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Sea squirts and immune tolerance

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Transplantation specificity and protective immunity occur in both adaptive and innate branches of the vertebrate immune system. Understanding the mechanisms that underlie specificity and self-tolerance of immune function has major significance, from preventing a rejection reaction after transplantation to dissecting the causes of autoimmune disease. The core of vertebrate immunity is the ability to discriminate between highly polymorphic ligands, and this process is also found in allorecognition systems throughout the metazoa. *Botryllus schlosseri* is a tunicate, the modern-day descendents of the phylum that made the transition between invertebrates and vertebrates. In addition, *B. schlosseri* undergoes a natural transplantation reaction, which is controlled by a single, highly polymorphic locus called *fuhc*, reminiscent of major histocompatibility complex (MHC)-based allorecognition. The life-history characteristics of Botryllus make it an excellent model to dissect the functional and developmental mechanisms underlying allorecognition, and have the potential to reveal novel insights into issues from innate recognition strategies to the evolution of genetic polymorphism. In addition, we hypothesize that allorecognition in Botryllus must be based on conserved processes that are fundamental to all immune function: education and tolerance, or the ontogeny and maintenance of specificity.

Historically, studies of allorecognition in non-vertebrate organisms were motivated by three findings. The first was the important role of allorecognition in dissecting functional aspects of cell-based immunity in the adaptive immune system. Allorecognition in the vertebrates is a function of immunity, and transplantation was a key assay that lead to the discovery of processes such as major histocompatibility complex (MHC)-based presentation of peptides to T cells (Medawar, 1945), as well as identification of the role of natural killer (NK) cells in the rejection of transplanted bone marrow (reviewed by Yu et al., 1992). The second finding is that allorecognition is universal: in plants and nearly all metazoan phyla there are examples of species that demonstrate highly polymorphic allorecognition responses, including sponges, cnidarians, echinoderms, tunicates and jawless fish (Burnet, 1971). This indicates the presence of complex and discriminatory recognition systems in lower organisms, and initial questions in the field revolved around the potential evolutionary relationships between the vertebrate and non-vertebrate allorecognition systems and, particularly before the discovery of the T-cell receptor (TCR), whether these organisms would be good models for dissecting cellular immunity. In other words, were the origins of the vertebrate adaptive immune system found in allorecognition systems of non-vertebrate organisms? And, if so, could these systems provide novel insights into immune function?

A well-studied allorecognition system exists in the colonial ascidian (or sea squirt) *Botryllus schlosseri*, a simplified and experimentally accessible model that can be used to approach these questions empirically. A member of the chordate subphylum Tunicata, the ascidians cycle between two distinct body plans during their lifetime and occupy a key phylogenetic position in vertebrate evolution (Dehal et al., 2002). Ascidian embryogenesis results in a chordate tadpole with a notochord; a dorsal, hollow nerve tube; a pharynx with gill slits; and a striated musculature. After hatching, the tadpole finds a suitable substrate, settles, and metamorphoses into a sessile invertebrate form called an oozooid. The oozooid has a complex body plan including a gastrointestinal tract, heart, central and peripheral nervous system, and a complex hematopoietic system with multiple cell types. There are approximately 500 species of ascidian and, in the majority of these, the oozooid increases in size, eventually reaches sexual maturity, and can live for several years. However, in a subset of colonial species, including *Botryllus schlosseri*, growth occurs not by an increase in size, but by an asexual budding process whereby the oozooid duplicates itself, eventually giving rise to a colony of genetically identical individuals called zooids, which are united by a common vasculature (Manni et al., 2007). The source of the budding resides in a group of mobile stem cells that can be enriched and transplanted and, as described below, these cells play an important role in the phenomenon of allorecognition (Laird et al., 2005a). Each zooid is a complete and independent body and, despite a shared vasculature, individual pieces of a colony can be surgically separated and will continue to grow. Thus, naïve subclones can be generated, allowing for multiple experiments on a single genotype (Milkman, 1967). The zooids and vasculature are embedded in a gelatinous, cellulose-based tunic, and an extracorporeal vascular network ramifies throughout the tunic, terminating at the periphery of the colony in small protrusions called ampullae, which are the site of the allorecognition response.

