 2. Similarly, only two transcripts were differentially expressed as a result of immune stimulation to samples pretreated with

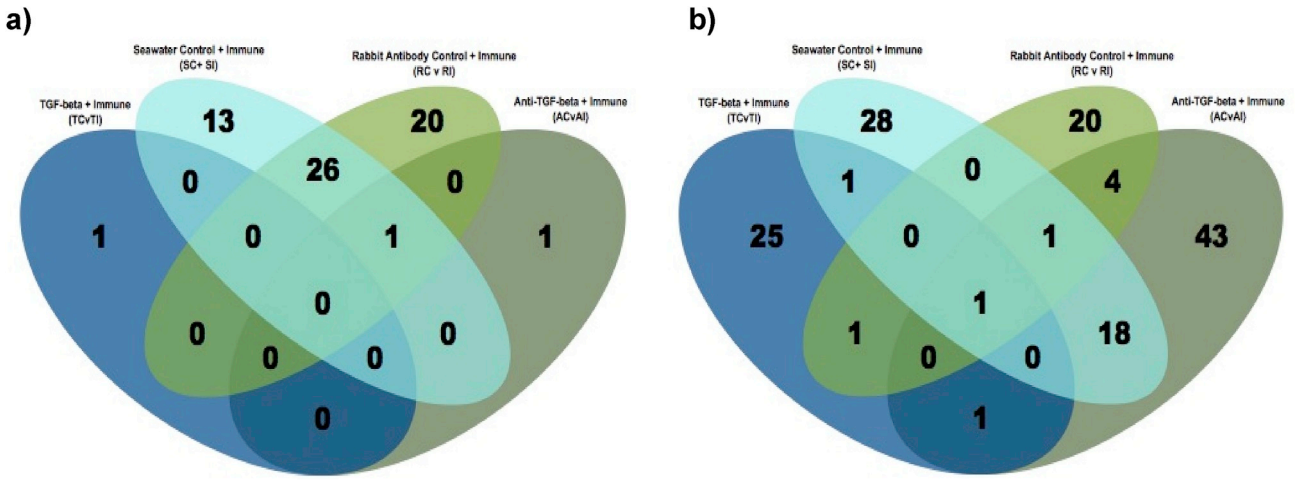

Fig. 1. Ovelap of significantly differentially expressed genes and expressed GO terms between comparisons
a) Summary of overlap of differentially expressed transcripts as a result of immune stimulation within each of the four TGFβ pretreatment groups. Numbers represent amount of transcripts differentially expressed as a result of each contrast, or groups of contrasts.
b) Summary of overlap of significantly enriched biological process GO terms as a result of immune stimulation within each of the four TGFβ pretreatment groups. Numbers represent amount of enriched GO terms as a result of each contrast, or groups of contrasts.

anti-TGFβ (contrast between **A**nti-T**G**F**β** pretreatment and **C**ontrol immune buffer (AC) and **A**nti-T**G**F**β** pretreatment and **I**mmune stimulation (AI)). Neither of these transcripts were annotated (Supplementary File 1).

3.2. Expression of putative TGFβ pathway components

A total of 38 transcripts with annotations involved in TGFβ signaling were expressed in one or more of our coral samples (Supplementary File 2). This included nine copies of various SMAD signaling molecules, and three transcripts annotated as a bone morphogenic protein. While none of these transcripts was significantly differentially expressed as a result of enhancement or inhibition of the TGFβ pathway, several demonstrated contrasting patterns of differential expression as a result of enhancement or inhibition of the TGFβ pathway (Fig. 2

3.3. Gene ontology analyses

Gene ontology (GO) analysis confirmed that manipulation of the TGFβ pathway had limited effects on host corals in the absence of immune stimulation (Supplementary File 3). A total of three biological process GO terms were significantly enriched as a result of enhancement of the pathway via the addition of TGFβ (Supplementary Fig. 1). In contrast, no biological process (BP) GO terms were differentially expressed as a result of inhibition of the pathway by addition of anti-TGFβ. It should be noted that the addition of the rabbit antibody control (SCvRC) did result in significant enrichment of six BP GO terms, none of which were involved in immune or stress response (Supplementary Fig. 2).

