A basal chordate model for studies of gut microbial immune interactions

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INTRODUCTION

Because of their phylogenetic position relative to vertebrates, deuterostome invertebrates (Figure 1) are compelling models for studies of immunity. Of the representative deuterostome invertebrates, echinoderms, which include sea urchins and starfish, diverged prior to chordates and have proven to be highly informative models in which to examine innate immunity (Rast and Messier-Solek, 2008; Messier-Solek et al., 2010). Protochordates are comprised of invertebrate chordates such as sea squirts (Urochordata or Tunicata) and amphioxus (Cephalochordata). These species, which share certain developmental features with vertebrates and possess competent innate immunity, diverged prior to the origins of the vertebrate adaptive immune system.

Ciona intestinalis (sea squirt), which has been the focus of our recent efforts, is relatively easy to maintain and propagate (Figure 2) at room temperature and continues to serve as a highly informative model for studies of animal development (Katz, 1983; Heinertzhagen and Okamura, 2001; Canestro et al., 2003; Shi et al., 2006). As a model of animal development (Satoh and Levine, 2005; Lemaire et al., 2008; Christiaen et al., 2009) Ciona has proven invaluable in: (1) unraveling details of Hox-gene influences on development (Ikuta et al., 2010), (2) mapping pathways in cardiac development (Davidson, 2007), (3) defining the roles of cis-regulatory networks (Kubo et al., 2010), (4) modeling the effects of maternally derived epigenetic silencing (Sasakura et al., 2010), and (5) defining the evolution of the cell death machinery (Terajima et al., 2003; Baghdiguian et al., 2007). Many of the involved processes utilize signaling pathways that are relevant to studies concerning immunity and immune homeostasis.

Deuterostome invertebrates possess homologs of a large number of vertebrate innate immune receptors, effectors, and their corresponding regulatory elements (Rast and Messier-Solek, 2008; Messier-Solek et al., 2010). The most surprising finding regarding immunity in sea urchin and amphioxus is the expansion of multigene families encoding homologs of different innate immune pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), scavenger receptors, and leucine-rich receptor (LRR)-containing intracellular sensors such as nucleotide-binding oligomerization domain-like receptors (NLRs; Rast et al., 2006; Holland et al., 2008). Novel evolutionary constraints most likely led to the lineage-specific expansions and functional divergence of the various gene families encoding these molecules in amphioxus and sea urchin. Detailed sequence analyses have revealed examples of parallel or convergent evolution relating to the expansions (Leulier and Lemaitre, 2008). Very little sequence similarity among presumed homologs (i.e., lack of one-to-one orthology) is evident between these receptors in invertebrate deuterostomes and their vertebrate counterparts. Innate immune genes in Ciona have not undergone the expansions seen in amphioxus and sea urchin (Dehal et al., 2002; Hughes and Friedman, 2005).
INNATE IMMUNITY AND INFLAMMATION IN CIONA

Comparative biological studies have revealed that most multicellular life is protected by conserved mechanisms of innate immunity which include: barrier defenses [i.e., host (mucosal) epithelium] and the associated non-specific secretory components (e.g., antibacterial peptides), PRRs, which are present on the surface and within the cytoplasm of phagocytes and other host cells, various phagocyte effector mechanisms (e.g., reactive oxygen species, induced cell death), and different enzymatically catalyzed cascades involved in clotting, melanization, and complement activation (Figure 3; Laville et al., 2010; Maldonado-Conteras and McCormick, 2011). A variety of innate immune genes and mechanisms have been identified in Ciona (Parrinello, 2009), which, based on histological and molecular characterizations have evolved complex barrier defense strategies. Challenge with microbe-associated molecular patterns (MAMPs) such as the Gram-negative lipopolysaccharide (LPS) induces inflammatory-like reactions that are characterized, in part, by tumor-necrosis factor (TNF)-like gene expression and cell recruitment to various body compartments (Di Bella and De Leo, 2000; Melillo et al., 2006; Parrinello et al., 2007, 2008, 2010; Cammarata et al., 2008; Bonura et al., 2009). These responses can recruit a variety of cell types (Parrinello et al., 1996; Arizza and Parrinello, 2009; Arizza et al., 2011) and induce a number of immunological phenomena (Smith and Peddie, 1992; Melillo et al., 2006; Parrinello et al., 2007), including the expression of characteristic innate immune receptors (Shida et al., 2003; Parrinello, 2009; Sasaki et al., 2009; Dishaw et al., 2011). Although a variety of different MAMPs induce Ciona TLRs, LPS does not activate TLR expression directly, as with TLR4 in vertebrates, suggesting the presence of alternative LPS sensors (Sasaki et al., 2009). A repertoire of innate effectors [e.g., TLRs, TNF, complement components, and the protochordate-specific variable region-containing chitin-binding proteins (V CBPs)] are expressed in the gut of Ciona (Marino et al., 2002; Sasaki et al., 2009; Skjoedt et al., 2010; Dishaw et al., 2011) and could play essential roles in the stable maintenance of host–microbial interactions (see below).

