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Immune-Directed Support of Rich Microbial Communities in the Gut Has Ancient Roots

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Abstract

The animal gut serves as a primary location for the complex host-microbe interplay that is essential for homeostasis and may also reflect the types of ancient selective pressures that spawned the emergence of immunity in metazoans. In this review, we present a phylogenetic survey of gut host-microbe interactions and suggest that host defense systems arose not only to protect tissue directly from pathogenic attack but also to actively support growth of specific communities of mutualists. This functional dichotomy resulted in the evolution of immune systems much more tuned for harmonious existence with microbes than previously thought, existing as dynamic but primarily cooperative entities in the present day. We further present the protochordate *Ciona intestinalis* as a promising model for studying gut host-bacterial dialogue. The taxonomic position, gut physiology and experimental tractability of *Ciona* offer unique advantages in dissecting host-microbe interplay and can complement studies in other model systems.

Keywords

gut immune evolution; gut host-microbe interactions; *Ciona intestinalis*; gut immune effectors; mutualism; gut biofilms

Introduction

Complex communities of microbes colonize nearly every epithelial surface of animals, including mucosal epithelial surfaces that become highly valued locales for microbial

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attachment and growth immediately upon birth (Neish, 2014). The gastrointestinal (GI) system, in particular, has emerged as a prime example of an epithelium-lined organ that sustains an abundant and complex community of closely associated, generally species-specific microbiota, which can exert enormous physiological influence on animal hosts (Savage, 1977; Falk et al., 1998; Neish, 2009; Lozupone et al., 2012; Yatsunenکو et al., 2012). The host-microbe interactions at the surface of the epithelium are benign or beneficial as a whole and generally are tolerated by the host, although pathogenic breach of the epithelium can result in strong host immune responses. Understanding the multiple types of microbe-host interactions in the gut may provide insight as to the types of selective pressures that spawned the emergence and elaboration of the immune systems of metazoan animals.

From an ecological standpoint, the alimentary canal (or GI system) is an external environment that passes through animals, generally via two openings (i.e., in and out). In the simplest terms, this canal evolved to deliver water and nutritional sustenance to an increasingly complex and generally sterile body. Although this function is essential for life in nearly all animals, such a direct juxtaposition of substances from the external environment inside the body nevertheless poses an enormous challenge for the host. Within the gut tube, the host encounters an essentially continuous microbiological load that can include pathogens as well as a myriad of either beneficial or innocuous species. This complexity is enhanced by the presence of distinctly differentiated compartments within the gut tube, each with potentially distinct and permanent commensal communities (Faust et al., 2012; Koren et al., 2013; Segata et al., 2012). One often studied example is the human colon, which contains several compartments, including the often controversial cecal appendix, and hosts up to 100 trillion (10^{14}) bacterial cells at any given time, in effect generating orders of magnitude greater intraorganismal genetic complexity than that of the host genome itself (Andersson et al., 2008; Costello et al., 2009; Eckburg et al., 2005; Gill et al., 2006; Human Microbiome Project, 2012; Ley et al., 2006; Macfarlane and Macfarlane, 1997; Savage, 1977;). Reduced diversity and/or shifts in the composition of microbiota among different epithelial compartments can dramatically affect homeostasis and host health, in some cases promoting infections that endanger host wellbeing (Chan et al., 2013; Fallucca et al., 2014; Luckey et al., 2013; Mondot et al., 2013; Petrof et al., 2013; Power et al., 2014; Shim, 2013). Further, in mammals, the direct epithelial dialogue with bacteria is generally critical for immune maturation (Cebra, 1999; Cebra et al., 1998; Sommer and Backhed, 2013), and often can provide tolerogenic signals to the host (Lee, 2009; McFall-Ngai et al., 2013; Shin et al., 2011). It might seem at the outset that the monumental task of managing the complex microbial milieu of the alimentary canal would be performed by a multilayered system of immune attack that is effectively repelling an ever changing array of invaders intent on circumventing its defenses. Indeed, the gut of animals evolved so that its epithelial surface is fully immunocompetent, serving as a barrier to potential pathogens while simultaneously recognizing and communicating with microbes in both the lumen (inside) of the gut and the intra-organismal tissue that is generally sterile. [e.g., (Bosch et al., 2009; Duerkop et al., 2009; Duerr and Hornef, 2012; Pott and Hornef, 2012)]. This function is carried out in large part by immunocytes, which migrate to tissue spaces adjacent to the epithelial barrier early during development. In some species, these immunocytes form dense

communities and produce an outward appearance of a defense system poised to attack, analogous to an army preparing to repel an invasion. In vertebrates, this tissue space is well recognized for the presence of gut-associated lymphoid tissue (GALT), one of the most prominent immune tissues (Pearson et al., 2012; Shields, 2000). Whereas certain aspects of host immunity certainly do involve these aggressive characteristics, an increasing amount of emerging data indicate that the system as a whole may be much more tuned for harmonious existence with the microbes it encounters and may have evolved such relationships in this cooperative manner (Eberl, 2010; Equileor and Ottaviani, 2011; Loker, 2012; Nyholm and Graf, 2012; Thomas and Parker, 2010).

In this review, we use an immune-phylogeny approach (Fig. 1) to argue that host defense systems have met the challenge of managing microbial communities by evolving strategies that not only directly protect the host's tissue from pathogenic infection but also actively support the growth of specific communities of microbes such that the maturation of these barriers becomes as much, if not more so, of a “welcome wagon” for mutualists in the lumen as opposed to a buildup for war against pathogens. This dichotomy serves to protect against pathogenic invasion, to ensure proper nutritional sustenance to the host and to train the host's immunological systems. The host-microbial dialogue, as part of the metaorganism or holobiont (Bosch and McFall-Ngai, 2011; McFall-Ngai et al., 2013; Rosenberg et al., 2010), has helped shape the evolution of gut immunity and exists as a continually dynamic but primarily cooperative system in the present day. Furthermore, we present the concept that protochordates, such as the tunicate *Ciona intestinalis*, can serve as unique and highly informative models for defining sets of chordate rules for host-bacterial dialogue in the gut. These organisms provide experimental advantages based on both their taxonomic position and specific gut physiology, and, when compared to vertebrates, can offer fascinating insights into the evolution of host-microbe relations in the gut.

Simplest Epithelium

The most basic and phylogenetically conserved interactions between complex metazoan hosts and the microbial world occur across epithelial barriers (Fig. 2A). *Hydra sp.* are solitary freshwater Cnidarians with a simple body architecture consisting of a thin gelatinous mesoglea separating an internal endoderm and external ectoderm (Fig. 2B). They have become an indispensable model system for defining the origins of the most basic interactions across epithelial barriers (Augustin et al., 2012; Bosch, 2013). Distinct bacterial communities have been shown to colonize the epithelium of two *Hydra* species cultured under identical laboratory conditions; the microbial communities observed are representative of those identified in *Hydra* isolated from the wild (Fraune and Bosch, 2007). These observations constitute the first compelling evidence that even the simplest of animals maintain species-specific microbial communities and give rise to several questions: What regulates these bacterial communities? Could it be that ecological factors at the surface of the epithelium such as nutrient composition and pH create conditions that favor some microbial communities over others? Alternatively, does host immunity play a much more specific and active role in defining the communities, and if so, could genetic factors outweigh environmental influences? Further insight into the mechanisms affecting this

unexpectedly high degree of selection can be gained by considering several key observations.

