# The echinoid immune system and the phylogenetic occurrence of immune mechanisms in deuterostomes

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*In this article, Courtney Smith and Eric Davidson reinterpret the published data on immune function in lower deuterostomes and primitive chordates. It leads them to a new model of immune system phylogeny in which MHC-directed T-cell responses are the last to evolve and are not derived from subchordate self–nonself recognition systems.*

Modern echinoderms are a sister group of chordates in the deuterostome assemblage of the Animal Kingdom. One might imagine that elements of the genetic program specifying the immune effector cells of echinoderms, and their ancient common ancestors with chordates, might form the core of the diversified genetic regulatory programs specifying the various immune effector cells of higher vertebrates. Indeed, in their responses to bacteria, to allogeneic stimulation and to injury, sea urchin coelomocytes execute many of the cellular functions that are essential to higher vertebrate immune effector cells. However, a re-examination of some earlier data (see below) leads to the conclusion that sea urchins lack any adaptive immunological memory, though they effectively reject allografts. Furthermore, the 'self–nonself' recognition systems they utilize in allograft recognition may operate in an entirely different way, and may have evolved for entirely different purposes, than those of higher vertebrates. Given the phylogenetic status of echinoderms, these arguments do not encourage the theory that it was from primitive allogeneic recognition systems *per se* and that vertebrate immune systems were elaborated in the course of evolution.

## Echinoid immune functions

### *Reactions to allogeneic challenge*

Sea urchins display effective chronic allograft rejection reactions. About a month is required for a primary allograft to be rendered acellular[1,2]. Coffaro[1] reported that every one of over 1000 allografts carried out with *Strongylocentrotus purpuratus* and *Lytechinus pictus* was rejected, while autografts on the same animals heal inefficiently. A major class of coelomocytes, those capable of phagocytic activity, are involved in graft rejection. Coelomocytes also display xenogeneic and allogeneic cytotoxicity reactions *in vitro*, as measured by $^{51}Cr$ release[3]. In mixed allogeneic cultures of *S. droebachiensis* coelomocytes, killing is reported to be extremely efficient, and requires immediate cell contact. However, only 70% of the allogeneic mixtures and 90% of the xenogeneic mixtures showed cytotoxicity, which is quite different from the 100% *S. purpuratus* and *L. pictus* graft rejection data of Coffaro[1]. Perhaps this is due to the significantly less polymorphic genome of *S. droebachiensis* compared to *S. purpuratus* (about 2% nucleotide sequence polymorphism in *S. droebachiensis* versus about 5% in *S. purpuratus*)[4]. Taken together these

experiments show that at least some classes of sea urchin coelomocytes, including phagocytes, are specifically stimulated to express cytotoxic functions on contact with allogeneic cells.

An intriguing observation of Coffaro and Hinegardner[2] is that allografts made among partially inbred *L. pictus* are often not rejected, in contrast to allografts among individuals drawn from the wild population. The data are inadequate for any inference as to mechanism since only 16 animals were utilized from each of $F_2$ and $F_3$ sibling mated stocks, of which 5/16 and 10/16 animals, respectively, accepted allografts. Thus, when the probability that two animals share alleles is increased by inbreeding, the otherwise invariant allograft rejection response is abrogated in some animals. Like the *in vitro S. droebachiensis* cytotoxicity studies, these results suggest that allograft recognition depends on a relatively small number of polymorphic loci, or perhaps even a single locus.

Figure 1 shows a reanalysis of Coffaro's data (Coffaro, K.A. (1979), PhD Thesis, University of California) on rates of rejection of second-set and third-party allografts in sea urchins. Clearly, the coelomocyte-mediated rejection system is greatly 'activated' by the experience of primary graft rejection (or merely by the accompanying injury; no allografts following autografts are described). Thus, the second-set allografts are much more rapidly rejected than the first. However, there is no indication of immunological memory in these data: the entirely unrelated third-party allografts are rejected with exactly the same enhanced kinetics. Thus, the effector mechanism of the rejection is not one that depends on amplification of an antigen-specific subset of responding cells.