Each week, zooids reproduce both sexually and asexually and, for the latter, each zooid can give rise to between one and four new zooids. Thus, under good growth con-

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ditions, colonies are constantly increasing in size and often grow into adjacent individuals. When two colonies of *B. schlosseri* come into proximity, the ampullae extend from each individual and contact each other (Fig. 1A). Two outcomes can result from this interaction: either the ampullae will fuse together and form a single chimeric colony with a common vasculature (Fig. 1B), or the two colonies will reject each other in an active, blood-based inflammatory reaction, during which the interacting ampullae are destroyed and the two colonies no longer interact (Fig. 1C,D). Allorecognition is controlled by a single locus called the fusion/histocompatibility (*fuhc*) locus (Oka and Watanabe, 1957; Sabbadin, 1962). In order to fuse, two colonies must share one or both *fuhc* alleles (the reactions are identical in both situations); individuals sharing neither allele will reject each other. Populations of *B. schlosseri* contain tens to hundreds of *fuhc* alleles, making it one of the most polymorphic genetic loci ever described. Importantly, no other loci contribute to this reaction; even wild-type crosses do not deviate from single-locus genetics (Oka and Watanabe, 1957; Sabbadin, 1962; Scofield et al., 1982).

Why is there allorecognition in Botryllus?

Once two colonies are united by a common vasculature, the stem cells that are responsible for regenerating new buds transplant between individuals and contribute to both somatic and germline development in the other genotype (Sabbadin and Zaniolo, 1979). In many cases, the two stem cell lineages will compete, with one lineage dominating the other and eventually replacing all germline and/or somatic tissues for the life of the chimera. This process, called stem cell parasitism (SCP) is a repeatable and heritable trait with winner and loser genotypes found in lab-reared and field colonies (Pancer et al., 1995; Stoner et al., 1999; Laird et al., 2005a). As an individual that loses its germline is dead in an evolutionary context, there is clearly tremendous selective pressure on both the creation of, and ability to correctly discriminate between, polymorphic histocompatibility ligands.

The natural transplantation of stem cells is not unique to Botryllus. In cattle, two or more fetuses in the same uterus can possess a common extra-embryonic vasculature, and this can result in adults that are somatic cell (blood) chimeras and that accept reciprocal skin transplants. The natural transplantation of long-term hematopoietic stem cells (HSCs) was the basis of the original concept of immunological tolerance (Owen, 1945; Billingham et al., 1953). Importantly, SCP is thought to be widespread and the driving force for the evolution of polymorphic allorecognition throughout the metazoa (Burnet, 1971; Buss, 1982; Buss, 1987).

Functional aspects of histocompatibility

The allorecognition system in Botryllus demonstrates remarkable specificity, and three observations provide clues to the possible mechanisms underlying this process. First, two *B. schlosseri* colonies sharing only a single *fuhc* allele are compatible, and the characteristics of the fusion reaction are indistinguishable from colonies that share both alleles (Sabbadin, 1962; Scofield et al., 1982). Second, a priori, it seems unlikely that specificity could be the result of homotypic interactions: this would predict that an *fuhc* allele could mutate randomly such that it could only bind to itself out of multiple competing specificities and, furthermore, that this has happened from hundreds to thousands of times. Finally, functional and genomic studies do not support the presence of recombination, somatic hypermutation or other characteristics of adaptive immunity, such as memory, in this reaction (reviewed by Saito et al., 1994; Dehal et al., 2002). *B. schlosseri* has a small genome (725 Mbp) (De Tomaso et al., 1998) and cannot somatically modify its germline-encoded genes, thus it seems unlikely that receptors exist that can specifically bind to non-self *fuhc*. This is consistent with the single-allele match rules of fusibility and, together, indicates the presence of an effector system that is based on recognition of self-*fuhc* alleles by non-rearranging,