First, in experiments lasting more than 15 weeks, colonization of the epithelial surface of *Hydra* was noted to occur in reproducible stages (Franzenburg et al., 2013a). Both experimental and mathematical concepts were applied to demonstrate that colonization is controlled first by competitive interactions among diverse bacteria for access to the host surface and resources. The initial colonization of the tissue appears to prime the response, which matures over some defined period of time. The result is reduced diversity of bacterial communities as well as a reduction in variation in the abundance of certain groups in a process that is influenced by host-derived factors. A key feature of the *Hydra* system (but unlike some other Cnidarians) is the apparent lack of mobile phagocytic cells. Microbial sensing in this species is mediated exclusively by the epithelium and involves the nuclear factor kappa B (NF- κ B), Toll-like receptor (TLR), and Myd88 signaling cascades (Bosch et al., 2009). Preliminary sorting of bacteria apparently is carried out by diverse antimicrobial peptides (AMPs) that influence composition of the microbiota from the onset of development onwards (Franzenburg et al., 2013a; Franzenburg et al., 2013b; Fraune et al., 2010). The association of stable microbial communities is governed by epithelial homeostasis and is influenced by the physical process of cellular turnover (Boehm et al., 2012; Bosch, 2013; Fraune et al., 2009). In mammals, for comparison, stem-cell guided gut epithelial turnover helps to promote not only healing of injured surfaces but to re-establish the complex layering of mucin (Johansson, 2012; Johansson et al., 2011a; Johansson et al., 2011b), re-concentrate host-derived factors such as AMPs and prompt re-colonization of some adherent bacterial communities (Eri and Chieppa, 2013; Marchiando et al., 2010; Mukherjee et al., 2008; Sommer and Backhed, 2013). Thus, even in the most basic epithelial surface of the *Hydra* system, a vastly complex interplay between an array of factors, including local physiological properties, host-derived immune mediators, and microbiome-derived factors, governs the establishment, maintenance and displacement of adherent microbial communities.

The Cnidarian Gastric Cavity

The Cnidarian body plan consists of a pair of tissue layers. In actively feeding Cnidarians such as *Hydra*, as well as in some species of corals, anemones, and jellyfish, a single opening acts as both a mouth and anus (Fig. 2B). The oral opening empties into a sac that is lined with endoderm (a specialized digestive epithelium known as gastrodermis), which is analogous to the gut epithelium of higher animals. Food is ingested into a pouch-like area (i.e., the gastric cavity) where it is metabolized, after which fecal remains are released into the environment through the same oral opening. However, just as with the gut of higher animals, the gastric cavity remains an external environment within the internal confines of the host organism (in this case, the Cnidarian polyp). Recently, Agostini and colleagues (Agostini et al., 2012) demonstrated that this most primitive example of a gut-like structure is not as simple as it may seem. Previous work in corals (Sorokin, 1973) and anemones (Herndl and Velimirov, 1986) has demonstrated distinct groups of bacteria in the gastric cavity. It was concluded that either the animals were cultivating bacteria as a backup carbon source during times of starvation (Herndl and Velimirov, 1986) or that the bacteria

contributed essential vitamins and nutrients for the host (Sorokin, 1973). The more recent analysis by Agostini and colleagues suggests that the corals manage communities of bacteria in distinct areas of the gastric cavity, forming a complex ecosystem of diet-, host-, and bacterial-derived factors that is associated with differences in pH, dissolved oxygen, essential nutrients, vitamin content and bacterial diversity within individual polyps (Agostini et al., 2012). The gastric cavity microbiota within the polyps were shown to differ from microbial populations of surrounding seawater, suggesting that this co-evolving ecosystem involves bacterial selection. Given what we know from the studies in *Hydra* and other Cnidarians, in which expression of immune factors has been shown in oral tissues (Kimura et al., 2009; Vidal-Dupiol et al., 2011), it is tempting to speculate that, host-derived immune mediators play a significant role in defining how the composition of the gastric cavity microbiome develops and is maintained in these ancient diploblastic organisms. Consistent with this view, strong immune gene expression is detected in endodermal tissues and/or oral ectoderm (Kimura et al., 2009; Vidal-Dupiol et al., 2011). Furthermore, complex expression patterns of immune molecules result when the animals are dosed and/or challenged with various bacteria. Such patterns, including the direct downregulation of a specific AMP (Vidal-Dupiol et al., 2011) by a known coral pathogen, point toward a complex dialogue between host and microbiota in the gastric cavity.

Ancient Alimentary Canals

The alimentary canal, which in the broadest sense is a feeding tube that on one end has a mouth and on the other an anus, is an ancient structure shared by all bilaterian animals (Fig. 2C). In triploblastic animals (three germ layers, a coelomic cavity, and bilateral symmetry; Fig. 1), the gut is derived from endoderm lining the yolk sac, which itself is enveloped by the developing coelom, a feature unique to animals with mesoderm (Hejnol and Martindale, 2008; Holland, 2000; Noah et al., 2011). Developmentally, some aspects of mesodermic formation of the coelom as well as the derivation of the gut from the earliest blastomere divide the bilaterians in two groups, the protostomes and the deuterostomes. The protostomes include the molting organisms (Ecdysozoans) such as arthropods and nematodes as well as the non-molting forms (Lophotrochozoans), which include annelids and molluscs. The deuterostomes include echinoderms, early chordates (the invertebrate protochordates) and the later chordates (the vertebrates) (Fig. 1). Three important aspects of the gut appear to be phylogenetically conserved: 1) compartmentalization so that along the alimentary canal, morphological, and in some cases biochemical, features separate the gut into distinct segments roughly within the confines of a foregut (mouth, esophagus, stomach), midgut (the middle intestines), and the hindgut (mostly the caecum, colon, anus), 2) the gut is a prominent immunological organ that exerts a strong influence on host physiology and 3) the gut has evolved in most taxa as an ecosystem to harbor microbial communities (in many cases including a fermentation chamber) that in most cases is of host benefit.

In general, many features of animal immunity have been conserved in phylogeny; however, there exists an astonishing diversity of specific immune mechanisms, consistent with the extensive variation among genomes and the enormous morphological complexity representative of the various phyla. In all animals, the expression of AMPs in the gut is a major effector response that is critical to the function and maintenance of homeostasis.

Recent work has demonstrated a remarkable array of multi-functional potential for these diverse effector molecules (Diamond et al., 2009; Semple and Dorin, 2012; Semple et al., 2010). Functions include the “neutralization” of lipopolysaccharide (LPS), an important component of the cell wall of many bacteria. Such neutralization, which appears to facilitate the induction of intestinal epithelial cell (IEC) tolerance (Pulido et al., 2012), reduces the host's response to LPS, which might otherwise result in bacterial killing and/or unnecessary host inflammation. However, neutralization apparently does not eliminate the biological function of LPS in mutualistic bacteria which grow quite readily within environments rich in the AMPs (Gruenheid and Le Moual, 2012; Gutsmann et al., 2005). Thus, in addition to selective anti-bacterial properties, some AMPs demonstrate immune-modulating potential (Choi et al., 2012; Scott et al., 2007; Territo et al., 1989). This dual function may serve to protect the host against collateral damage (i.e., unnecessary or misdirected inflammation) and facilitate colonization by harmless or beneficial microbiota. It also is possible that some of these AMPs mobilize immunity to actively regulate the composition of adherent bacterial communities. Simpler model systems (e.g., those lacking adaptive immunity) can help dissect the diverse roles of AMPs in the gut.