Documentation of these types of functional effects is critical to the design of experimental approaches for characterizing the interactions of immune receptors with gut microbiota. However, enumerating gene homologs and defining their expression patterns is one thing, understanding their dynamic interactions in a cellular and molecular context is a daunting challenge in invertebrate systems, particularly those that inhabit marine and aquatic environments. Despite a number of potential shortcomings, Ciona has the potential to reveal conserved mechanisms sustaining the evolution of host–microbial interactions (see below). Experimental manipulation of host–microbial interactions at the gut epithelial interface is critical to such studies and in order to approach these questions, it is essential to first understand the Ciona gut as both a physical barrier and as an immunological organ.

AN INVERTEBRATE CHORDATE GUT MODEL

Ciona is a highly successful, cosmopolitan solitary tunicate that has adapted to living in diverse marine environments (Caputi et al., 2007). Other tunicates include both colonial and solitary forms and spend their adult lives as sessile, filter-feeding, organisms. In addition, a few pelagic forms have been identified (Denoeud et al., 2010; Nishida et al., 2010). Because the tunicate feeding strategy involves siphoning seawater, the gut in Ciona is in continuous contact with both dietary and seawater microbiota where in addition to its essential physiological role, it acts as both a physical barrier and site of continuous immunological interaction. An immunocompetent gut, which includes mucosal immunity mediated by surface epithelium, is present even in the simplest metazoans (Bosch et al., 2009). In mammals, proper development of gut mucosal immune tissues is dependent on the proper timing and colonization of the gut by microbial communities (see below); however, details governing these events remain to be defined (Cebra et al., 1998; Cebra, 1999; Hooper and Gordon, 2001; Mazmanian et al., 2005; Edelman and Kasper, 2008; Kanther and Rawls, 2010). Intensive studies in comparative models, such as Ciona, have the potential for shedding light on the basic biology of gut immune homeostasis and in turn, may reveal basic mechanisms of dysfunctional gut immunity [e.g., inflammatory bowel diseases (IBD) in mammals]. As an initial step in adapting a urochordate as a new model system for understanding gut immune homeostasis and mucosal immunity, we have combined cellular, molecular, and microbiological approaches to characterize the Ciona gut and its microbiota. At this preliminary stage of investigation, basic aspects of complex microbial community dynamics can be identified that mirror many of the
FIGURE 2 | (A) *Ciona intestinalis* is a solitary urochordate that typically grows in close proximity on suitable substrates. (B) *Ciona* is relatively translucent and the gut can be visualized through the tunic (overlay depicts the branchial basket and gut; solid arrows depict water flow; dotted arrows indicate two routes of flow). (C) The entire gut can be readily dissected. (D) Juveniles are attracted and attach to standard tissue culture plastic plates. (E) *Ciona* can be reared to adulthood in a laboratory environment (Cirino et al., 2002; Joly et al., 2007). *Ciona* adults are hermaphrodites and release sperm and egg into the water column. Typically, fertilization leads to rapid development and hatching of swimming tadpole larva. After settlement, the animal undergoes metamorphosis into a permanent, sessile, filter-feeding lifestyle; colonization of the gut by microbes is likely immediate. Images (D,E) are courtesy Dr. Paola Cirino.