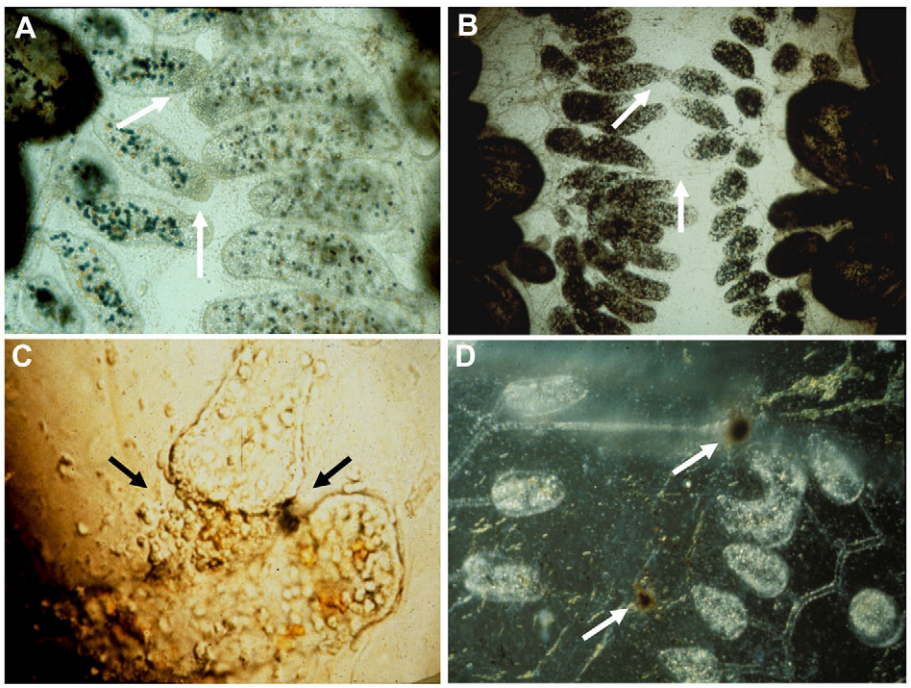

Fig. 1. Allorecognition in *Botryllus schlosseri*. (A) When two colonies grow close together, the ampullae reach out and interact (white arrows), resulting in one of two outcomes: either the two ampullae will fuse (B), allowing the circulation of the two colonies to interconnect (white arrows), or they will reject each other (C,D). Rejection is a localized inflammatory reaction where blood cells leak from the ampullae. (C) A close-up view of rejecting ampullae showing cell leakage. Figure modified and reprinted with permission from the Marine Biological Laboratory, Woods Hole, MA (Scofield and Nagashima, 1983). Once outside the circulation, cells discharge their vacuoles initiating a prophenoloxidase pathway that eventually forms dark melanin scars, called points of rejection (POR) (C, black arrow on right; D, white arrows). The ampullae then disintegrate (left of the POR that is indicated by the top white arrow in D) and the colonies no longer interact. The reaction takes around 24-48 hours to occur and is controlled by a single gene called *fuhc* (fusion/histocompatibility). Colonies will fuse if they share one or both alleles, and will reject each other if no alleles are shared.

germline-encoded receptors that are analogous to the missing-self recognition in vertebrate NK cells (Karre et al., 1986). The discriminatory ability of this system is significant. Colonies from California reject those from Japan and the Mediterranean at high frequencies (>90%), suggesting that the Botryllus effector system can pinpoint a self-allele from hundreds, to potentially thousands, of competing specificities (Rinkevich et al., 1992; Rinkevich and Weissman, 1991).

Further insight into the functional aspects of histocompatibility comes from studies in xenorecognition. There are nine closely related botryllid species but, in most xeno-pairings, the two individuals do not react to each other, neither fusing nor rejecting (Hirose et al., 2002; Saito, 2003). This implies the presence of an activation signal that initiates the reaction, a process that is further supported by two other experiments. First, if two incompatible colonies are placed briefly into contact and then separated, scarring will often occur 24-48 hours later at the site where the ampullae were in contact, demonstrating that contact with a conspecific individual activates a downstream reaction that occurs even if the colonies have been separated (Tanaka, 1973). Second, ampullae do not react when they touch other objects. This suggests that allorecognition in Botryllus consists of an activation step that leads to a default rejection reaction; however, if the colonies are compatible, an inhibitory step later blocks rejection and initiates a remodeling step resulting in vascular fusion.