Drosophila melanogaster (fruit fly) has been used widely and successfully as a model system to dissect host-microbe interactions. The genomes of various *Drosophila* species have been determined (Gilbert, 2007; Hahn et al., 2007; Rubin, 1996; Song et al., 2011), knockouts are available and knockdowns easily generated, and more aspects of their biology are understood at the genetic level than for any other species (e.g., see special Nature web focus: <http://www.nature.com/nature/focus/drosophila/>). *Drosophila* possess a long gut (several times their body length) that is compartmentalized into fore-, mid-, and hindgut. Intestinal stem cells that can facilitate the renewal of gut barriers are present (Buchon et al., 2013b; Lemaitre and Miguel-Aliaga, 2013; Micchelli, 2012; Micchelli et al., 2011). The *Drosophila* immune system has been studied extensively, frequently using systems biology approaches (Bodenmiller et al., 2007; Cuomo and Bonaldi, 2010; de Morais Guedes et al., 2005; Engstrom et al., 2004; Lilley and Griffiths, 2003; Stuart et al., 2007; Vierstraete et al., 2005), and has been thoroughly described elsewhere (Charroux and Royet, 2010; Kingsolver et al., 2013; Kuraishi et al., 2013; Lemaitre and Hoffmann, 2007; Wang et al., 2014). Briefly, *Drosophila* relies on NF- κ B modulation of antimicrobial defenses in response to recognition and activation of either immune deficiency (IMD) or TLR signaling pathways. In gut epithelial cross-talk with bacteria, *Drosophila* also utilizes (like many other animals) the dual oxidase (DUOX) system to modulate inflammation and discriminate among potential pathogens (Kim and Lee, 2014; Lee et al., 2013; Ryu et al., 2010). Immune homeostasis of gut microbial communities, in *Drosophila*, largely is governed largely by the activity of AMPs and the modulation thereof (Kuraishi et al., 2013).

Complex communities of bacteria, strongly affected by diet and environment, can be sampled from the *Drosophila* gut, particularly in wild-type flies (Broderick and Lemaitre, 2012; Buchon et al., 2013a). The gut hosts a much lower compositional diversity than is found in surrounding environments, suggesting that *Drosophila* modulate the communities of bacteria that persist in their gut (Chandler et al., 2011), likely by creating either an environment that is not hospitable to most microbes or one that supports the growth of some

species over others. However, the inability to consistently recover identical bacterial operational taxonomic units (OTUs, typically equivalent to bacteria matched to a sequence identity of >97%) across individual flies and populations calls into question the possibility that flies select for or are dependent on a limited core set of microbiota (Wong et al., 2013). Intriguingly, *Drosophila*, like bumblebees and honeybees (Martinson et al., 2011) as well as fireflies (Sudakaran et al., 2012), does maintain particular species of bacteria in the gut. These gut microbiota have now been shown to modulate various aspects of gut biology, including host innate immunity (Buchon et al., 2013a; Glittenberg et al., 2011; Lee et al., 2013) as well as intestinal epithelia cell turnover (Buchon et al., 2009a; Buchon et al., 2009b; Lee, 2009). Modulation of host responses is not limited to the gut, and likely can exert enormous influences on host behavior and physiology (Engel et al., 2012; Koropatnick et al., 2004; Salem et al., 2013; Sharon et al., 2011; Storelli et al., 2011). This phenomenon demonstrates a co-evolving partnership between host and gut microbiota, despite the observation that many of the microbial species in the gut of *Drosophila* do not colonize the animal permanently, but rather need to be replenished periodically to preserve the composition of the gut flora (Blum et al., 2013). Some microbes may find it difficult to establish permanent communities, possibly as a consequence of epithelial turnover and peritrophic matrix renewal in the midgut (Kuraishi et al., 2011). Additional difficulty could arise via the activity of a broad spectrum of AMPs expressed in each compartment of the gut.

In the simple nematode, *Caenorhabditis elegans*, the entire gut is descended from a single embryonic cell (Deppe et al., 1978) and results in a simple intestine (20 somatic cells) lacking stem cells, and subsequently, renewal potential, which in turn influences lifespan. This trait is likely evolutionarily derived in *C. elegans*, and other organisms (Rost-Roszkowska and Undrul, 2008), as renewal of gut epithelium is a common feature among most animals. Because *C. elegans* feeds primarily on microorganisms, host-microbe interactions are initiated in the intestines and colonization of the gut can occur there; pathogens can also attempt to colonize and reduce lifespan of the worm either by adhering to the outer cuticle and causing damage or by secreting toxins that cause intestinal damage and/or systemic injury (Zhang and Hou, 2013). When confronted with pathogens, *C. elegans* can utilize avoidance behaviors that involve signaling through its single TLR, Tol-1 (Pradel et al., 2007). However, protection against pathogenic invasion of the intestines also is dependent on Tol-1 (Tenor and Aballay, 2008) and can involve a variety of complex protective defense strategies, including detoxification of bacterial products (Marsh and May, 2012; Zhang and Hou, 2013). These responses rely heavily on the expression of various immune mediators such as: lectins and lysozyme (Mallo et al., 2002), AMPs (Roeder et al., 2010), orthologs of Toll-interleukin-1 receptor (TIR) domain protein sterile alpha and armadillo-motif containing protein (SARM) and mitogen activated protein kinases (Marsh and May, 2012; Shivers et al., 2009). Olfactory-driven avoidance behaviors are also present (Zhang et al., 2005). Studies in *C. elegans* were among the first to suggest that gut-bacterial interactions could influence longevity in the host (Garigan et al., 2002; Gusarov et al., 2013; Ottaviani et al., 2011; Portal-Celhay et al., 2012; So et al., 2011). Whereas persistence of gut microbiota likely is completely dependent on diet, host fitness and outcome depend on relationships developed with certain bacteria (Cabreiro and Gems, 2013).

Among the non-molting protostomes and much like other animals, the gut of the earthworm (a segmented worm, or annelids) is highly compartmentalized and relies on bacteria as part of its diet and/or to: help metabolize food, process calories, establish bioavailable vitamins and nutrients, and protect against pathogens (Drake and Horn, 2007; Idowu et al., 2008; Parthasarathi et al., 2007; Thakuria et al., 2010). However, the bacterial groups seen most consistently in surveying earthworm gut microbiota often do not represent those found in the diet or experimental bedding, suggesting that some bacteria likely are retained for some time in the gut and then proliferate in response to certain diets (Rudi et al., 2009). It seems that some bacteria are resistant to or can tolerate the antimicrobial activity of digestive fluids derived from the earthworm gut (Khomiakov et al., 2007) and, not surprisingly, the immune system of this species demonstrates both specificity and memory components (Kvell et al., 2007). The earthworm gut likely has the capacity to discriminate among beneficial and harmful microbiota.

At least one species of polychaete worm (also an annelid) has been shown to maintain specific communities of bacteria within distinct regions of the gut (consistent with anterior, middle, and posterior sections, as referred to by the authors)(Li et al., 2009). Surprisingly, the deep-sea annelid tubeworms lack a gut but instead have an anterior “plum” organ that is highly vascularized and used for exchanging dissolved molecules and capturing material from seawater. Within the coelomic cavity is a specialized organ, the trophosome, which houses symbiotic bacteria responsible for nearly all of the dietary metabolic activity sustaining the animals (Cavanaugh et al., 1981). A detailed transcriptomics study under *in situ* conditions in *Ridgeia piscasae* (the deep sea tubeworm) (Nyholm et al., 2012) identified innate immune pattern recognition receptors (PRRs) as major players in helping to establish and maintain homeostasis through a carefully orchestrated dialogue between host and bacterial symbionts of the trophosome. Another marine invertebrate filter-feeder, the oyster *Crassostrea virginica*, also has been shown to also maintain distinct bacteria in distinct compartments of the gut (King et al., 2012). Here, as in other examples noted above, the gut likely is functioning as a co-evolved system in which successful partnerships emerge from a continuous dialogue involving local ecological properties of the gut compartments and an active innate defense system utilizing PRRs (including TLRs), AMPs, and the specific recognition and effector responses mediated by epithelial cells as well as immunocytes (Bachere et al., 2004; Buchon et al., 2013a; Nyholm and Graf, 2012; Rosa et al., 2012; Schmitt et al., 2012; Zhang et al., 2014).