By comparison patterns of gene expression associate with immune stimulation alone, and in combination with manipulation of host TGFβ pathway, resulted in significant enrichment of many BP GO terms. A large portion of these were significantly enriched as a result of multiple treatments (Fig. 1). Immune stimulation alone resulted in significant enrichment of 49 BP GO terms, all but two of which were positively enriched (Fig. 3). Of these enriched terms, 20 were related to immune system processes, all of which were positively enriched. The combination of rabbit antibody and immune stimulation resulted in significant enrichment of 27 terms, many of which were involved in RNA processing (Supplementary Fig. 2).

The combination of TGFβ pathway manipulation and immune stimulation had significant effects on gene ontology enrichment. Immune stimulation in samples pretreated with TGFβ (TCvTL) resulted in enrichment of 29 BP GO terms, only one of which was involved in immune response (Fig. 3). Additionally multiple terms related to reactive oxygen species processes were negatively enriched as a result immune stimulation following TGFβ pretreatment. Samples which had been pretreated with anti-TGFβ (ACvAL) prior to immune stimulation had contrasting patterns of gene ontology enrichment. The immune response of corals treated with anti-TGFβ prior to immune stimulation resulted in enrichment of 68 BP GO terms, 62 of which were positively

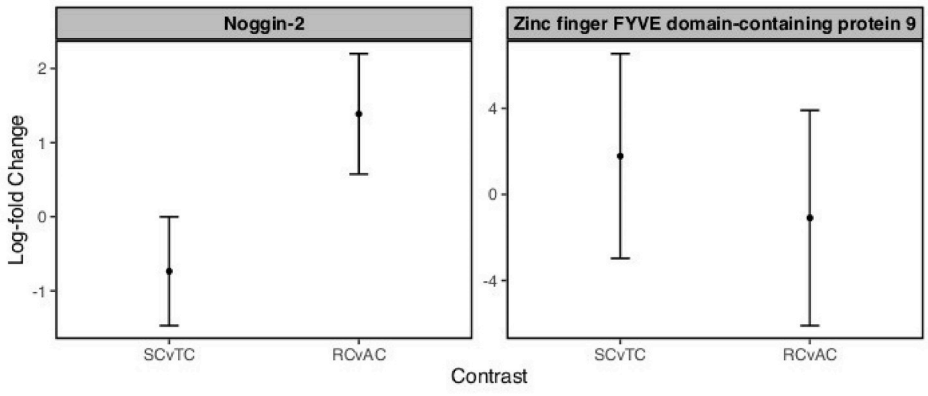

Fig. 2. Expression changes of genes associated with TGFβ Plot of log_2fold change of putative TGFβ contigs as a result of the addition of exogenous TGFβ and anti-TGFβ. Error bars display standard error of log_2fold change. Filtering parameters were used to select displayed transcripts. Those shown are transcripts that had a standard error of greater than 1 (i.e. the most variable) between log_2fold change values for the effects of the addition of TGFβ (SCvTC) and addition of anti-TGFβ (RCvAC).