Molecular mechanisms underlying allorecognition

Using a forward genetic approach, a candidate *fuhc* gene was identified, the polymorphisms of which correctly predict the outcome of histocompatibility assays in both lab-reared and wild-type pairings. Although fuhc is a member of the immunoglobulin (Ig) superfamily, it has no orthologous relationship to any mammalian protein and is clearly not the forbearer of the MHC. Fuhc has no recognizable signaling domains, and the average difference between any two *fuhc* alleles is 40 amino acids, which are spread throughout the ectodomain. The latter is intriguing in the context of how this polymorphism is interpreted to provide specificity (De Tomaso et al., 2005).

In addition to the *fuhc*, another gene was identified within the locus, named *fester*, which probably represents an allorecognition receptor (Nyholm et al., 2006). *fester* encodes a membrane-bound protein with no homology to any vertebrate proteins, but is highly polymorphic; however, these polymorphisms do not contribute to histocompatibility outcomes. Fester also undergoes somatic diversification by alternative splicing: 64 alternative splice variants of the 11-exon gene have been identified. All individuals express a full-length version plus three splice variants. In addition, each individual expresses a unique subset of between eight and 24 different splice variants, alluding to an individual-specific function.

Independent functional assays suggest that *fester* appears to play roles in both initiating the reaction as well as recognition of fuhc. For the latter, an anti-fester monoclonal antibody (mAb) interfered with reactions in an allele-specific fashion. When added to a compatible pairing, there was no effect and colonies fused normally; however, in rejecting pairs, the presence of the mAb turned a rejection into a fusion, but only if both partners carried the *fester* allele that the antibody was specific for (Nyholm et al., 2006). This finding suggested that *fester* encodes an inhibitory receptor for fuhc, and that the mAb was mimicking ligand binding. These results also suggested that both ampullae must be stimulated for fusion to occur, as both individuals needed to carry the correct allele for the phenotype.

B. schlosseri is highly amenable to reverse genetics using RNA interference (RNAi) (Laird et al., 2005b). When all *fester* expression was knocked down, completely opposite results were observed from mAb interference: the colonies became unreactive, neither fusing nor rejecting. This suggests that *fester* is also involved in activating the rejection reaction. We speculate that the splice variants that are expressed by all individuals may be involved in activation, whereas the individual-specific repertoire may play a role in binding to fuhc and constitute an inhibitory receptor. Specificity may be achieved by an overall avidity integrated from multiple splice variants binding to the polymorphic regions of fuhc. Thus, mAb interference and *fester* knockdown results would be consistent because *fester*-deficient ampullae could neither initiate a rejection reaction in incompatible pairings, nor recognize the *fuhc* locus in compatible pairings. This also suggests that fusion and rejection are not mutually exclusive events. A recent unpublished study identified a new member of the *fester* family that is encoded near the *fester* locus, and functional studies support these conclusions.

What can we learn from allorecognition in non-vertebrates?

The phylogenetic relationship of the ascidians and vertebrates predicted that *fuhc*-based allorecognition was controlled by an ancestral, MHC-type molecule (Scofield et al., 1982). However, there is clearly no orthologous relationship between the two systems. In addition, recent results in the cnidarian Hydractinia describe a candidate allorecognition gene, called *alr-2*, which has no relationship to the MHC or *fuhc* (Nicotra et al., 2009). Furthermore, the jawless vertebrates (lampreys and hagfish) have a completely unique adaptive immune system that is not based on Ig superfamily members, but rather on the recombination of genes encoding leucine-rich repeat proteins called variable lymphocyte receptors (VLRs) through mechanisms that are not based on recombination-activating gene (RAG) or activation-induced deaminase (AID); this system is probably responsible for the allorecognition reactions described in this species (Pancer et al., 2004). Thus, it appears that the proteins that control allorecognition are unique to each phylum and have evolved independently. Moreover, there can be completely unrelated mechanisms within a phylum. For example, ascidians are hermaphroditic and multiple species show self-sterility, an allorecognition event between egg and sperm. There are two candidate loci involved in this process that are unrelated to the *fuhc* locus (Harada et al., 2008). This rapid evolution is also found within the mammalian NK cell inhibitory receptors: different species use completely divergent proteins (C-type lectins or Ig superfamily members, or even a combination of both) to accomplish missing-self recognition of the MHC class I molecules (reviewed by Parham, 2005).

