CHAPTER 15

UROCHORDATE IMMUNITY

Masaru Nonaka*[,1] and Honoo Satake[2]

[1]*Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Hongo, Tokyo, Japan*
[2]*Suntory Institute for Bioorganic Research, Mishima, Osaka, Japan*
**Corresponding Author: Masaru Nonaka—Email: mnonaka@biol.s.u-tokyo.ac.jp*

Abstract: This chapter provides a short review of the immune system of urochordates, the closest living relative of vertebrates. Since adaptive immunity is a unique property of vertebrates, urochordates rely exclusively on innate immunity to recognize and eliminate pathogens. Here we discuss three immune systems of urochordates which show different evolutionary relationship with the vertebrate immune system. Urochordate Toll-like receptors (TLR) show a clear orthologous relationship with vertebrate counterparts, although they show unique characteristics most likely gained in the urochordate lineage. The urochordate complement system also shows orthologous relationship with the vertebrate complement system. From the structural and functional viewpoints, it seems to represent a more primitive state of the vertebrate complement system without any major deviation. In contrast, the allorecognition systems of urochordates show no evolutionary relationship with any invertebrate or vertebrate systems, suggesting that they were invented in the urochordate lineage.

INTRODUCTION

Vertebrates developed two types of the adaptive immune system, a well-defined one based on conventional lymphocytes and the major histocompatibility complex (MHC) of jawed vertebrates and an emerging one based on novel lymphocytes of jawless fish.[1] No evidence for the presence of adaptive immunity has been reported from invertebrates thus far, whereas various types of innate immunity, some common with vertebrates and others specific to certain phylogenetic groups, have been reported. To understand the origin and evolution of vertebrate immune system, it is essential to analyze the immune system of the closest relatives of vertebrates. Vertebrates, urochordates and cephalochordates constitute the phylum Chordata. Traditionally, cephalochordates are considered as the closest living

Invertebrate Immunity, edited by Kenneth Söderhäll.

relatives of vertebrates, with urochordates representing the earliest chordate lineage. This view is mainly justified by overall morphological similarities and an apparently increased complexity in cephalochordates and vertebrates relative to urochordates. However, recent molecular phylogenetic analyses provided compelling evidence that urochordates and not cephalochordates, represent the closest living relatives of vertebrates.[2] Comprehensive search for immune-related genes from draft genome information of one urochordtae species, *Ciona intestinalis*, indicated that the pivotal genes for adaptive immunity, such as the MHC class I and II genes, T-cell receptors, or dimeric immunoglobulin molecules, are missing from the *Ciona* genome.[3] In contrast many genes possibly involved in innate immunity are identified. Among them, here we discuss three well-characterized systems, TLR (Toll-like receptor), complement system and allorecognition. The former two are evolutionary related to their vertebrate counterparts, whereas the last one is unique to urochordate lacking the vertebrate counterpart.

TOLL-LIKE RECEPTORS (TLR)

What Are Toll-Like Receptors?

Toll-like receptors (TLRs) play pivotal roles in host defenses via the innate immune system. All TLRs are Type I transmembrane proteins which harbor an intracellular Toll/Interleukin-1 receptor (TIR) domain and extracellular leucine rich repeat (LRR) motifs.[4,5] LRRs exhibit specific pathogenic ligand recognition and TIR participates in the activation of downstream signaling pathways. Nine functional human TLRs (hTLRs) have been identified. As summarized in Table 1, each hTLR directly recognizes their specific ligands (or pathogen-associated molecular patterns, PAMPs). Molecular diversity in the number and organization of LRRs enables the specific and sensitive recognition of PAMPs by respective TLRs. TLRs are expressed not only in immune cells such as lymphocytes, macrophages and dendritic cells but also nonimmune tissues including lung, small intestine, stomach and testis. TLR1, TLR2, TLR4, TLR5 and TLR6 recognize extracellular microbial pathogenic components on plasma membranes, whereas TLR3, TLR7, TLR8 and TLR9 respond to viral DNA or RNA on endosomes. In addition, TLR4 requires an extracellular associated protein, MD2, to recognize lipopolysaccharide (LPS).[4-6] Interaction of TLRs with specific PAMPs triggers signal transduction pathways via adaptor proteins (MyD88, TIRAP, TRIF and TRAM) followed by activation of a wide range of inducible transcriptional factors such as NF-κB, AP-1 and IRF, leading to production of a inflammatory cytokine TNFα, chemokines and/or Type I interferon.[4,5] TLRs or their related genes have also been detected in fish,[7-9] cyclostomes,[10] amphioxus,[11] sea urchin,[12] annelid[13] and cnidarian,[14] although their functions, except for several fish TLRs, have yet to be elucidated.

TLRs of *C. intestinalis*

A *Ciona* genome survey and molecular cloning revealed the presence of two TLRs in *C. intestinalis*, namely, Ci-TLR1 and -2.[3,15] Ci-TLR1 and Ci-TLR2 are composed of a TIR, transmembrane and LRR domain, which is typical of TLRs. Moreover, 7 and 13 LRRs are found in Ci-TLR1 and Ci-TLR2, respectively. Ci-TLR1 and -2 were most homologous to hTLR7 (26%) and hTLR8 (26%), respectively.[15] However, the sequence homology is inconsistent with PAMP recognition and intracellular localization of Ci-TLRs (Table 1).