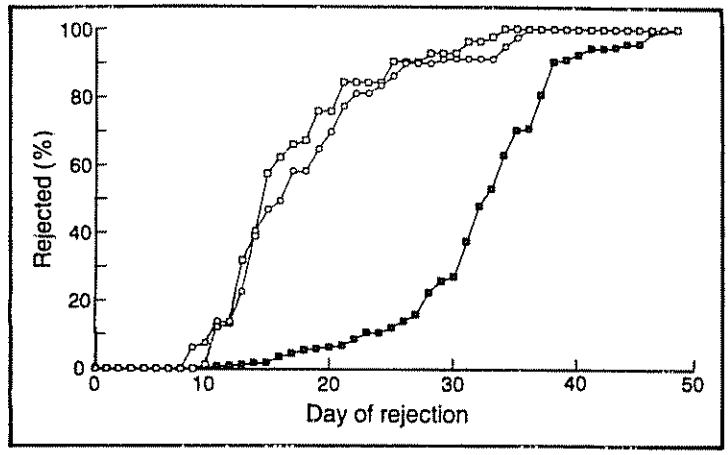


**Fig. 1.** *Allograft rejection in sea urchins. The data, taken from Coffaro (Tables 3–1 to 3–15 of Ref. 5), were compiled from eight allograft experiments on outbred* L. pictus *and converted to percentage of animals that reject over time. Primary allografts (n = 213, filled squares); second set allografts (n = 83, open squares); third-party allografts (n = 70, circles).*

*Other coelomocyte response reactions of echinoderms*

We recently discovered a remarkable molecular response of *S. purpuratus* coelomocytes to minor body wall injury[5]. Transcripts of the *profilin* gene of this organism are elevated several-fold within a day in response to a needle hole injury to the peristomial membrane, to withdrawing and reinjecting coelomic fluid, or to injecting coelomic fluid from another congeneric species. Profilin acts at the intersection of the signal transduction and cytoskeletal mobilization systems of the cell[6]. When the injury is inflicted a diffusible signal must be emitted, since most coelomocytes are remote from the site of injection. This implies a receptor and a transduction system, which, via profilin, would cause the global cytoskeletal reorganization that activated sea urchin coelomocytes undergo[7]. The injury signal evidently affects genomic expression of a battery of response genes, of which *profilin* is only one.

Starfish coelomocytes also produce interleukin 1 (IL-1), similar enough to mammalian IL-1 to be active in assays on mammalian cells and to react with antisera against mammalian IL-1 (Ref. 8). Furthermore, starfish coelomocytes respond mitogenically to IL-1 (Ref. 9). Whether or not this molecule structurally resembles IL-1, the evidence[9] indicates that echinoderm coelomocytes must express receptors for cytokines, and their signal transduction apparatus.

Finally, echinoderm coelomocytes respond to bacteria in interesting ways. They move chemotactically to the site of an infection, again implying a receptor apparatus. The red spherule class of coelomocytes, which accumulates quickly at the site of any injury, releases by degranulation a potent, broad spectrum bacteriocidal napthaquinone pigment, called echinochrome[10]. Bacteria are sequestered within cellular clots by coelomocytes, and Gram-positive bacteria induce phagocytosis in certain coelomocytes[11]. In *S. purpuratus*, inoculated bacteria are reduced 100-fold within a few hours, and are totally eliminated within a few days[12]. These processes are apparently specific, since neither a freshwater *Aeromonas* nor a terrestrial *Serratia* pathogen could be effectively attacked[11,12]. The response to bacteria is clearly not adaptive, however, as the rate of clearance is the same whether or not the sea urchins have been previously inoculated with the same or different bacteria.

In summary, echinoderm coelomocytes possess many of the basic functional attributes of vertebrate immune effector cells. They are responsive, motile, chemotactic cells that produce and are affected by cytokines; they evidently mount various receptors for diffusible factors that probably activate signal transduction systems; they have innate sensitivity to various bacterial products; they elaborate cytotoxins by degranulation; they carry out phagocytosis and encapsulation. These responsive activities require profound cytoskeletal transformations. They can recognize their own cells and kill foreign cells by contact-dependent cytotoxic processes. But it is extremely unlikely that echinoderms are capable of any form of specific immunological memory.