These recent discoveries demonstrating an absolute lack of conservation of allorecognition molecules amplify one of the most intriguing mysteries in evolution: the origins of the vertebrate adaptive immune system. Adaptive immunity appeared on the scene abruptly in nearly its present form in the jawed vertebrates (Flajnik et al., 2003), with no precursors of any

of the major genes, including the MHC, TCR or B-cell receptor (BCR), found in any other non-jawed vertebrate genome sequenced to date. Thus, one of the most intricate and complex organs in the vertebrate body seems to have evolved incredibly fast and left no clue of its origins in extant organisms: a phenomenon that is completely at odds with the evolution of any other complex biological system. The same appears to be true for allorecognition between, and even within, each phylum.

If there is no conservation of the ligands and receptors used in these various polymorphic recognition systems, are there any commonalities? One mechanism that all of these systems absolutely require is the ability of effector cells to monitor the specificity of recognition events, a process referred to recently as quality control (Boehm, 2006). This phrase encompasses both the processes that create specificity during development, a mechanism that is also termed education, as well as its maintenance over the lifespan of the individual, or tolerance. However, from a functional standpoint, education and tolerance are probably two sides of the same coin. As outlined below, recent data suggest that it may be these quality-control processes that are conserved in the metazoa.

The idea that quality control, not ligands and receptors, is the conserved process of allorecognition is best seen within the vertebrates. Recognition of polymorphic ligands is the basis of both adaptive and some innate immune function, and is carried out by a diversity of receptors including the TCR, BCR, Ly49, killer cell Ig-like receptors (KIRs) and novel immune-type receptors (NITRs), among others (Parham 2005; Cannon et al., 2008). However, each one of these systems has a common theme: the presence of activating and inhibitory receptors, coupled to signaling through immunoreceptor tyrosine-based activation motif (ITAM) and immunoreceptor tyrosine-based inhibitory motif (ITIM) domains, respectively. In other words, evolutionarily unrelated ectodomains (many of which recognize the same ligand, the MHC), all converge onto equivalent signaling pathways. And, although no evidence has yet linked these signaling pathways to allorecognition in the non-vertebrates, the key genes are conserved. Membrane proteins with ITIM and ITAM domains, as well as signal transduction molecules Zap70, SHIP and a shp1/2 homolog, have been found in genome sequences throughout the metazoa, including marine sponges (B. Degnan, personal communication), cnidarians (Steele et al., 1999), sea urchins (Rast and Messier-Solek, 2008) and protochordates, including Botryllus (Azumi et al., 2003). Allorecognition has been described in species in each of these phyla.

We hypothesize that the core of polymorphic recognition strategies may reside in intracellular processes that integrate and respond to external stimuli, and have an early evolutionary origin. The mix and match of divergent ectodomains to common signaling domains, which in turn converge onto these unknown but conserved processes, would allow rapid evolution of the domains involved in recognition at the cell surface – a necessary characteristic of an immune system. This could also provide an explanation for the sudden appearance of adaptive immunity in the jawed vertebrates, as these quality-control mechanisms would have pre-dated the ability to diversify germline-encoded genes. This also explains the complete lack of conservation of ligands and receptors both between and within phyla.