core features and symbiotic intricacies of host–microbe interactions that are recognized in the mammalian gut ecosystem (Savage, 1977; Hooper and Gordon, 2001; Willing et al., 2011). The interactions between host and gut microflora are not simple, but instead involve complex mutualisms (Bischoff, 2011) that ultimately help govern immune development, and the establishment and maintenance of immune homeostasis (Artis, 2008; Chung and Kasper, 2010; Hooper and Macpherson, 2010).

COMPLEX BIOLOGY OF THE CIONA GUT

*Ciona* filters microbe-rich seawater through a modified pharynx where ciliated cells push food particles into a gut, which is divided into esophagus, stomach, mid-gut, and hind-gut; the latter two are referred to collectively as intestines. The esophagus connects the branchial basket to the stomach in which both cilia and mucous glands are highly developed for the efficient transfer of foods. The stomach epithelium forms many cilia-rich ridges and grooves and is composed of at least two major epithelial cell types, as well as an undifferentiated cell population (Burighel and Cloney, 1977). The stomach epithelium, which is presumed to be the site of most digestive enzyme secretion, contains secretory cells, and is coated by a thin layer of mucus. The mid-gut is distinguished by an interior typhlosole that runs the entire length and is rich in testicular acini. Three types of largely granular epithelial cells, absorptive, mucous, and large round (or elliptical), define the mid-gut. Glycogen stores are concentrated in the mid-gut and to a lesser degree in the stomach. Energy is stored as both fat and glycogen (Yonge, 1925). The sexual ducts exit the atrial siphon adjacent to the hind-gut. Although absorption is most prominent in the mid-gut, diffusion of dissolved substances occurs throughout the alimentary track. The *Ciona* gut demonstrates complex epithelial cell renewal traits (Ermak, 1981), which are of particular importance to several aspects of mucosal physiology, including immune function. The highly developed and compartmentalized stomach and distinct intestinal region in *Ciona* morphologically resemble that of more recently diverged chordates (Millar, 1953; Burighel and Cloney, 1977).

COMPLEX MICROBIAL COMMUNITY DYNAMICS DEFINE THE CIONA GUT

Details surrounding the relationships between filter-feeding invertebrates and the microbial communities colonizing their guts are lacking but may be broadly relevant to gut immunity in vertebrates for determining: (1) the role of diet in gut microbial ecology, (2)
Barrier defenses across the epithelium are governed by innate immune phenomena characterized by the secretion of mucus, antimicrobials, and soluble immune molecules. On the basolateral surface and deeper, host innate immunity consists of various proteolytic-coagulation cascades for wound healing and microbe trapping, as well as complement defense pathways. Distinct phagocytes populate this area as well other cell types [e.g., dendritic cells (DC) in vertebrates]. Gut DCs sample luminal antigens and present them to the adaptive immune system which includes gut-specific lymphocytes of both T and B cell lineages. This results in the maturation of immunity and the recruitment of additional cell types. A parallel, more simplified, system in Ciona has evolved to also include barrier defenses across distinct epithelial lineages in the gut and the secretion of immune mediators, including antimicrobial peptides, into the lumen. In the basolateral side, distinct populations of hemocytes, including granular amoebocytes, populate the laminar connective tissues. Immunological competence in this area is mediated by coagulation/immobilization cascades and microbe trapping, complement defenses, distinct antigenic sampling via PRRs, secretion of pro-inflammatory signals, and recruitment of specialized hemocytes. However, as opposed to the vertebrate gut, the Ciona gut generates immune amplification within an innate immune context and does not couple to a distinct, more specialized, system such as adaptive immunity.