Table 1. Ligands of human and *Ciona* TLRs

TLR	Ligands	Intracellular Localization
TLR1/2	triacylated lipoprotein	PM
TLR2	Zymosan (yeast cell wall 1, 3-β-glucan Lipoarabinomannan Heat-killed *Legionella pneumophila* (HKLP, Gram-negative) Heat-killed *Staphylococcu aereus* (HKSA, Gram-positive) Glycosylphosphatidylinositol (GPI)-anchored glycoprotein	PM
TLR3	poly(I:C) (double-stranded RNA)	ES
TLR4 (with MD2)	LPS (lipopolysaccharide from Gram-negative bacteria) Lipid A (lipid component of LPS)	PM
TLR5	Flagellin (bacterial flagellar filament)	PM
TLR6	MALP-2 (mycoplasma-derived macrophage-activating lipopeptide) FSL1 (micoplasma-dereived lipoprotein)	PM
TLR7	Imidazoquimod (imidazoquinolone amino acid analog), single-stranded RNA	ES
TLR8	Single-stranded RNA	ES
TLR9	Unmethylated CpG DNA	ES
Ci-TLR1	Zymosan (yeast cell wall)	PM and ES
Ci-TLR2	Heat-killed *Legionella pneumophila* (HKLP, Gram-negative) poly(I:C) (double-stranded-RNA) Flagellin (bacterial flagellar filament)	

PM, plasma membrane; ES, endosome.

The Ci-TLR1 and Ci-TLR2 genes were expressed intensively in the stomach, intestine and numerous hemocytes and, to a lesser degree, the central nervous system.[15] These findings indicate that Ci-TLRs function mainly in the alimentary tracts and hemocytes. Intriguingly, both of Ci-TLRs, unlike any vertebrate TLRs, were present on both the plasma membrane and a number of late endosomes.[15] Moreover, Ci-TLR1 and Ci-TLR2 activated NF-κB in response to multiple TLR ligands (Table 1), which are recognized by different mammalian TLRs. Zymosan (*Saccharomyces cerevisiae* cell wall) for hTLR2, heat-killed *Legionella pneumophila* (HKLP, a Gram-negative bacterium) for hTLR2, double-stranded RNA, poly(I:C) for hTLR3, *Salmonella typhimurium* Flagellin (the major component of the bacterial flagellar filament) for hTLR5 elicited a dose-dependent transactivation of NF-κB in the *ci-tlr1-* or *ci-tlr2*-expressing cells.[15] Poly(I:C) also elicited approximately 4-fold and 10-fold Ci-TNFα expression in the anterior and middle intestine, respectively.[15] Likewise, induction of 4-fold and 10-fold Ci-TNFα expression by Flagellin was observed in the stomach and middle intestine, respectively.[15] In contrast, no Ci-TNFα induction was detected in the posterior intestine.[15] These profiles of the Ci-TNFα induction are compatible with the tissue-distribution of *ci-tlr* expression; *ci-tlr1* and *ci-tlr2* are abundantly expressed in the stomach, anterior and middle intestine, but not in the posterior intestine.[15] These data lead to two important conclusions. Firstly, Ci-TLRs, like vertebrate TLRs, directly recognize their PAMPs and trigger the transactivation of NF-κB. Secondly, Ci-TLRs are 'functionally hybrid TLRs' of vertebrate cell-surface TLRs and endosome TLRs:

poly(I:C) is recognized by hTLR3 on endosomes TLR, whereas hTLR2 and hTLR5 respond to Zymosan, HKLP and Flagellin on the cell surface, respectively (Table 1). In addition, the PAMPs of Ci-TLRs are in good agreement with their cellular localization to both the plasma membrane and endosomes.

C. intestinalis possesses only two TLRs,[15] whereas other deuterostome invertebrates, amphioxus and sea urchin, were found to possess a great number of TLRs or their related genes: 72 genes in amphioxus[11] and 222 genes in sea urchin.[12] Furthermore, the molecular phylogenetic analyses demonstrated that most of these genes were generated via species-specific gene duplication, suggesting that these deuterostome invertebrates expand TLRs or their related genes in unique lineages of innate immunity, if most of the genes are functional. These findings lead to two scenarios of evolution of TLR or their related genes. First, only a few TLR or their related genes might have existed in a common deuterostome antecedent and *C. intestinalis* conserves the ancestral characteristics. Alternatively, a common deuterostome antecedent might have numerous TLR family genes. If this is true, *C. intestinalis* should have lost a large part of ancestral TLR family genes. Instead of such a gene deletion, Ci-TLRs are highly likely to have acquired multiple PAMP recognition and intracellular localization as mentioned above. Unfortunately, Ci-TLRs are at present the only invertebrate TLRs of which intracellular localization, PAMP recognition and signaling have been investigated. Elucidation of PAMPs and intracellular localization of sea urchin, amphioxus and cyclostome TLRs is expected to contribute not only to understanding of their biological roles but also to the investigation of molecular and functional divergence of the invertebrate TLR family.