Although it is clear that further complexity exists (e.g. Kharitonenkov et al., 1997; Carlyle et al., 2008), effector function depends ultimately on the correct interpretation of external stimuli, which requires cells to measure the strength of signaling pathways and compare that with a threshold response value. In turn, this threshold must be set during development in an education process, and may also be dynamic throughout the life of a cell (Grossman and Paul, 1992). The mechanisms that underlie integration and education are unknown, but are key to understanding clinical issues such as tolerance. Moreover, over the last few years, multiple studies have demonstrated the regulation of signaling strength in the immune system. Studies in T cells and macrophages have revealed that calcineurin (which acts through an unknown mechanism), miR-181 and miR-155 (which regulate the translation of phosphatases, thereby affecting inhibitory signaling) all function to modify the signaling strength of ligand engagement on the cell surface (Gallo et al., 2007; Li et al., 2007; O'Connell et al., 2009). Obviously, the ability to manipulate signaling strength or response thresholds may be excellent targets for future clinical intervention, such as inducing tolerance.

Botryllus provides an excellent model to study both the structural basis of innate recognition as well as these quality-control processes. Unlike other invertebrate models, allorecognition in Botryllus follows simple Mendelian genetics from which even wild-type crosses do not deviate. In addition, in laboratory and wild genotypes, the polymorphisms of a single protein within this locus determine the outcome, irrespective of genetic background. Recognition occurs on the surface of a single-cell layered epithelial sheet, on the tips of the ampullae, which is macroscopic and sits outside of the body and can be easily visualized and manipulated. Thus, *B. schlosseri* offers a model where the entire allorecognition system is segregated spatially and focused on recognition of polymorphisms of a single gene, with few inputs and a binary outcome. Studies can be done in a wild-type background comparing the outcomes from multiple *fuhc* alleles, allowing the sensitivity of the system to be tested directly. Botryllus uses germline-encoded receptors to achieve this specificity, analogous to NK cell recognition of polymorphic MHC, but how this occurs is unknown. The relative simplicity of this system will allow multiple in vivo and ex vivo approaches to dissect the structural and functional mechanisms that underlie specificity.

In addition, Botryllus juveniles are competent to undergo allorecognition immediately following metamorphosis; thus, education must occur during embryogenesis, which is a 1-week process in a tadpole larva that develops in a mosaic fashion and consists of around 5000 cells. In addition, our functional assay in adults is based on the surgical removal and regeneration of the ampullae, which re-develop with no loss in specificity (Nyholm et al., 2006). Together this will allow characterization and comparisons of gene expression and alternative splice variants throughout embryogenesis and regeneration, and this in turn can be compared between individuals that are homozygous for any *fuhc* allele in different genetic backgrounds.

Botryllus is also amenable to small-molecule screens. In proof-of-principle experiments, the vascular endothelial growth factor receptor 2 (VEGFR2) inhibitor PTK787 was used to block vascular regeneration, and this effect could be

Advantages of Botryllus as a model for allorecognition

- Allorecognition occurs through the polymorphisms of a single gene via a missing-self recognition process that is analogous to the vertebrate NK-MHC inhibitory interaction
- Recognition takes place on the surface of a macroscopic extracorporeal vasculature consisting of a single-cell layered epithelium, which can be easily isolated and manipulated
- Education processes occur during embryogenesis, and specificity is maintained during vascular regeneration
- Botryllus is highly amenable to both reverse genetic analysis and small-molecule screening

phenocopied by VEGFR2 knockdown (Tiozzo et al., 2008). Given the genetic and physical properties of this interaction, coupled to the ability to take multiple redundant approaches for functional analysis, perhaps the most exciting potential use of Botryllus is the ability to take a quantitative proteomic approach to dissect allorecognition responses. As outlined above, multiple aspects of this system make it amenable to quantifying the proteome, phosphoproteome and cell surface expression during embryogenesis, regeneration and allorecognition in vivo, and for comparing values between different genetic backgrounds. This will allow quantitative analysis of the regulation of specificity.

Besides the functional aspects described above, the relative genetic simplicity of this system will allow analyses of the evolution of polymorphism, both within *B. schlosseri* as well as among related botryllid species (Saito et al., 1994). Allorecognition proteins have also been hypothesized to have an effect on larval behavior, which can now be tested directly (Grosberg and Quinn, 1986). In summary, *B. schlosseri* has a life history that links together a number of components of allorecognition from disparate fields that are experimentally accessible and can be studied empirically.

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