FIGURE 3 | Simplified schematic of mucosal immunity emphasizing barrier defense strategies of vertebrates and Ciona. Barrier defenses across the epithelium are governed by innate immune phenomena characterized by the secretion of mucus, antimicrobials, and soluble immune molecules. On the basolateral surface and deeper, host innate immunity consists of various proteolytic-coagulation cascades for wound healing and microbe trapping, as well as complement defense pathways. Distinct phagocytes populate this area as well other cell types [e.g., dendritic cells (DC) in vertebrates]. Gut DCs sample luminal antigens and present them to the adaptive immune system which includes gut-specific lymphocytes of both T and B cell lineages. This results in the maturation of immunity and the recruitment of additional cell types. A parallel, more simplified, system in Ciona has evolved to also include barrier defenses across distinct epithelial lineages in the gut and the secretion of immune mediators, including antimicrobial peptides, into the lumen. In the basolateral side, distinct populations of hemocytes, including granular amoebocytes, populate the laminar connective tissues. Immunological competence in this area is mediated by coagulation/immobilization cascades and microbe trapping, complement defenses, distinct antigenic sampling via PRRs, secretion of pro-inflammatory signals, and recruitment of specialized hemocytes. However, as opposed to the vertebrate gut, the Ciona gut generates immune amplification within an innate immune context and does not couple to a distinct, more specialized, system such as adaptive immunity.

the nature of host selection of gut microbiota, and (3) the role of microbiota in gut immune homeostasis. These features of gut physiology are essential to our defining of homeostasis, even if a model organism such as Ciona does not share all the innate receptor orthologs found in mammals. Because the composition of gut microbiota is relevant to host physiology for a variety of reasons not limited to their metabolic output, we suspect that the gut microbial communities in Ciona will not simply reflect diet and/or environmental availability, but will reveal species-specific communities that determine or influence various aspects of overall immune competence.

Gut bacteria from four Ciona populations (Woods Hole, MA, USA; San Diego and Monterey Bay, CA, USA; and Naples, Italy) have been partially sampled using PCR-amplified 16S ribosomal genes recovered in small clone libraries derived from whole gut homogenates (and/or recovered fecal matter). The 16S products were then characterized by sequencing of individual clones as well as by screening of restriction fragment length polymorphisms
A fraction of the recovered microflora (~25 bacterial species) was cultured successfully. The *Ciona* gut revealed distinct communities of bacteria that were affected by both diet and environment, including species of metabolic significance (e.g., *Chitinophaga*). Starvation induced reproducible dysbiosis (i.e., disruption or displacement of microbial communities) and revealed bacterial families and genera that were conserved across populations and between two *Ciona* species (e.g., Gammaproteobacteria such as *Vibrio* sp. and *Shewanella* sp., as well as various *Oceanospirillales* genera; Dishaw, unpublished observations).

The successful recovery, identification, and growth of native gut bacteria from wild *Ciona* adults provides the basic background to inoculate the developing gut of juveniles grown in controlled laboratory environments (e.g., under semi- or sterile conditions or colonized by complex mixtures of microbes). Changes in community structure subsequent to experimental manipulation can be monitored using real time quantitative PCR. These experiments, which currently are in progress, have the potential to define the onset and normal timing of microbial colonization in the development of the *Ciona* gut in general, and more specifically, characterize how this interaction affects the maturation of the hemocyte-rich laminar spaces (i.e., immune tissues). Determining how interactions with microbiota affect maturation of the gut immune tissues will utilize dysbiosis techniques including antibiotic treatments and/or development of juveniles under germ-free or semi-germ-free conditions. In this regard, two features of the *Ciona* model are particularly attractive: (1) it is relatively easy to produce and maintain hundreds of *Ciona* juveniles and (2) transparency of tissues makes it feasible to visually characterize gut development and gauge luminal content (*Figure 2*) and make possible the tracking of host–microbe interactions (e.g., the use of labeled bacteria) from early in development through adulthood.