**Allogeneic responses of urochordates**

Allogeneic responses have been studied in tunicates in three ways: by zooid fusion in colonial tunicates, particularly *Botryllus*[13,14]; by allograft rejection in the solitary ascidian *Styela*[15]; and by mixed hemocyte killing reactions in experiments carried out on *Styela*[16] and *Halocynthia*, another solitary ascidian[17,18]. A point of major importance on which genetic and transplantation studies in *Botryllus* and *Halocynthia* agree, is that the allorecognition effector cells of urochordates kill allogeneic targets unless they share one allele of one (*Botryllus*) or two (*Halocynthia*) polymorphic recognition loci. That is, killing, which requires cell contact, occurs unless the interacting cells share an allele. This system thus detects

similarity, not difference, unlike the adaptive major histocompatibility complex (MHC)–peptide recognition systems utilized by the killer T cells of higher vertebrates. In wild populations of the species studied, there is an easily observed frequency of randomly selected pairs of individuals that apparently share the same recognition allele: in *Halocynthia* this is about 15% (Ref. 18), in *Styela* about 25% (Ref. 15) and in *Botryllus* 3–11% (Ref. 19).

In *Botryllus*, the same allogeneic recognition system is used to prevent self-fertilization within the hermaphroditic colonies. From this fact the argument is made that a fertilization barrier against inbreeding provided the selective force for the phylogenetic development of the allogeneic immune response systems of higher deuterostomes[14]. However, one might heed Burnet's[20] cautionary remark: "My chief quarrel with much current work is the effort to force results obtained in invertebrates into the pattern of vertebrate immunology without regard to the extreme differences in physiology." Indeed, such a difference in 'physiology' is evident even within urochordates. Thus, Fuke[17] showed that the fertilization of *Halocynthia* gametes is entirely independent of the alloreactivity of their coelomocytes. Fertilization in this species takes place between gametes of any pair of animals, whether their coelomocytes are compatible or not. The use of allorecognition by *Botryllus* is considered to be a specialization of *Botryllus* for colonial existence.

A vexing issue is whether urochordates possess an immunological memory. Raftos *et al.*[15] reported a complicated series of experiments on *Styela* allograft rejection that has a bearing on this problem. First-set allografts are rejected in a little over a month, and second-set grafts from the same donors are rejected in a shorter time. Of third-party allografts, 25% behaved like the second-set, that is, they were rejected at an accelerated rate. Another 25% were not rejected at all, presumably because they were derived from genetically compatible animals; and the remaining 50% were rejected at the slower rate of the primary set. Further allografts suggested that most of the rapidly rejected third-party allografts were from animals that, because of the relatively low polymorphism, happened to share a marker with the initial, first-party allograft donors, while the majority of the other third-party donors did not. This result, which unfortunately rests on a rather small number of animals, would imply some ability of the *Styela* system to distinguish between different allogeneic donors. Raftos and Briscoe[21] have produced genetic evidence showing that the *Styela* allorecognition system functions somewhat differently from that of either *Halocynthia* or *Botryllus*. They conclude that in *Styela* there is a single locus involved in recognition, just as in *Botryllus*, but that for graft acceptance in *Styela* it is required that both codominant alleles be held in common. Hence, this system appears formally equivalent to one which detects difference, as does the higher vertebrate MHC allorecognition system.