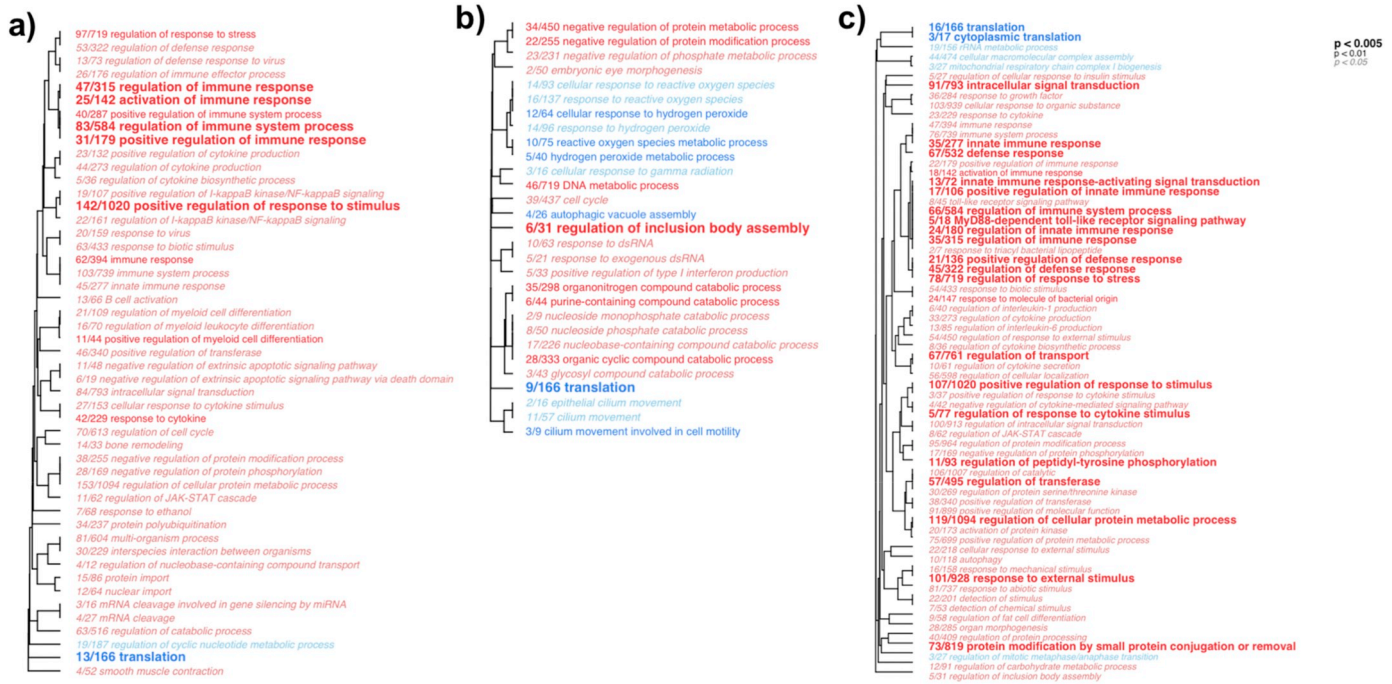

Fig. 3. Gene ontology enrichment as a result of immune treatment
Hierarchical clustering of differentially expressed biological process gene ontology terms that were significantly enriched as a result of immune stimulation in samples that were pretreated with **a)** seawater control (SCvSI) **b)** exogenous TGFβ (TCvTI) and **c)** anti-TGFβ (ACvAI). Terms in red were positively enriched, terms in blue were negatively enriched. Ratios on each branch indicate the ratio of statistically significant transcripts within a term compared to the total number of transcripts included in that term. Font style (bold/italicized) indicates significance of each term, as indicated by the legend

enriched (Fig. 3). This included 26 positively enriched terms involved in various aspects of the immune response such as cytokine and Toll-like receptor signaling.

In total, 40 immune related BP GO terms were significantly enriched as a result of immune stimulation in one or more pretreatment groups. A total of 1397 transcripts were involved in one or more of these significantly enriched immune biological processes. Included in this list of transcripts were those with functions related to a variety of immune processes, including inflammation and Toll-like receptor signaling processes (Fig. 4). Many of these transcripts, while not significantly differentially expressed, did show contrasting patterns of expression as a result of both immune stimulation and manipulation of the TGFβ pathway. Specifically several positive regulators of immune response increased in expression following immune stimulation in corals pretreated with anti-TGFβ, but decreased in expression, or increased to a lesser magnitude, in response to immune stimulation when pretreated with TGFβ (Fig. 4).