**GUT MICROBIAL IMMUNE INTERACTIONS**

The circulatory system of *Ciona* is open and continuous with histologically defined blood lacunae. Gut-associated lacunae, which...
we have termed the *Ciona* gut lamina propria (Dishaw et al., 2011), share blood cells between the various tissue spaces and are richly infiltrated by a variety of hemocyte types. A complex developmental maturation of this subepithelial lamina that likely is influenced by luminal antigen exposure is indicated. Notably, the hemocytes are not restricted to the gut as morphologically indistinguishable cell types can be detected in other parts of the body. Adult *Ciona* injected with MAMPs (e.g., in the tunic) can be induced to generate localized inflammatory responses, which include an active recruitment of these and other immunocompetent cells (Di Bella and De Leo, 2000; Pinto et al., 2003; Bonura et al., 2009; Parrinello et al., 2010). In this regard, variable (V) region-containing chitin-binding proteins (VCBPs), which are expressed by distinct gut epithelial cells in amphioxus (Cannon et al., 2002), also have been identified in *Ciona* (Figure 5). VCBPs have been shown to be secreted by discrete cells of the stomach and intestinal epithelium into gut lumen where they interact with bacteria via their V-type immunoglobulin domains (Dishaw et al., 2011). The functional relevance of the VCBP chitin-binding domain remains unclear. *Ciona* granular amoebocytes, which also express VCBPs, are present in both blood and the lamarine spaces of the gut. *In vitro* experiments have demonstrated that granular amoebocytes, recovered from blood, recognize (phagocytose) bacteria coated with VCBPs (Dishaw et al., 2011) *in vitro*. We have hypothesized that the morphologically indistinguishable cells found in the lamina propria function in an equivalent manner and play a major role in the dynamics of gut immunity in both *Ciona* and amphioxus. This hypothesis is supported further by the finding that native VCBPs bind luminal bacteria (Dishaw et al., 2011) and it is entirely likely that VCBPs enhance phagocytic recognition of gut bacteria coated with VCBPs that traverse epithelial barriers (e.g., in instances of epithelial damage). An emerging functional role for VCBPs places them in the broader context of our hypothesis by suggesting that some innate receptors, secreted into the gut lumen, may be serving still undefined roles as symbiosis factors. Penetration of the mucosal barrier would trigger an immunological event by the tagged (i.e., opsonized) microbe to protect the integrity of self (i.e., phagocytosis, inflammation, and cell recruitment). Broad analogies can be drawn between this process and immune recognition in the vertebrate mucosal environment, albeit involving different effectors. MAMP challenge across the gut mucosal barriers also may recruit cells from distant tissue spaces; however, this may be unnecessary since the subepithelial lamina is densely populated with many hemocyte types along the gut length (Figure 5). Several preliminary observations (Dishaw, De Stantis, and Pinto, unpublished) suggest that lamina-associated gut hemocytes from *Ciona* may be exposed to luminal contents (e.g., through injury of epithelium or exposure to factors that affect epithelial tight junctions). Electron microscopic analysis will be critical to demonstrate if hemocytes actively cross the epithelium and interact with gut microbiota and if luminal antigens enter the laminar spaces.