### Allogeneic responses might reveal a developmental, rather than an immune defense, mechanism

We feel that there is, as yet, no overwhelming argument that urochordate allorecognition systems differ significantly from echinoderm allorecognition systems. If it is unlikely that a self–nonself recognition system to avoid inbreeding provides a general explanation for allorecognition reactions in solitary tunicates, this is even less an acceptable hypothesis to explain allorecognition in echinoderms. There is no evidence for colonial ancestry in echinoderm evolution[22], nor is there any intraspecific restriction on sperm–egg interaction in echinoids. Gametes from any individuals cross-fertilize freely, including eggs and sperm from *S. purpuratus* hermaphrodites, which occur spontaneously in nature (authors' unpublished data). It is a fantasy to suppose that control of inbreeding could provide an adaptive explanation for the existence of graft rejection in sea urchins. A different way to think about allogeneic rejection in subchordate deuterostomes is that this experimental manipulation reveals (in a particularly bizarre way) a common mechanistic aspect of cell interaction required in all developmental morphogenesis, and also in wound healing.

In development, cells participating in morphogenesis recognize each other as a prerequisite for integration into a tissue by means of a variety of specific cell surface molecules. Some of these are homophilic, as exemplified by the tissue-specific cadherins[23], and others heterophilic, for example the integrin–fibronectin family of interactions[24]. Perhaps some of these molecules, or others that function like them, are polymorphic in these marine invertebrates, just as is much of the single copy genome[4]. In normal development, cells that fail to form an appropriately balanced set of connections could be targeted for destruction; a similar fate might await cells that fail to form normal connections in wound healing. If, in sea urchins, lack of specific cellular adhesion molecules required for integration of an allograft into the surrounding tissue triggered coelomocyte killing reactions, it is possible that shared possession of a single allele would suffice for acceptance (for example, see the cadherin transfection experiments)[23,25,26]. It would be likely that some organisms (or some tissues in some organisms) are less tolerant of unmatched (that is, only haploidentical) polymorphic adhesion molecules than others. From this point of view, the difference between the *Botryllus* and the *Halocynthia* and *Styela* systems may not be fundamental or important. The basic issue is whether certain urochordate hemocytes are able to recognize differences among allogeneic cell surfaces, and then amplify, providing an immunological memory, as well as recognizing the presence of (at least) a single self marker, for which the evidence seems clear. This issue is unresolved, and further data are obviously needed.

How, then, does the lower deuterostome immune defense system actually work? It is not the intention here to propose a series of mechanisms, but rather to examine the somewhat meagre evidence, considering Burnet's caution, with respect to those aspects that are, and those that are probably not, analogous to the immune systems of higher deuterostomes. As we noted above, many of the nonadaptive basic defense functions of mammals, including cytotoxic, chemotactic, phagocytotic and 'natural' cellular immunity against bacteria are already present. In addition, the accelerated allogeneic responses, even if nonspecific, suggest the possibility of cytokine-

mediated proliferation of effector cells. Injection of allogeneic cells into *Styela* causes proliferation of lymphocyte-like coelomocytes in certain body wall 'crypts', that apparently serve as hematopoietic tissues[27]. An IL-1-like molecule is also present in tunicates[28], and has been shown directly to induce proliferation in *Styela* hematopoietic cells[29].

**Where in chordate phylogeny does the MHC–peptide-based T-cell recognition system arise?**

Much evidence indicates that teleost fish possess immune systems that are essentially similar to those of all tetrapod classes of vertebrates (for reviews see Refs 30–35). Teleosts apparently have B and T cells, they are capable of acute allograft rejection and an adaptive circulating antibody response, and their immunoglobulin genes are organized in essentially the same way as vertebrates. However, none of these statements is obviously applicable to elasmobranchs (sharks and rays) or agnathans (lampreys and hagfish).

The arguments in the previous sections suggest that invertebrate deuterostomes may not use primitive versions of the allorecognition systems of fish and tetrapods; they might recognize only cellular adhesion molecules that are, within a more or less limited range of polymorphism, self-specific. This mode of allorecognition function may be so different from that of higher vertebrate systems, which are adaptively sensitive to thousands of different nonself peptides, that there could well be no direct evolutionary relationship. Thus, it is of interest to re-examine the evidence with respect to allorecognition in elasmobranch and agnathan vertebrates. Specifically, we would like to ask the iconoclastic question of whether the differences between the allorecognition systems of lower chordates and those of echinoderms do imply the presence in lower chordates of allorecognition mechanisms basically similar to those of mammals.

