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Marine invertebrates cross phyla comparisons reveal highly conserved immune machinery

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ABSTRACT

Naturally occurring histocompatibility responses, following tissue-to-tissue allogeneic contacts, are common among numerous colonial marine invertebrate taxa, including sponges, cnidarians, bryozoans and ascidians. These responses, often culminating in either tissue fusions or rejections, activate a wide array of innate immune components. By comparing two allorejection EST libraries, developed from alloincompatible challenged colonies of the stony coral *Stylophora pistillata* and the ascidian *Botryllus schlosseri*, we revealed a common basis for innate immunity in these two evolutionary distant species. Two prominent genes within this common basis were the immunophilins, Cyclophilin A (CypA) and FK506-binding protein (FKBP). *In situ* hybridizations revealed that mRNA expression of the coral and ascidian immunophilins was restricted to specific allorecognition effector cell populations (nematoblasts and nematocytes in the coral and morula cells in the ascidian). The expressions were limited to only some of the effector cells within a population, disclosing disparities in numbers and location between naïve colonies and their immune challenged counterparts. Administration of the immunosuppression drug Cyclosporine-A during ascidian's allogeneic assays inhibited both fusion and rejection reactions, probably through the inhibition of ascidian's immunocytes (morula cells) movement and activation. Our results, together with previous published data, depict an immunophilins-based immune mechanism, which is similarly activated in allogeneic responses of distantly related animals from sponges to humans.

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Introduction

Allorecognition, the ability to discriminate between conspecific self and non-self, has probably evolved over 600 million years ago, concurrently with the development of multicellularity, allowing the demarcation of the very first multicellular organisms (Srivastava et al. 2010). Colonial marine invertebrates, including taxa of sponges, cnidarians, bryozoans and ascidians, express allorecognition proficiencies that exemplify high specificity and accuracy, including extensive allotypic diversity that in some cases, abide by strict mendelian inheritance rules (Scofield et al. 1982; Grosberg 1988; Rosengarten and Nicorta 2011). The sessile life style of these organisms and the frequently aggregated larval settlements generate densely populated communities in which intra-species encounters are common, usually resulting in two archetypal allogeneic responses. The first is the morphological fusion of interacting genotypes and the formation of chimerical entities. The second is a destructive immune rejection response, carried out by specific effector cell-populations, which lead to the physical separation between allogeneic parties. Its cellular origin and its characterization vary between different phyla from archeocytes and granulated gray cells in sponges (Humphreys 1994), nematocytes in the cnidarians (Buss et al. 1984; Lange et al. 1989) to morula cells in the ascidians (Rinkevich et al. 1998; Ballarin et al. 2001). In these phyla and in other invertebrate taxa, the activated effector cells reach and converge into allogeneic contacting areas, where they often discharge their harmful contents, culminating in tissue damage, necrosis, and even whole organismal death (Buss et al. 1984; Ballarin et al. 1998; Rinkevich 2005).

Based on the continuously extending genomic data and new molecular tools now available, recent studies have attempted to scan invertebrates genomes, including Cnidaria and Urochordata (*i.e.* Miller et al. 2007), addressing the crux of innate immunity. Yet, little is known about the actual roles of the identified genetic elements in immune defense and allogeneic responses.

Here we compared allogeneic rejection transcriptomes in representatives of two disparate vertebrate phyla, the cnidarian *Stylophora pistillata*, a branching coral, representing one of the earliest multicellular animal phyla and the colonial tunicate *Botryllus schlosseri*, a urochordate, closely related to the vertebrates. This comparison revealed common expression patterns of specific immune-related genes as well as shared functional attributes

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expressed during allogeneic rejection in the two species. Two genes from the list, the immunophilins FK506-binding protein (FKBP) and Cyclophilin A (CypA), exemplified parallel expressions in the allorejection effector cells; the coral's nematocytes and the ascidian's morula cells. Cyclosporine A, an immunosuppression drug, which targets CypA, inhibited fusion and rejection in *B. schlosseri*. Based on these results and previous studies, we portrayed here both functional and molecular cross-phyla resemblances, indicating the existence of a highly conserved innate immune mechanism in the animal kingdom.

Materials and methods

Allorecognition assays and the administration of Cyclosporine A (CsA)

For allorecognition assays we used young *S. pistillata* colonies (10–18 months old) originated from field collected planulae, as described (Oren et al. 2010) and laboratory-bred *B. schlosseri* colonies, offspring of founders collected from several coastal locations (Oren et al. 2007). Allorecognition assays were performed on both organisms as described (Rinkevich 1995).

In order to study the physiological effects of CsA on *Botryllus* allogeneic interactions, allogeneic compatible and incompatible pairs of *Botryllus* colonies were submerged in 1 mg CsA in 100 ml seawater (10 μg/ml) just after initial tunic contacts were established between allogeneic pairs. As control, we used clones of the interacting genotypes (ramets), which were kept in 100 ml of seawater in identical conditions.

Light and electron transmission microscopy

Histological preparations were made on tissue samples taken from allogeneic interacting and naïve *Stylophora* and *Botryllus* colonies. Samples were fixed in 4% formaldehyde for 2 h, embedded in paraffin, serially cross-sectioned (5 μm) and stained by hematoxylin and eosin. Observations were performed under Olympus B×50 Upright microscope, equipped with Color View camera (Soft Imaging System, Munster, Germany).