4. Discussion

While it is well-documented that there is considerable overlap between symbiosis and immunity in a variety of systems (Akamatsu et al., 2016; Toth and Stacey, 2015; Yasuda et al., 2016), these potential relationships in cnidarians are understudied (Correa et al., 2009; Detournay et al., 2012; Detournay and Weis, 2011). Here we describe the effects of manipulating host TGFβ pathway in the scleractinian coral *O. faveolata*. We document the limited impact of manipulating the TGFβ pathway under control conditions, which is in stark contrast to the effects of enhancing or inhibiting the TGFβ pathway on subsequent host immune response. Our findings indicate that variation in TGFβ pathway activity has significant effects on the coral immune response, and potentially subsequent disease susceptibility. Here we summarize our findings and highlight the potential associated ecological consequences of these results.

While previous studies have documented aspects of the TGFβ pathway in other cnidarians (Berthelier et al., 2017), limited resources exist regarding their genomic presence and expression in scelaractinian corals. The use of pharmaceutical TGFβ-3 and anti-TGFβ allowed us to explore the effects of pathway manipulation (Berthelier et al., 2017; Detournay et al., 2012). While we do not have direct evidence that the pharmaceuticals bound directly to *O. faveolata* TGFβ pathway components, both Berthelier et al. and Detournay et al. successfully used the same compounds to enhance and block TGFβ in corals and anemones (Berthelier et al., 2017; Detournay et al., 2012), and similar techniques have been used in a wide range of other non-model systems (Padgett et al., 1993; Sampath et al., 1993; Zoccola et al., 2009). As evidence of the direct effects of these compounds on the *O. faveolata* pathway, we observed response of TGFβ pathway components to these treatments. Two pathway components, noggin 2 and zinc finger FYVE domain-containing protein 9 (ZFYVE9), displayed contrasting changes in expression as a result of the addition of TGFβ and anti-TGFβ. In particular, ZFYVE9, which promotes TGFβ signaling and SMAD recruitment (Tsukazaki et al., 1998), increased in expression with the addition of TGFβ and decreased with addition of anti-TGFβ. In contrast, Noggin-2, which can negatively regulate TGFβ and related pathways (Groppe et al., 2002), showed opposite trends of expression when the pathway was enhanced or inhibited. These findings are consistent with effective enhancement or inhibition of the TGFβ by these compounds in *O. faveolata*. This is in agreement with the above mentionsed studies which have used similar commercially available compounds to measure functionality of these and related pathways in other non-model organisms (Berthelier et al., 2017; Detournay et al., 2012; Padgett et al., 1993; Sampath et al., 1993; Zoccola et al., 2009). Thus, based on the preponderance of previously published literature using this methodology, and the consistency of our results, we are confident that our data reflect the effects of changes in TGFβ signaling on host coral immunity.

Addition of exogenous TGFβ or anti-TGFβ had limited effects on host coral gene expression under control conditions, as evidenced by the low number of significantly differentially expressed genes and few