*Allogeneic responses in agnathans and elasmobranchs*

We are again forced to rely on sparse data, a fact that constitutes a reminder of the insecurity of the traditional view that these lower vertebrates mount responses to allogeneic challenge which, in mechanism, are homologous with those of higher vertebrates. Allograft rejection in sharks and lampreys is chronic[36,37]; although in the shark *Heterodontus* second-, third- and fourth-set grafts are rejected more rapidly[36]; unfortunately, no third-party grafts were carried out (compare with Fig. 1 of Ref. 36).

Acute allograft rejection occurs only in those vertebrate taxa in which *bona fide* T cells have been identified, that is, teleosts and tetrapod classes. In elasmobranchs and agnathans there is no functional or other evidence for killer T cells, or for T-cell regulation of the response to allogeneic challenge[30]. Adult lampreys lack a thymus[38], and they do not possess spleen or lymph nodes[39]. In the lamprey, there is also a sharp reduction in allograft survival time comparing second-set to primary allografts, but again there are no adequate third-party data, so whether this is simply a nonspecific activation of the cellular rejection system or an adaptive immunological memory, as claimed, is unclear[37,40].

Even if there is an immunological memory in the elasmobranch response to allogeneic challenge, this may depend on an entirely different mechanism than that used in the T-cell mediated responses of mammals. Elasmobranchs differ from subchordate deuterostomes as they produce antibodies. In sharks, purified IgM binds to lymphocytes (rather than to target cells) via Fc receptors, thereby endowing these lymphocytes with specific cytotoxic capabilities[41,42]. It is not known whether this form of cytotoxicity can function adaptively, and the experiments cited were carried out with 'natural' antibody. Sharks also have potent spontaneous killer macrophages that are targeted *ab initio* against many different cell surface determinants, but which require no priming or immunization[43]. Chronic allograft rejection in elasmobranchs could be mediated by FcR-bound antibodies. An interesting further light on this is shed by a recent observation of Haynes and McKinney[44]. The spontaneous killer macrophage activity of elasmobranchs is downregulated by suppressor lymphocytes at higher temperatures, but allogeneic suppressors work just as well as do autologous suppressors. Thus, even in a situation that superficially seems to resemble a mammalian T-cell-mediated process, histocompatibility and restriction are not involved.

*Humoral responses of agnathans and elasmobranchs*

Recent studies have revealed that both the heavy and light chain immunoglobulin (Ig) genes of sharks and their closest sister group, the rays and skates, are organized entirely differently from those of all teleosts and tetrapods[31,45–47]. Each germ line *V*-region gene has its own *J* and C coding sequence. While many of these genes undergo recombination, producing some junctional diversity, about half are already partially or fully recombined in the germ line. Perhaps these genes encode natural antibodies that are already as fully effective against evolutionarily selected pathogen targets as any likely to be formed by an insertion of a new junctional sequence. Since shark *V*-region genes do not necessarily exclude one another from productive rearrangement, any given shark B cell may produce many different immunoglobulins. Thus there is a reduced role for the selectional function carried out by helper T cells in higher vertebrates, since in elasmobranchs there is no choice to be made among the many different possible recombinational products of each Ig gene cluster. Ig gene organization in elasmobranchs in itself provokes the issue of whether helper T cells are involved in the humoral immune response of these animals. Furthermore, in sharks there is no heavy chain class switching, nor affinity maturation and it is not clear if clonal amplification of B cells occurs at all[31,45,47]. Thus, in shark, serum antibody levels are always remarkably high, irrespective of immunization. Even if in elasmobranchs there is B-cell clonal amplification, this does not necessarily imply any helper functions exercised by T cells or their like.