The simplest deuterostomes include diverse groups of highly derived echinoderms, some of which demonstrate exceptionally complex immune systems (Smith et al., 2010). One of the most dramatic findings from the complete genome of the purple sea urchin (*Strongylocentrotus purpuratus*) (Davidson, 2006; Sea Urchin Genome Sequencing et al., 2006) was the presence of massively expanded repertoires of innate immune receptors and effectors (Hibino et al., 2006; Rast et al., 2006). The sea urchin immune system has undergone extensive expansion and diversification of genes primarily related to pattern recognition of microbial products, including TLRs, nucleotide-binding oligomerization domain receptors (NLRs, cytoplasmic receptors) and the scavenger receptor cysteine-rich (SRCR). Other key elements of innate immunity, including some components of the

complement system, peptidoglycan recognition proteins (PGRPs) and Gram-negative binding proteins (GNBPs), are present in the sea urchin genome in equivalent numbers to their homologs in protostomes or other deuterostomes (Hibino et al., 2006). The sea urchin also expresses, in immunocytes and other tissues, an unusual gene family that is potentially unique to these animals [Sp185/333, (Ghosh et al., 2010)]. Sp185/333 gene products exhibit exceptional diversity in immunocytes (Majeske et al., 2013) and it is likely that Sp185/333 proteins play an integral role in echinoderm immunity, possibly through coupling interactions among other PRRs.

Not surprisingly, most of the expanded and diverged genes are expressed in the gut of the sea urchin (Hibino et al., 2006) and, like many of these PRRs in mammals (Abreu, 2010; Cario et al., 2002; Rakoff-Nahoum et al., 2004), may be involved in helping to define physiologically distinct regions of the gut. As in numerous other animals, the gut of the adult echinoderm is highly compartmentalized and includes anaerobic chambers. Diverse communities of bacteria, many of which are not common in the animal's surroundings, reside within these compartments in sea urchins (Meziti et al., 2007; Thorsen, 1998; Thorsen et al., 2003), as well as in sea cucumbers (Zhang et al., 2012; Zhang et al., 2013) and a deep sea holothurian (Amaro et al., 2009); some of these bacteria may represent members of the permanent gut flora. Holothurians present an interesting case and a unique opportunity to study bacterial (re)colonization of guts from adult animals as they can undergo autotomy (i.e., self amputation), in most cases resulting in a nearly complete loss of internal organs (Byrne, 2001; Mashanov and Garcia-Ararras, 2011; Wilkie, 2001); under the proper conditions, these species can regenerate their internal organs (e.g., reconstruct the entire intestinal tract). It is likely that immunity plays vital roles in the regenerative process as development leads to re-exposure to microbes. Regeneration is also associated with a unique immune profile in some tunicates (an invertebrate chordate group, see below) (Rinkevich et al., 2007). Because of the complexity of interactions that involve innate immunity and frequently occur across mucosal barriers, invertebrate defense systems are far from simple (Loker et al., 2004). It is now apparent that the host immune dialogue with the external environment and associated microbiota is essential in maintaining tissue homeostasis, influencing renewal processes and ultimately driving immune evolution and innovation (Loker, 2012). Although it remains to be determined, the unusual expansion of innate immune receptors among some deuterostome invertebrates suggests the existence of a complex dialogue with microbiota, particularly in the gut where most of them are expressed. Complex mutualisms, as well as pathogen discrimination, within the gut lumen could drive the diversification of these PRRs (Dishaw et al., 2012b; Hibino et al., 2006; Rast et al., 2006).

Tunicates: representatives of the early chordate gut

Protochordates are invertebrate chordates such as sea squirts (Urochordata or Tunicata) and the lancelet, amphioxus (Cephalochordata). These species diverged prior to the vertebrates (Fig. 1) and as such lack classical adaptive immunity; nevertheless, they retain and share key, chordate-specific, developmental features that include a notochord, hollow dorsal nerve cord, pharyngeal slits, endostyle and post-anal tail (Brown et al., 2008; Cameron et al., 2000; Cañestro et al., 2003b; Katz, 1983; Kubo et al., 2009; Meinertzhagen and Okamura,

2001; Paris and Laudet, 2008; Shi et al., 2005; Swalla et al., 2000; Swalla and Smith, 2008). *Ciona intestinalis*, a tunicate with a compartmentalized gut (Burighel and Cloney, 1997; Hirano and Nishida, 2000; Nakazawa et al., 2013) that undergoes epithelial turnover and renewal (Ermak, 1981), is being adapted by our laboratory as a model to help define the evolution of bacterial-immune dialogue in the chordate gut (Dishaw et al., 2012a; Dishaw et al., 2011). *Ciona* is a suspension (filter) feeder that siphons water continuously, a process that results in the exposure to and consumption of ample amounts of dietary microorganisms. This basic process exposes the gut to potential colonizers and/or pathogens. A variety of cell types can be discerned within the stomach epithelium of tunicates (Fig. 2D), including absorptive, zymogenic, endocrine, ciliated mucous and undifferentiated cells (Koyama et al., 2012) as well as various lineages of blood-derived hemocytes. The gut also produces copious amounts of mucus (Goddard and Hoggett, 1982). Whereas the amphioxus genome experienced a considerable expansion of innate immune receptors almost as extensive as that described in the sea urchin (Dishaw et al., 2012b; Holland et al., 2008; Huang et al., 2008) and, through domain duplication, may have even resulted in novel signaling pathways (Zhang et al., 2008), the same is not true of *Ciona intestinalis*. Rather, *Ciona* appears to have undergone a considerable genome reduction and likely represents a core immune gene set (Cañestro et al., 2003; Dehal et al., 2002; Kubo et al., 2009). At a minimum, *Ciona* could represent the basic functional requirements for homeostasis in the gut of chordates. *Ciona* possesses a competent innate immune system (Azumi et al., 2003; Shida et al., 2003) and includes AMPs (Di Bella et al., 2011; Fedders and Leippe, 2008), two TLRs with multi-ligand binding potential (Sasaki et al., 2009), GNBPs, lipopolysaccharide binding protein (LBP), a variety of C-type lectins and other PRRs, tumor necrosis factor (TNF) (Azumi et al., 2003), mannose binding lectin (MBL) (Skjoedt et al., 2010), complement protein C3 (Marino et al., 2002), as well as the variable region-containing chitin-binding proteins (VCBPs), a relatively small family of immunoglobulin domain-containing proteins (Dishaw et al., 2011).

Like most other animals, *Ciona* likely uses AMPs as a first line of defense (Di Bella et al., 2011; Fedders and Leippe, 2008; Lu et al., 2014); however, response to specific bacterial products also can be viewed as being 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). Although the *Ciona* genome encodes only two TLRs, both are expressed, along with VCBPs, in distinct locations along the gut. Having only two TLRs, each interacting with multiple ligands, could be interpreted to be disadvantageous. However, multiple PRRs may be coupled to downstream pathways, which may facilitate more discriminating ligand recognition.