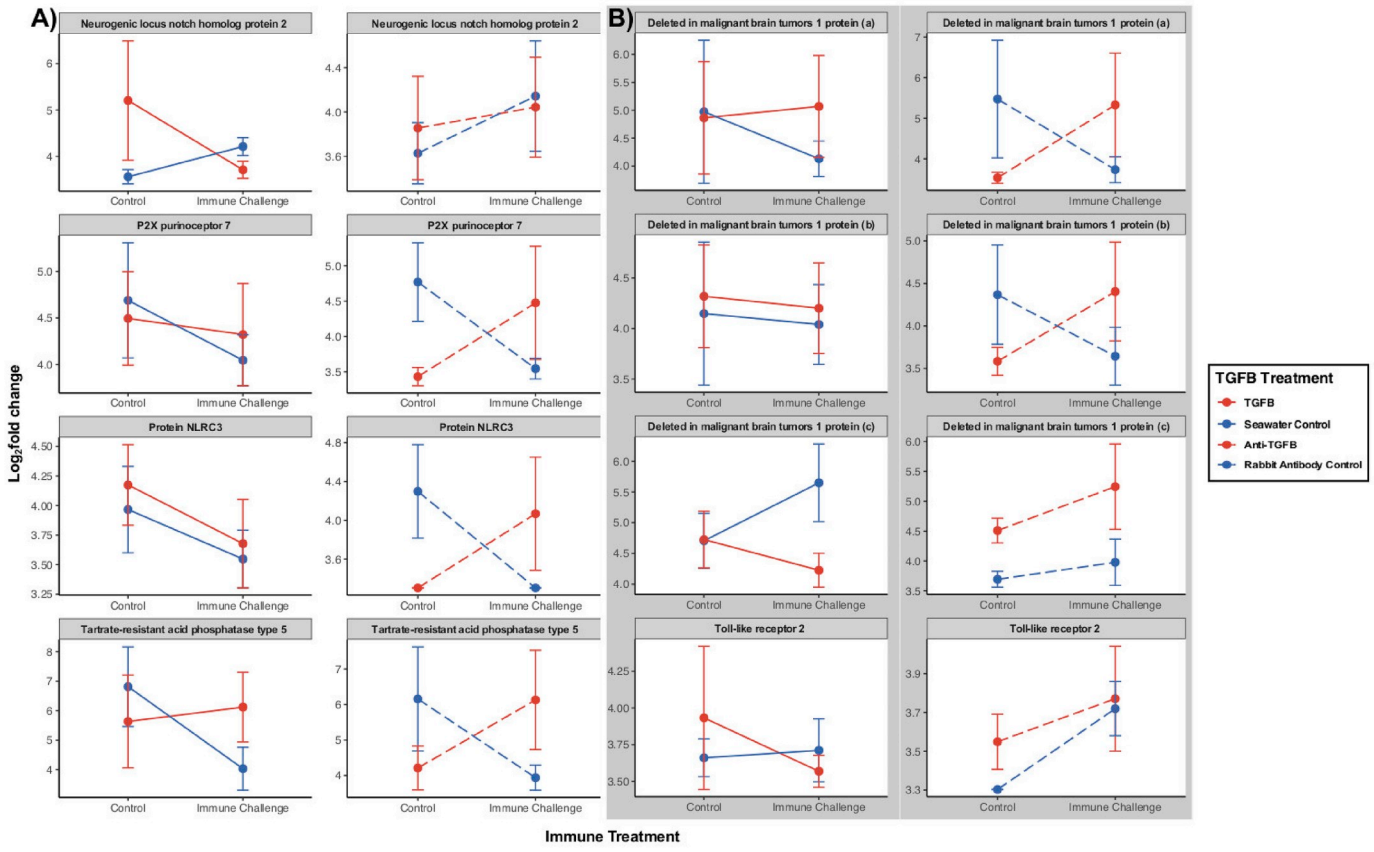

Fig. 4. Differential expression of genes involved in inflammation and Toll-like receptor signaling
Paired interaction plots showing the effects of both immune stimulation and manipulation of the TGFβ pathway on expression of genes involved in **A)** inflammatory processes and **B)** Toll-like receptor signaling. Genes show opposing trends in response to immune stimulation following TGFβ pathway enhancement (solid lines) or inhibition (dashed lines). Responses in gene expression are more pronounced when corals are pretreated with anti-TGFβ, inhibiting the pathway. Filtering parameters were used to select the displayed transcripts. Those shown are transcripts which were involved in a significantly enriched immune GO term, were expressed in all five colonies, and underwent similar changes in expression as a result of both seawater and rabbit antibody control (direction of change was the same). Genes shown are those with the highest variation (highest standard error) in log_2fold change when comparing TCvTI and ACvAI.

significantly enriched GO terms as a result of these treatments. Enhancement of the pathway (by addition of exogenous TGFβ) predominantly increased microtubule and ciliary action. Interestingly, both microtubule networks and cilia may regulate TGFβ activity in different capacities (Batut et al., 2007; Christensen et al., 2017; Dong et al., 2000; Labour et al., 2016). Microtubule networks may serve to sequester SMAD molecules and thus reduce downstream TGFβ signaling (Dong et al., 2000). Other microtubule-associated proteins such as kinesin are necessary for the transport and activation of SMADs (Batut et al., 2007). Similarly, primary cilia have been implicated in many types of TGFβ signaling (Christensen et al., 2017; Labour et al., 2016). Therefore observed enrichment of these two processes is likely a result of increased downstream signaling and regulation following addition of exogenous TGFβ. In contrast, addition of anti-TGFβ to reduce or block TGFβ signaling resulted in almost no effects on host gene expression under constitutive conditions.