In summary, there is no clear evidence implying either killer or helper T-cell functions in lower chordate immune systems. Recently, a short sequence, possibly homologous to an exon of mammalian MHC was recovered by a polymerase chain reaction (PCR) from shark genomic DNA[48] (see Fig. 2). There is no information on the functional nature of the expressed molecule. Whatever its

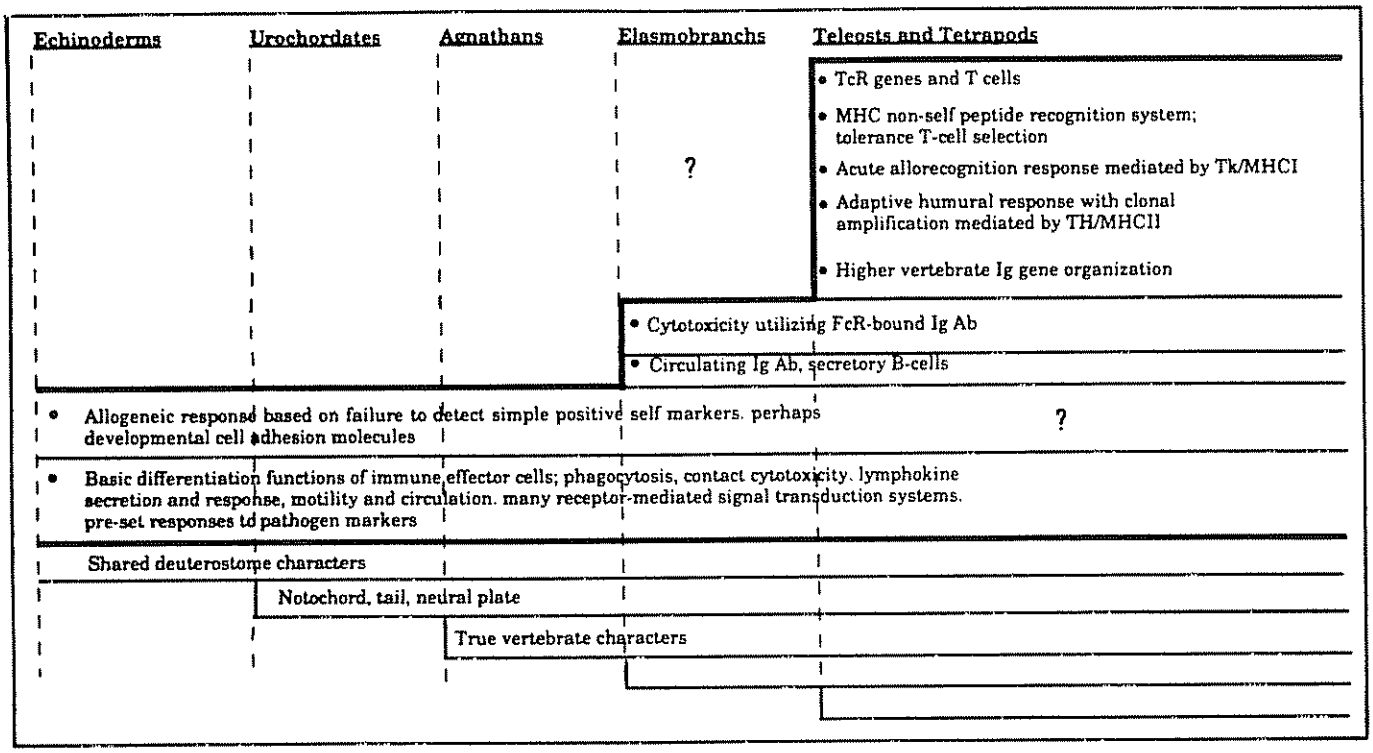


Fig. 2. *A stepwise phylogeny of immune system function. The diagram is constructed as an inclusive arrangement of shared higher deuterostome characters. These are merely indicated by empty brackets at the bottom of the figure; characters shared between elasmobranchs and higher vertebrates but not present in agnathans (for example, the jaw), and the large number of specifically tetrapod–teleost characters are omitted from the figure (for detailed cladistic character analyses of higher deuterostomes, see references in Ref. 55). The dashed line with question mark in the elasmobranch area of the chart opposite MHC is based on a recent report[48] of a genomic fragment that might encode an MHC class I molecule. The sequence in question, however, is of an α3 domain, and there is no evidence for the presence of the peptide interaction region of the molecule. Furthermore, while the sequence displays a great many similarities with canonical C heavy chain family features, there are only four amino acids in this sequence that seem relevant to the assertion that it represents an MHC class I sequence. Of these, three are not in any position in the α3 chain for which there is structural evidence for important intra- or inter-chain interactions, while the fourth (Gln242) might interact with $\beta_2$-microglobulin in mammalian MHC class I α3 domains; we are indebted to Dr Pamela Bjorkman for this analysis. It may be concluded that, at present, the identification of this molecule as a shark MHC class I remains tentative. TK; cytotoxic T cells; TH; helper T cells.*

structural homologies, we argue on available biological and molecular evidence that the T-cell restriction function of the mammalian MHC system is unlikely to exist in elasmobranchs.

## A stepwise immune system phylogeny

Marchalonis and Schluter[49] describe the evolutionary appearance of Ig genes as an evolutionary 'big bang'. With apologies to these authors, a second big bang was the appearance of the MHC-restricted T-cell system, which is evidently present in all tetrapods and bony fish. On the basis of perhaps a less than customarily optimistic survey of the scattered evidence on lower deuterostomes, we are inclined to place the occurrence of this second major event of immune system evolution near the point at which the teleost–tetrapod group diverges from their sister chordate groups, and no earlier. We believe the allograft rejection data for lower deuterostomes have traditionally been overinterpreted.