Current laboratory investigations (Dishaw et al., unpublished) suggest that *Ciona* likely maintains a state of balance (i.e., homeostasis) between tolerance and protection through host epithelial–microbe interactions that promote the maintenance of stable, adherent, microbial communities (Dishaw et al., 2014). *Ciona* TLRs (Sasaki et al., 2009), as well as additional PRRs [e.g., MBL (Skjoedt et al., 2010)], are differentially expressed in unique sections of the gut. The innate immune repertoire of *Ciona* likely is augmented by VCBPs. At least one of these proteins (VCBP-C) is secreted into the gut lumen by specialized

epithelial cells, can bind bacteria, and acts as an opsonin (Dishaw et al., 2011). It is likely that these innate immune molecules interact with the gut flora and modulate adherence patterns. VCBPs are expressed in distinct regions along the gut at the onset of developmental maturation and as well as at the onset of feeding, and are highly responsive to microbial products (Liberti et al., 2014). *Ciona intestinalis* from distinct populations have now been shown to harbor a core set of microbiota in the gut (Dishaw et al., 2014) in which adherent communities likely play key functional roles. How the *Ciona* immune system helps to discriminate among colonizing bacteria and regulate the population of gut symbionts is unclear, given the reduced complexity of its innate immune repertoire. Mariculture of *Ciona* is well established; animals can be grown under microbe-free conditions by the hundreds on standard tissue culture dishes that facilitate the production of large clonal replicates (Fig. 3) (Dishaw et al., 2012a). Current efforts are focused on characterizing the biology of the compartmentalized gut in *Ciona* and in determining the roles of VCBPs in modulating the composition of bacteria in the gut and facilitating homeostasis. More global studies of the mechanisms of temporal and developmental regulation of microbial populations in this species are in progress.

Vertebrates: immune management of complex flora

The epithelial barriers and underlying mucosal tissues of the intestine are integral components of the highly complex and integrated vertebrate immune system (Bates et al., 2006; Buchon et al., 2009a; Duerr and Hornef, 2012; Mazmanian et al., 2005; Pott and Hornef, 2012; Round and Mazmanian, 2009). The gut in general, and the distal gut (i.e., the lower intestines) in particular, possess a number of distinct compartments each housing complex communities of microbes. Each compartment within the gut is defined by a complex set of microbial, physiologic and immunologic parameters, and these compartments act together as a major site of immune detection and response (Abreu, 2010; Clarke et al., 2010; Hooper et al., 2001; Lentle and Janssen, 2008; Lentle et al., 2013; Ley et al., 2006; Macdonald and Monteleone, 2005; Ortega-Cava et al., 2003; Savage, 1977;), where host survival requires achieving a balance between resistance and tolerance (Fujimura et al., 2010; Hill and Artis, 2010; Medzhitov, 2010). This balance involves a continuous crosstalk, vital to immune development and gut homeostasis, between the gut microbial community and the immune system (Cebra, 1999; Edelman and Kasper, 2008; Hooper and Gordon, 2001; Kanther and Rawls, 2010; Mazmanian et al., 2005). This crosstalk varies considerably along the length of the alimentary canal (e.g., distinct compartments) where physical properties such as flow rate (Pacha, 2000), which itself is affected by diet (Hammer et al., 1998), as well as localized mucus production can vary significantly (Johansson et al., 2011a; Johansson et al., 2008; Ley et al., 2006). Shifts in the population structure of the microbiota can have significant downstream consequences (Bates et al., 2006; Rawls et al., 2006; Chow and Mazmanian, 2009; Round and Mazmanian, 2009; Kanther and Rawls, 2010; Vijay-Kumar et al., 2010) e.g., the breakdown of immune tolerance, resulting in chronic inflammatory conditions (Cario and Podolsky, 2005; Schreiber et al., 2005; Round and Mazmanian, 2009; Round et al., 2010a).

Despite the enormous complexity of microbiota encountered by and maintained within the gut of mammals, a single cell layer of IECs provides the interface for communication with

this immense array of microbial and food components (Duerkop et al., 2009; Vaishnava et al., 2008). Innate immune receptors (e.g., TLRs and NLRs), which are expressed by IECs (Abreu, 2010; Artis, 2008) along with a diverse set of mucosal-derived factors (Hooper and Macpherson, 2010; Macfarlane and Macfarlane, 1997), provide the front line of communications between the innate immune system and the resident gut bacteria. While recognition complexity of adaptive immune receptors involves somatic rearrangement of immunoglobulin genes, it now seems clear that this essential dialogue with resident microbiota is not to be re-learned each generation and thus is governed by heritable, germline-encoded, innate immune receptors. In vertebrates, homeostasis in the gut is likely maintained further via complex interactions with the adaptive immune system across the basal lamina propria (Round and Mazmanian, 2009). Further details of the rules that govern symbiotic interactions and sustain homeostasis in the intestines remain largely unknown (Hooper, 2009; Medzhitov, 2010). It is well established that the proper developmental ontogeny of the mammalian gut is dependent on the proper timing and exposure to appropriate microbiota. In the absence of proper colonization, appropriate maturation of the gut immune system, and in particular, gut-associated lymphoid tissue (GALT) is lacking (Atarashi et al., 2011; Cebra, 1999; Chung et al., 2012; Deplancke and Gaskins, 2001; Ivanov et al., 2009; Tlaskalova-Hogenova et al., 2002; Umesaki and Setoyama, 2000; Williams et al., 2006). Studies in various model organisms demonstrate a significant influence on host epithelial behavior and gut immune responses by the colonizing flora, via microbe-specific metabolic products or small molecules.

Bacterial recruitment and colonization is also influenced significantly by physical properties of the gut, which includes mucus type and availability (Johansson et al., 2011a), which together with host diet, can help attract bacteria to the epithelial surface (Derrien et al., 2010; Falk et al., 1998; Fischbach and Sonnenburg, 2011; Juge, 2012; Kashyap et al., 2013a; Kashyap et al., 2013b; Marcobal et al., 2013; Martens et al., 2008; Sonnenburg et al., 2005). This co-evolved mutualism, consequently, involves host physiological responses to bacterial-derived capsular polysaccharides that influence microbial colonization and host immune tolerance (Macfarlane and Macfarlane, 1997; Mazmanian, 2008; Mazmanian and Kasper, 2006; Mazmanian et al., 2005; Mazmanian et al., 2008; Round and Mazmanian, 2009, 2010; Round et al., 2010b). These examples demonstrate a co-evolved mutualism with gut flora that is hundreds of millions of years old and consistently, across diverse taxonomic lineages, modulates host tolerance and influences the process of homeostasis (Dalmaso et al., 2011; Hooper et al., 1998; Ley et al., 2008; McFall-Ngai et al., 2013).

Our understanding regarding the overall function of the vertebrate GALT has undergone a revolution within the past 10 years. The idea that the immune system evolved, in part, to support symbiotic bacteria has been most fully developed in mammals, particularly humans and laboratory rodents. However, developments in the field of immunology have always been strongly driven by the need to prevent infectious disease, leading to a misdirected early view of the immune system as being antagonistic toward all microbes, even those that normally colonize the epithelial surfaces of the host. As will be discussed below, the idea that the immune system is antagonistic toward all non-host species is still influential, with unfortunate consequences for human health.

The realization that the immune system evolved, in part, to actually support the growth of mutualistic bacteria in mammals originally derived from studies in xenotransplantation (Parker and Leshner, 2005). Researchers investigating the possibility of implementing clinical xenotransplants found that “natural” antibodies, or those antibodies present without a known history of infection or immunization, were responsible for xenograft rejection. Subsequent investigation revealed that: (a) the natural antibody repertoire is very stable in the population, not changing over the course of time (Galili et al., 1999; Parker et al., 1999), (b) mutualistic bacteria express antigens recognized by those antibodies (Casali and Schettino, 1996; MacDonald and Pettersson, 2000; Shimoda et al., 1999; Sonnenwirth, 1979; Tagliabue et al., 1983), and (c) the expression of those antigens by mutualistic bacteria does not appear to be obligatory (Stowell et al., 2010); i.e., the bacterial antigens recognized by xenoreactive antibodies are not constitutive in nature, and tended to vary depending on the bacterial isolate. These three observations, when combined, suggested that the immune system is not antagonistic toward the entire microbiome, as was thought at the time. If such antagonism existed, expression of bacterial antigens recognized by natural antibodies, particularly non-constitutive antigens, should decrease over time. Indeed, in the face of a potent immune response, natural selection should drive the microbiome to mimic the host in terms of antigen expression. With this in mind, the stable expression by gut bacteria of the antigens recognized by natural antibodies suggested that the traditional view in immunology was misguided: that immune recognition is beneficial rather than detrimental to survival of some bacteria (Everett et al., 2004). These observations, combined with earlier work demonstrating that intestinal bacteria produce their own receptors for host immune molecules (Friman et al., 1996; Schalen, 1993; Wold and Adlerberth, 2000; Wold et al., 1990), led to a series of studies indicating that the immune system supports the growth of mutualistic bacteria in the gut of mammals (Bollinger et al., 2003) (Fig 4).