For EM analyses, naïve and allogeneic interacting ramets were fixed in 2.5% glutaraldehyde in seawater and stored at 4 °C for 10 days. Next, they were washed twice in 0.2 M cacodylate buffer (pH 7.2–7.4) and postfixed by 0.1% OsO_4 in the same buffer for 1 h, at room temperature. After three buffer washes, samples were dehydrated by ethanol series and embedded in Epon 812. Zones of interest were selected and positioned properly in flat embedding molds. Ultrathin sections of 80 nm were cut with Leica Ultracut R ultramicrotome. Finally, the sections were stained with uranyl acetate and lead citrate. Grids were observed under a Hitachi 7500 electron microscope. Alternatively, thin sections of 1 μm were stained by a mixture of toluidine blue and azure II and observed under a Leica DME photonic microscope.

In situ hybridization

Interacting histoincompatible *B. schlosseri* and *S. pistillata* pairs of colonies and their naïve counterpart ramets were fixed for 2 h in 4% paraformaldehyde following 70% ethanol overnight, dehydrated in 70% methanol, embedded in paraffin, and cut into 5 μm sections. Total RNA was extracted, separately, from interacting pairs and from corresponding naïve ramets ($n = 4$) by Epicentre MasterPure^{TM} RNA Purification kit (cat. no. MC85102, Madison, Winconsin, USA). The integrity of the total RNA was verified by 1% agarose gel electrophoresis. First strand cDNA was synthesized by DNA synthesis kit (cat. no. K1622, Fermentas, MD, USA). Based on our EST database (Oren et al. 2007, 2010), we designed primers that were used to obtain sense and antisense DIG-labeled RNA probes. The probes were cloned into pdrive plasmid (cat. no. 231124, Qiagen, Valencia, CA) and used as templates for synthesizing the appropriate DIG-labeled RNA probes (sense and antisense) using DIG RNA Labeling Kit (SP6/T7; Roche Diagnostics, Penzberg, Germany). Hybridization of probes to tissue sections was performed according to Breitschopf et al. (1992) for paraffin-embedded tissues. DIG-labeled RNAs on samples were observed using anti-DIG antibody (cat. no. 11277073910, Roche, Mannheim, Germany), Nitro Blue Tetrazolium (cat. no. N5514, Sigma Aldrich, St. Louis, Missouri, USA) and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (cat. no. b0274, Sigma Aldrich, St. Louis, MO, USA) as substrates for peroxidase activity.

Quantitative RT PCR

RNA was extracted from three pairs of interacting *Botryllus* colonies and their naïve counterparts as described in '*In situ* hybridization' section. First strand cDNA was synthesized in a total volume of 20 μl with the Thermo Scientific Verso cDNA synthesis kit. The PCR amplification was performed using designed sets of primers (IDT Inc.). The real time PCR mixture consisted of 40 nM cDNA sample, 70 nM of each primer and 12.5 μl of SYBR Green mix (Abgene, Epsom, UK), in a final volume of 25 μl. RT PCR was carried out in the GeneAmp 5700 PCR thermocycler (PE Applied Biosystems, Foster City, CA) under the following conditions: 95 °C for 15 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. Amplification of cDNAs was performed in triplicates for each interacting pair and the relative abundance of each selected mRNAs was normalized in reference to 18S rRNA (accession no: AB211066). Data analysis was performed according to Pfaffl (2001).

Results

S. pistillata and B. schlosseri allorejection repertoires

Many of the allogeneic interactions in *S. pistillata* and *B. schlosseri* resulted in incompatible responses. *Stylophora* histoincompatible interactions developed into necrotic fronts at contact areas (Amar et al. 2008; Oren et al. 2010; Fig. 1a) while those of *Botryllus* developed cytotoxic necrotic lesions along the contact line between the interacting colonies (points of rejection, PORs; reviewed in Rinkevich 2005; Fig. 1b). The effector cells in *Botryllus* allorejection are the morula cells; berry-shaped blood cells equipped with several, transparent to yellowish-green vacuoles (approx. 2-μm in diameter), hosting the phenol-oxidase activity (Rinkevich et al. 1998; Ballarin et al. 2001; Fig. 1c and d). During the rejection process, morula cells accumulated at the tips of interacting ampullae (Fig. 1c) then infiltrated through the ampullar epithelium to the tunic matrix where they released their content and degenerated, digested or phagocytized by circulating phagocytes (Fig. 1d).

Two subtraction libraries were prepared for the allogeneic rejection processes in *S. pistillata* and in *B. schlosseri*, using the same methodology (Oren et al. 2007, 2010). The libraries were based on total RNA extracted from rejecting *Botryllus* and *Stylophora* colonies and from their correlated genetic naïve clones. During the subtraction protocol, ESTs unique to the rejecting clones were enriched by selectively eliminating the ESTs that were shared between the two extractions. The coral library contained 1760 high quality sequences including 230 contigs and 1530 singlets (redundancy of 22.6%). The ascidian library contained 1693 high quality sequences including 217 contigs and 1479 singlets (redundancy of 21.6%). A blast analysis revealed that 51% of the ascidian sequences

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