The limited effects of manipulation of the TGFβ pathway in the absence of immune stimulation is particularly surprising considering the diverse functions of TGFβ signaling in metazoans (Massague, 1998). However, it is possible that the dosage used and/or duration of the experiment was insufficient to observe significant effects of manipulation of the TGFβ pathway under constitutive conditions. An additional explanation is that under control conditions corals are capable of adapting to variation in the TGFβ pathway using moderate changes in a diversity of genes. This type of response would be difficult to detect using common statistical approaches and therefore explains our lack of observed effect.

While manipulation of the TGFβ signaling pathway had minimal effects on corals under constitutive conditions, its effects on subsequent immune response were notable. Control corals exposed to immune stimulation mounted a strong response marked by positive enrichment of multiple general and specific immune GO terms, including those involved with NF-κB signaling and apoptosis. Both NF-κB (Williams et al., 2018) and apoptotic (Fuess et al., 2017) signaling have been demonstrated to be key components of the cnidarian immune response. The responses of control coral to immune stimulation are generally comparable to past studies of both *O. faveolata* and other scleractinian corals (Fuess et al., 2016, 2017; van de Water et al., 2018; Wright et al., 2015, 2017). Addition of exogenous TGFβ so as to enhance downstream signaling resulted in a notable reduction of this immune response, as evidenced by gene ontology analysis. Multiple invertebrate studies in other systems have found TGFβ to be a potent immune regulatory cytokine (Letterio and Roberts, 1998; Travis and Sheppard, 2014). Similar patterns have been preliminarily reported in other model cnidarians (Detournay et al., 2012), but our results here are by far the most comprehensive documentation of these effects. In addition to reduction of immune response, we also observed negative enrichment of multiple GO terms associated with antioxidant responses following immune stimulation. This is particularly relevant as antioxidant production is a demonstrated component of both the coral immune response (Mydlarz et al., 2010; Palmer et al., 2009, 2011; Pinzon et al., 2014), and host response to thermal stress and breakdown of symbiosis resulting in bleaching (Barshis et al., 2013; Jin et al., 2016). Previous studies in the sea anemone *Exaptasia pallida* have indicated that enhancement of

TGFβ signaling using similar methods results in both decreases in bleaching susceptibility and production of nitric oxide following heat stress (Detournay et al., 2012). Therefore it is possible that the addition of exogenous TGFβ increases host stress tolerance, therefore increasing thresholds for activation of responses such as antioxidant production.

In contrast, addition of anti-TGFβ to inhibit downstream signaling not only preserved innate immune responses, but also appeared to strengthen the response of corals to immune stimulation as evidenced by a larger number of immune-related GO terms that were positively enriched. These GO terms were also related to more specific types of immune response such as interleukin and Toll-like receptor signaling, both of which are key components of innate immune response (Mydlarz et al., 2008, 2016; Palmer et al., 2008; Williams et al., 2018). Furthermore, comparison of a subset of immune-annotated transcripts involved in these GO terms indicates contrasting patterns of expression between corals pretreated with anti-TGFβ compared to those pretreated with TGFβ. While these patterns were not statistically significant, they contributed to larger patterns of GO enrichment which were indeed significant. Transcripts involved in inflammation and TLR signaling generally showed increases that were consistent with strengethen immune responses in anti-TGFβ pretreated coral samples, whereas these transcripts were either downregulated or weakly upregulated in corals treated with TGFβ. Inflammation and TLR signaling are two immune processes which are intimately linked with TGFβ signaling (Clark et al., 2011; Lee et al., 2011; Yoshimura and Muto, 2011), therefore it is unsurprising to observe effects of manipulating TGFβ on expression of components of these pathways. Additionally it is interesting to note the upregulation of anti-inflammatory NLRC3 in samples pre-treated with anti-TGFβ, compared to downregulation of this same transcript in samples treated with TGFβ. Related to its roles preventing excessive immune response, the TGFβ pathway often regulates inflammation (Yoshimura and Muto, 2011; Yoshimura et al., 2010). Therefore the observed patterns of NLRC3 expression are indicative of a secondary immuno-protective mechanism that host corals may employ to prevent excessive inflammatory responses to stimulation independent of TGFβ signaling.