The current view regarding molecular mechanisms underpinning this host-microbe association is that both host and symbiont produce an array of recognition molecules that involve both the innate and adaptive immune system of the host in a mutually beneficial way. Most of the work directed at understanding the complex relationships between gut microbes and immunity has focused on specific antigens recognized by the antigen binding domain of particular antibodies and on the expression of the type I pilus, a mannose dependent receptor found in enterobacteria such as *E. coli*. Studies in mammalian hosts have focused on immunoglobulin, particularly IgA, and on mucin. The model resulting from these studies (Fig. 4B) is based in part on the findings that a substantial amount of host-derived immunoglobulin is adherent to mucus (in distinct gut compartments) and assists in the formation of biofilms among select bacterial communities (Bollinger et al., 2003; Bollinger et al., 2006; Lee et al., 2011; Thomas and Parker, 2010). In a direct physical sense, the successful maintenance of biofilms protects the host against aggressive penetrating pathogens.

It seems clear that the view of the immune system as actively supporting the growth of symbiotic microbiota should no longer be a concept at the fringes of mainstream immunology. In fact, mainstream immunology may currently be witnessing a paradigm shift in recognizing that host immune protection was born as a consequence of a system that

evolved initially to facilitate and manage symbiotic interactions. The immune systems of modern animals have evolved to establish harmonious interfaces with the associated microbiota, if not to be the host's primary ambassador in the complex dialogue between host and associated microbiota. The gut remains the most pivotal organ from which to study how these diverse immune systems evolved.

Application of the *Ciona* system to future studies

As defined above, epithelial barriers and associated innate immune functions are phylogenetically ancient and have evolved diverse languages to help sustain a stable dialogue with adherent microbiota. As a result of this long collaboration, normal animal development and many aspects of tissue physiology (i.e., gut homeostasis) are highly dependent on microbial interactions (McFall-Ngai, 2002; Sommer and Backhed, 2013); the animal gut serves as a primary location of this interplay (Hooper and Gordon, 2001; O'Hara and Shanahan, 2006). For example, some microbial capsular polysaccharides and/or metabolic by-products readily interact with or traverse the epithelium and are sensed by innate immunocytes in vertebrates. The resulting co-evolved dialogue can produce a cascade of interactions that shape not only gut development and maturation of distinct lymphocyte lineages (Cebra et al., 1999; Koropatnick et al., 2004; Edelman and Kasper, 2008; Sommer and Backhed, 2013) but also immunological tolerance and gut homeostasis (Peterson et al., 2007; Mazmanian et al., 2008; Chow and Mazmanian, 2010; Aychek and Jung, 2014; Mortha et al., 2014).

While it is now recognized that microbes colonize the gut of most animals almost immediately after birth producing richly diverse and stable microbial communities (Savage, 1977;), the details of this carefully orchestrated dialogue are only now being revealed. In vertebrates, the distal gut possesses the most complex communities of microbes, which can vary dramatically among individuals (Hooper and Gordon, 2001; Qin et al., 2010). The respective roles of interindividual variation and effects of diet, environment and lifestyle in defining the host-microbe interactions in the gut of mammals are furthermore difficult to dissect owing to the coordinate influences of both innate and adaptive immunity along the length of the gut. Several mechanisms underlying the homeostasis of the gut have been defined (Duerkop et al., 2009) and include (a) a compartmentalized gut with epithelial renewal properties and a mucin-glycocalyx barrier (Johansson, 2012; Johansson et al., 2011a; Johansson et al., 2011b; Johansson et al., 2008), (b) complex epithelial interactions with the luminal microbial communities (Artis, 2008; Saenz et al., 2008) including the polarized expression patterns of PRRs (Gewirtz et al., 2001; Vijay-Kumar et al., 2008), and (c) multifunctional innate effectors (Diamond et al., 2009; Scott et al., 2007; Semple and Dorin, 2012). Although much is known about how the host protects itself from pathogenic invasion, much less is understood about how symbiotic communities are tolerated and cultivated by the immune system. With this in mind, model systems such as the invertebrate chordate, *Ciona intestinalis*, hold great promise for future investigations targeted at the mechanism(s) involving host support of mutualistic microbial growth in the gut.

Research in host-microbial relations has intensified in the face of dramatically increasing rates of occurrences of gastrointestinal-associated diseases, including inflammatory bowel

disease and a wide range of food allergies in westernized human cultures. The gut ecosystem, the major site of immune detection and response to a potentially overwhelming antigenic load, is failing in its ability to maintain a balance of resistance and tolerance (Rook and Brunet, 2005). The continuous crosstalk that has evolved between the immune system and the microbiome is breaking down, as is the closely associated development of host immunity (Bickler and DeMaio, 2008). To a significant extent, the root cause of these immune problems can be traced to a protracted history of concern about infectious agents, which has reinforced the traditional (and overly simplistic) view that the immune system is more of a defense system than an interface with the environment. As a result, a profound depletion of the diversity of the “human biome,” the life associated with the ecosystem of the human body, has impacted human health dramatically (Bilbo et al., 2011; Parker and Ollerton, 2013). Relevant animal models, particularly less complex models such as *Ciona*, will serve as invaluable means to address not only the ramifications of this “biome depletion” for host-microbe interactions, but also the anticipated biome enrichment procedures (Parker et al., 2012) that are expected to help resolve the problem. And since accumulation of immunocytes and lamina thickening (i.e., immune maturation) in the gut of *Ciona* appears to be intimately linked to exposure to microbiota (Dishaw et al, unpublished), the *Ciona* system offers an unprecedented opportunity to dissect the role of microbiota in directing immune maturation which may inform some processes that shape GALT formation in vertebrates.

In recent years, efficient isolator systems, which allow the researcher to completely control the timing of microbial colonization as well as the microbial community structure, have become increasingly popular (Gordon, 1960; Wagner, 2008). Mouse and zebrafish are the primary models for studying the interplay between gut microbiota and the host immune system using these isolator systems (Rawls et al., 2006). Since gut microbiota often reflect flora that are most readily acquired from the environment, critical differences likely exist between the microbiota of aquatic and terrestrial animals; this disparity may become important when comparing experimental findings between the systems. Marine environments, for example, possess abundant taxa (Pedros-Alio 2006, 2012; Munn 2011; Gibbons et al, 2013) that may influence symbiotic relationships. However, comparative studies hold enormous potential to reveal both complex and conserved features that sustain stable host-microbial partnerships (Rawls et al, 2004). For example, reciprocal microbial transplants between germ-free zebrafish and mice indicated the existence of selective local environments within each recipient that could shape the composition of related taxa, resulting in predictable assemblages of microbiota (Rawls et al, 2006). Furthermore, utilization of the zebrafish model has resulted in the finding that specific bacterial phyla within the zebrafish gut microbial community either can directly increase or decrease the absorption of dietary lipids by the gut, with clear implications for translational studies of probiotics in obesity (Semova et al., 2012). While these studies have been highly informative, a simpler chordate system may be necessary to further elucidate some of the basic underlying mechanisms conserved in the phylum to which vertebrates belong. Although most marine invertebrates do not thrive and/or develop properly in germ-free environments, *Ciona* adapts to these conditions for sufficient time to explore the biology of colonization and thus may serve as an excellent complementary model system.