Figure 2 presents a different phylogeny for immune system functions, which incorporates the results of the arguments made in this article. The echinoderms and urochordates possess a nonspecific, activation-based defense system that involves many basic cellular mechanisms required of higher vertebrate immune effector cells. Though agnathans have been thought to produce Ig-like molecules[50], recent sequence data indicate that the putative Ig molecule is closely related to complement C4 and it lacks any clear Ig homology[51]. Thus, the elasmobranch grade of vertebrate organization displays the major evolutionary advance represented by the appearance of secreted Ig, and of Fc receptors. Igs add the capability of recognizing nonself molecules to the nonspecific, self-recognition capabilities of the phagocyte category of immune cells of subvertebrate deuterostomes. However, in organisms of this grade, the generation of Igs is probably still not regulated by an MHC–helper T-cell mechanism. We think the set of processes required for the higher vertebrate (teleost and tetrapod) immune system (listed in the top right of Fig. 2) appeared together at this point in vertebrate evolution, and that they are not simply progressive refinements or diversifications of mechanisms already present; the mechanisms, and the genes they require, are novel, as are many other aspects of organ system, structure and function in the teleost–tetrapod clade.

Of course, nothing in evolution springs forth fully blown, at any level of organization. Among the features of the mammalian immune system that suggest the nature of evolutionary functional intermediates are NK cells. Some of these recognize MHC signals on their targets, and their effector functions are similar to those of killer T cells[52] although they do not utilize rearranging TCR. Similarly γδ T cells display much less receptor diversity than the αβ T cells, are not MHC restricted, and appear almost like cellular mediators of a natural immunity or an injury response system[53]. Consistent with this is a cladistic analysis of Ig family *V*-region sequences carried

out by Hubbard and Marchalonis[54]. From this it can be concluded that TCR *V* regions (α and β) diverged after the gene families encoding Ig light chain *V* regions and Ig heavy chain *V* regions diverged from one another. That is, if the presence of TCRs defines T cells, these arose after Ig-secreting B cells that utilize Ig heavy and light chains.

A take-home lesson from this exercise is that modern data regarding the cellular and molecular biology of lower chordate and subchordate deuterostome immune systems are clearly required, if these issues are to be resolved. There could be good news here too for molecular evolutionary immunologists. If we are at least partly right, major steps in immune system evolution occurred relatively late in vertebrate phylogeny, and thus organisms representing all grades of this phylogenetic process are accessible for analysis, rather than shrouded in the mysteries of Cambrian origins.

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