Here we report contrasting effects of TGFβ signaling on host gene expression dependent on host immune state that may have ecological implications. A growing number of studies have indicated a role of the TGFβ pathway in the establishment and maintenance of symbiotic relationships in cnidarians (Berthelier et al., 2017; Detournay et al., 2012). Furthermore, there is indication that symbionts may directly manipulate and increase host TGFβ signaling (Berthelier et al., 2017; Detournay et al., 2012). It is reasonable to predict that variation in symbiotic relationships (symbiont types, density, etc.) may result in variation in constitutive TGFβ signaling within and between coral species. Our findings here suggest ecological context for the consequences of this variation in a changing environment.

While we observed limited effects of manipulation of the TGFβ pathway on corals under non-stressful conditions, this relationship changes considerably following immune stimulation. Our results suggest that variation in TGFβ signaling impacts the ability of a coral to respond to pathogenic threats that may ultimately result in infection or disease. In particular the elimination of an immune response following increases in TGFβ signals suggests a potential ecological trade-off between symbiosis and immune response. If indeed increased TGFβ signaling results in reduced capacity to respond to immune stimulation, and symbiont density is positively correlated to TGFβ signaling as would be expected from previous findings (Berthelier et al., 2017; Detournay et al., 2012), symbiosis and its benefits may come at the cost of immune capabilities. Furthermore, these reduced immune capabilities would likely result in increases in disease and disease-related mortality as the prevalence and severity of coral disease continue to increase (Riegl and Purkis, 2015; Sokolow, 2009). The idea that increased symbiont density is actually a detriment to the host is not a new concept; numerous studies have suggested that excessive amounts of symbionts may increase host susceptibility to bleaching (Cunning and Baker, 2013, 2014). Here our results indicate that a similar pattern may be true in disease-contexts. Interestingly however, our results indicate that this trade-off occurs predominantly in the context of active immune stimulation, potentially presenting a new evolutionary conundrum. The rapid increase in disease severity and prevalence on reefs in recent decades (Daszak et al., 2001; Harvell et al., 1999; Sutherland et al., 2004) would indicate that the trade-off documented here between symbiosis and immunity occurs in a relatively new context. Therefore continued increases in disease prevalence may produce a selective event that will affect future coral-symbiont relationships. Consequently, it is important to further explore the relationship between symbiosis, TGFβ signaling, and immunity in cnidarians so as to increase understanding of factors contributing to coral disease susceptibility.

In conclusion, here we document effects of manipulating coral TGFβ signaling on subsequent induced immune response. To our knowledge this is the first empirical evidence of the immune-suppressive roles of the TGFβ pathway in a scleractinian coral. As the TGFβ pathway is known to contribute to the maintenance of a range of parasitc and mutualistic relationships, understanding its roles in scelaractinian corals which depend on symbiotic relationships is paramount. Here we lay the groundwork for increasing understanding of the TGFβ pathway, and highlight potential ecological considerations that warrant further investigation. Specifically, when coupled with existing research implicating the roles of TGFβ in symbiosis establishment and maintenance, our results suggest potential ecological trade-offs between symbiosis and immunity. It is therefore important to continue to explore the role of the TGFβ pathway in both the context of both symbiosis and immunity in corals to better predict reef community trajectories in the coming years.

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Author contributions

LDM, MEB, and LEF planned the experiments. MEB collected samples. All authors participated in conducting the experiments. LEF and CCB processed samples. LEF conducted all statistical analyses. LEF wrote the manuscript, with assistance from all authors.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2020.103639.

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