Ciona intestinalis has been shown previously to be a relevant model system for studies of development (Baghdiguian et al., 2007; Cañestro et al., 2003; Davidson, 2007; Katz, 1983; Meinertzhagen and Okamura, 2001; Sasakura et al., 2009; Shi et al., 2005) and immune defense (Fujita et al., 2004; Parrinello et al., 2008; Sasaki et al., 2009; Zucchetti et al., 2009) and presently is being developed for studies of gut host-bacterial interactions (Dishaw et al., 2012a; Dishaw et al., 2011). By permitting the experimental dissection of the conversation between host and microbiota at the onset of colonization, the *Ciona* model promises to reveal chordate rules that govern the biology of colonization. Experimentally, cultured *Ciona* is adaptable to address questions such as:

- What signals do bacteria send during colonization of previously sterile tissue?
- Do most stable bacterial communities exist as biofilms on or within epithelial-associated mucus?
- How are the first biofilms formed and what roles do they play?
- What role does the immune system play in that dialogue, in supporting biofilm formation and stability?
- What are the relative contributions between physiologic versus immunologic factors that define distinct gut compartments?
- How have factors such as flow rate, compartment diameter, and physiological criteria such as pH, coevolved with the expression of various immunologic factors to produce distinct GI compartments?

Addressing these questions across a broad spectrum of animal models will help identify chordate-specific details of the host-microbial dialogue that achieve and maintain lifelong homeostasis. As discussed previously, *Ciona* has undergone considerable reductions (gene loss) in many of the innate immune gene families (Dehal et al., 2002; Hughes and Friedman, 2005) that have expanded in other deuterostome invertebrates, implicating a streamlined version of phenomena that modulate host-bacterial interactions in the gut of of this chordate. This gene reduction can be highly advantageous in defining the first steps in the dialogue by reducing the set of candidate effectors in the system. For example, while *Ciona* only appears to possess two TLRs (Sasaki et al., 2009), recent experimental observations suggest that the gut is likely discriminating among microbes in a process coupled to immune mediators possessing immunoglobulin domains (Dishaw et al, 2011; Liberti et al, 2014) to selectively maintain a core microbiota (Dishaw et al, 2014) quite possibly in a fashion reminiscent of vertebrate immune inclusion-exclusion (Everett et al, 2004).

With a completed genome, well-developed mariculture methods and exceptional experimental tractability, development of *Ciona* as a host-microbe interaction model not only will complement the studies being performed in mouse and zebrafish but also will provide a system that: (1) can produce very high numbers of offspring with a very short generation time (i.e., sample and experimental replicates), (2) can be conveniently manipulated, unlike other marine invertebrates, into a specific pathogen-free or gnotobiotic (bacteria free) system, (3) allows extremely rapid shifting of microbial exposure due to its filter-feeding, (4) performs its immunological discrimination of microbes outside of the

influence of adaptive immunity and (5) may include a single molecular species (the VCBP family) that is unique to the protochordate system but may function in a manner analogous to other classes of immune effectors found in the vertebrates. In summary, the *Ciona* model can help address some of the fundamental questions that remain in this field such as how do we define homeostasis in the gut, as governed by innate immunity, and what role(s) do stably associated microbiota serve in that process and in shaping the maturation of host immune responses?

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Highlights

- We review the evolution of host-microbe dialog in the gut
- We review the role of host immunity in recognition of gut microbes
- We provide evidence that host immunity supports the growth of gut bacteria
- We argue that *Ciona intestinalis* is a viable model in studies of gut homeostasis

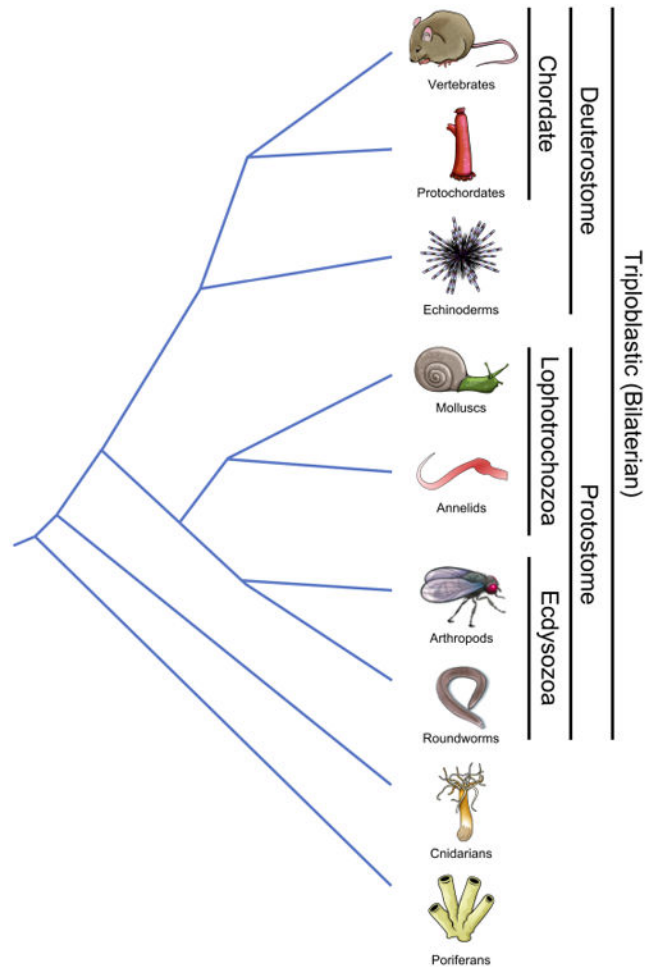


Figure 1. Phylogenetic relationships of the taxonomic groups discussed in this review. Relative times of divergence are not to scale. Groupings of particular phyla are indicated by text at right.

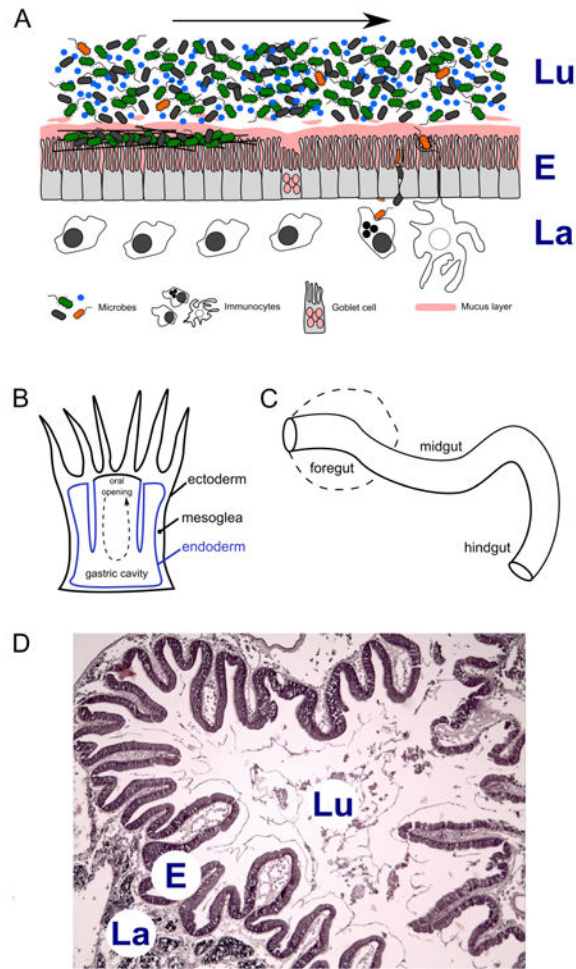


Figure 2.