**CONSERVED SENSORS IN HOST–MICROBE INTERACTIONS**

The extent to which innate receptors are expressed by gut epithelium and the functional implications of their interaction with symbiotic or pathogenic gut microbes, as well as virulence factors, are of central interest. Innate immune receptors in vertebrates, primarily TLRs, are expressed selectively in a polarized fashion on intestinal epithelial cells (IECs) of the small and large intestines (Abreu, 2010). TLRs provide indirect signals to the adaptive immune system by first providing innate immune signals to the underlying (basal lamina) tissues. In *Ciona*, the expression, function, and regulation of a variety of immune molecules (Azumi et al., 2003; Shida et al., 2003), including antimicrobial peptides (Fedders and Leippe, 2008), TLRs, Gram-negative binding proteins (GNBPs), lipopolysaccharide binding protein (LBP), TNF, MBL, complement protein C3, as well as VCBPs, are of potential interest. *Ciona* responds to bacterial ligands in a manner consistent with the patterns of expression of TLRs and other PRRs in gut tissues, resulting in the induction of pro-inflammatory molecules, e.g., TNF (Sasaki et al., 2009). Whereas *Ciona* only has two TLRs, both are expressed in distinct locations along the gut and along with other innate immune molecules likely play a significant role in discriminating among gut commensals and sensing pathogens (Sasaki et al., 2009; Abreu, 2010). The presence of only two TLRs, which interact with more than one ligand, could be seen as a disadvantage of the *Ciona* system over mammals, in which multiple TLRs discriminate among distinct ligands. Multi-functional PRRs, such as the TLRs, may be coupled to downstream pathways which may serve to better discriminate ligands; such pathways could differ significantly in *Ciona* and investigations in this system will help reveal how various organisms discriminate among TLR ligands. Under normal conditions, the gut in *Ciona* likely maintains a state of balance (i.e., homeostasis) between tolerance and protection through host epithelial–microbe interactions, as has been seen in vertebrates.
However, most such interactions are designed to sustain ancient symbiotic relationships and are not necessarily immune-restricted (Hooper and Macpherson, 2010). Innate receptors play significant roles in these basic processes that govern homeostasis and studies that define conserved mechanisms governing host–microbial interactions in the gut are of fundamental biomedical interest.

**DISCUSSION**

The animal gut is host to a massively populated dynamic ecosystem of microbes (Savage, 1977) with enormously complex antigenic diversity. Such gut microbial communities in mammals are linked intimately to the maturation and development of mucosal immunity and represent an important determinant of health and disease (Mazmanian et al., 2005; Fujimura et al., 2010; Hooper and Macpherson, 2010). A particularly complex physiological challenge is posed to the mucosal immune system of the host, which must differentiate distinct populations of commensal (i.e., possibly useful) microorganisms from pathogenic communities. Specifically, some symbionts are recognized and tolerated, and subsequently form a cooperative system in the gut, whereas pathogens, which in many cases are invasive, are not well tolerated and are cleared. Traditional views of gut immunity are complicated further by numerous commensals that although beneficial, can induce a pathogenic state in the host (i.e., pathobionts, Round and Mazmanian, 2009). Breakdown of commensal-immune suppression and tolerance mechanisms can lead to disruptions of homeostasis and in turn to inflammation, resulting in a range of distinct clinical phenotypes that define acute and/or chronic IBD (Chung and Kasper, 2007; Marchiando et al., 2010), e.g., tolerance to endotoxin is likely governed at the earliest exposure in development (Lotz et al., 2006). It has been suggested that immune systems, as defined currently, evolved first to manage complex symbiotic relationships, while the preservation of “self” became an inevitable adaptation (Bosch and McFall-Ngai, 2011). By this account, mechanisms (later acquired by innate immunity) that govern host–microbial interactions are of ancient phylogenetic origins.

Investigations in *Ciona*, which are focused on the interaction of microbes at the epithelial surface, may help reveal: (1) if certain bacterial species effect barrier function (Lyczak, 2003; Obland and Macnaughton, 2010); (2) if the degree of microbiome complexity influences epithelial response to infections (Mans et al., 2009); (3) how polarized expression of PRRs help maintain microbial recognition, immune integrity, and homeostasis; (4) how bacteria modulate intercellular tight junctions, which are key to barrier integrity (Turner, 2009); and (5) how secretory pro-inflammatory molecules, like TNF, affect barrier integrity by increased intercellular permeability. Much like mammals, rich microbial communities of distinct phyla and genera are selected in *Ciona* from an exceptionally vast range of choices of dietary and seawater microbes. Various commensal bacteria likely have co-evolved with the species and may have become integral to nutritional acquisition and gut homeostasis. As such, *Ciona* offers a unique opportunity to study and characterize not only the molecular events surrounding gut microbial engagement with mucosal immunity but to define the symbiotic ecosystem required for gut homeostasis and thereby host well-being in a controlled, systematic manner using a novel, tractable model system.
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