(A) Schematic diagram of a generic animal gut ecosystem. Dietary intake and microbes in the lumen (top) flow through the alimentary canal alongside the gut epithelium (middle), with immune and other host cell types occupying the lamina space interior to the gut wall (bottom). Bacteria in the lumen may be transient occupants (blue), commensal/biofilm residents (green), pathogenic invaders (orange) or opportunistic invaders (black). Flow of gut contents is indicated by arrow. (B) Schematic cross section through a Cnidarian polyp showing the simple gastric cavity; oral opening on top. Tissue layers and gut anatomical features are labeled. Flow of food into and out of the oral opening is indicated by arrow. (C) Segmented gut found in most bilaterian animals. Foregut may be a simple tube or elaborated into a stomach (dashed line). (D) Haematoxylin-eosin stained section of *Ciona intestinalis* stomach. Lu, lumen; E, gut epithelium; La, lamina.

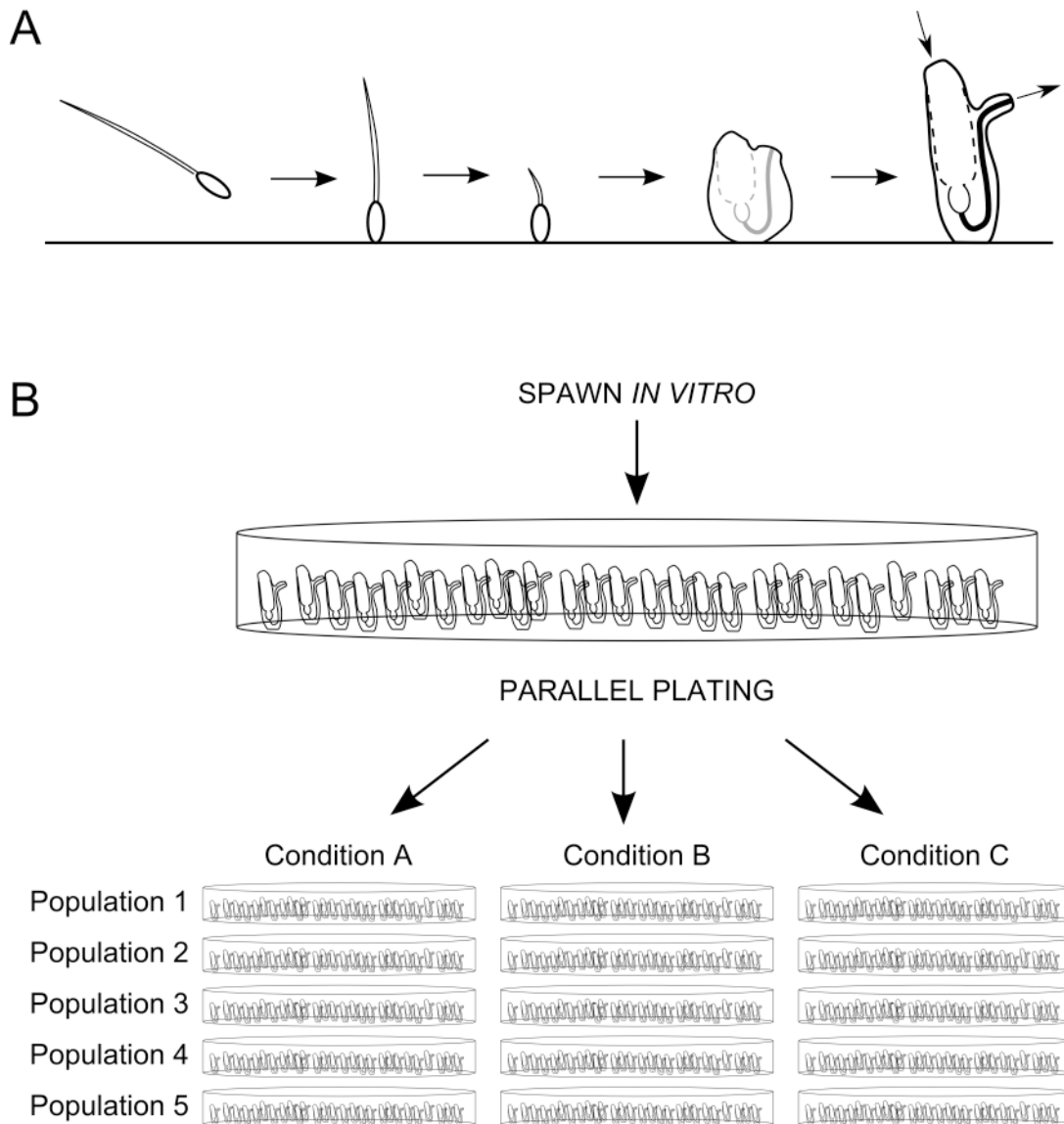


Figure 3.

(A) Life cycle of *Ciona intestinalis*, including from left to right: swimming tadpole larva stage, attached larva, tailbud retraction and onset of metamorphosis, early juvenile (not feeding; unopened siphons) and adult (with siphon flow indicated by arrows). (B) Illustration of high-throughput, parallel experiments using the *Ciona* system to investigate the response of five parallel populations of individually attached *Ciona* to three experimental conditions (standard tissue culture dishes shown). “Populations” are interchangeable with “replicates;” additional replicates allow the production of more genetic material for study. Pooled replicates from different genetic backgrounds can also be performed to strengthen observations.

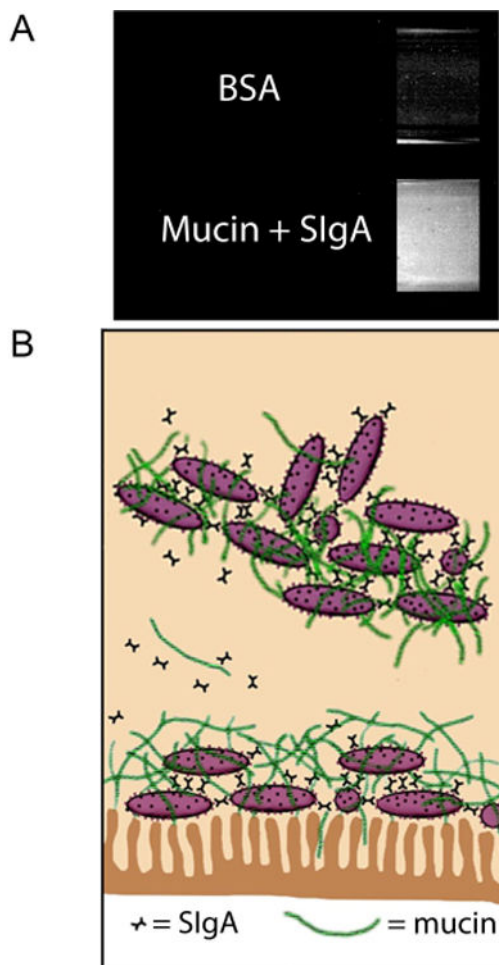


Figure 4.

Biofilm formation mediated by the human immune system. (A) side views of two test tubes (top, bottom) coated with human epithelial cells are shown. In each tube, *Escherichia coli* bacteria were incubated with nutrient rich media that was replenished on a regular basis. In the top tube, 0.5 mg/ml bovine serum albumin (BSA; a serum protein unrelated to immune function) was added to the media, and in the bottom tube, porcine gastric mucin (50 mg/ml) and human secretory IgA (SIgA; 0.5 mg/ml) were added to the media. Biofilm growth was evident in the tube containing immune-related molecules as shown by increased staining (bottom), but not in the tube without immune related molecules. (B) Schematic diagram demonstrating host-mediated biofilm growth (bottom, attached to epithelium, i.e., immune inclusion), as well as shedding of biofilm fragments (top, unattached to epithelium, i.e., immune exclusion).