COMPLEMENT SYSTEM

The mammalian complement system is a powerful defense mechanism consisting of more than 30 plasma and cell-surface proteins interacting in the recognition and elimination of pathogens.[16] Three major physiological functions of the mammalian complement system are; opsonization of the foreign particles, induction of the inflammatory reactions and cytolysis. Evolutionary studies revealed that the origin of the multi-component complement system consisting of C3, Bf (factor B) and MASP (mannan-binding lectin associated serine protease) is traced back to the common ancestor of Eumetazoa.[17,18] In addition, marked development of the complement system by gene duplication of the key components and subsequent functional differentiation likely occurred at the early stage of vertebrate evolution.[18] Thus, the urochordate complement system represents the evolutionary stage just before this development and accumulating analyses made it the best-analyzed invertebrate complement system. Several complement genes have been identified mainly from two species, *Halocynthia roretzi* and *Ciona intestinalis*. Those genes are; C3,[19,20] Bf,[3,21] MASPs,[3,22] mannan-binding lectin (MBL),[23] ficolin[24] and CR3 alpha[25] and beta.[26] In addition, a glucose binding lectin (GBL) lacking the collagen domain was reported from *H. roretzi* as a possible functional substitute for MBL.[27] For the functional aspect, *H. roretzi* C3, ficolin and GBL proteins were isolated from the body fluid and were shown to act as a component of the opsonic complement system. Moreover, the C3a fragment of *C. intestinalis* C3 was shown to have a chemotactic activity,[28] indicating that the role of the complement system in inflammation is also conserved between mammals and urochordates. In contrast, the third activity of the mammalian complement system, cytolytic activity, has not been recognized in the urochordate complement system.

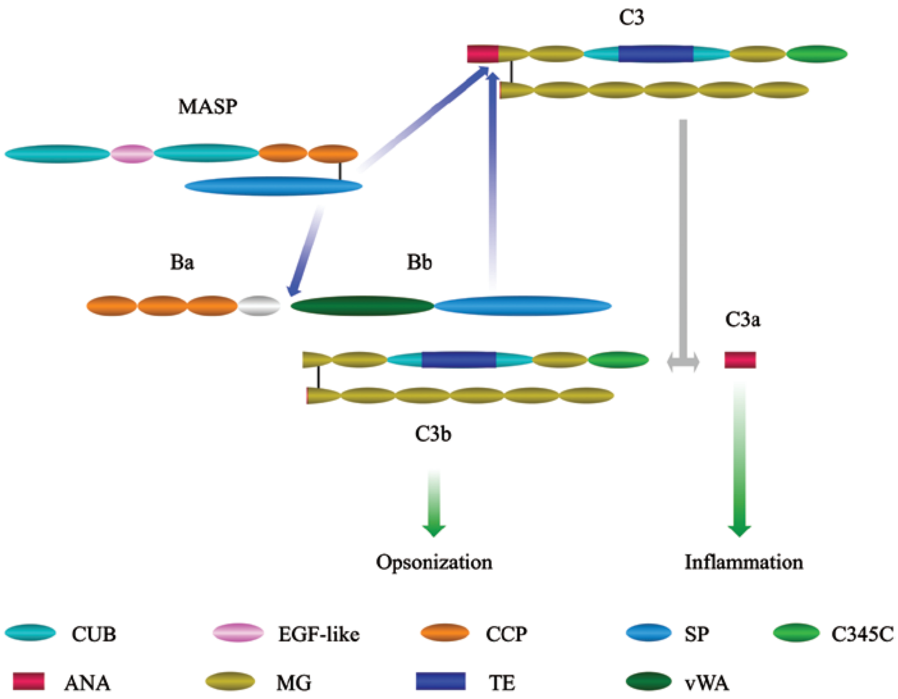

Figure 1. Schematic view of the core part of the urochordate complement system comprising C3, Bf and MASP. Conservation of the domain structure and functionally important residues of these components between mammals and urochordates suggests that the basic activation mechanism is also conserved. However, there is still no direct experimental evidence for proteolytic activation processes shown in blue arrows. The grey arrow indicates that C3 is cleaved by the C3 convertase (C3bBb) into two fragments, C3a and C3b and green arrows show biological functions of C3a and C3b. Abbreviations of domain names are: CUB, C1r, C1s, uEGF and bone morphogenetic protein; EGF-like, epidermal growth factor-like; CCP, complement control protein; SP, serine protease; C345C, C-terminal of C3, C4 and C5; ANA, anaphylatoxin; MG, macroglobulin; TE, thioester; vWA, von Willebrand factor Type A.

Although there are several C6-like genes with the membrane attack complex/perforin (MACP) domain in the *C. intestinalis* genome,[3] all of them lack the C-terminal short consensus repeat (SCR) and factor I/membrane attack complex (FIM) domains reported to be essential for interaction with other complement components. Thus, it is unlikely that these C6-like molecules are integrated in the urochordate complement system. All these results indicate that the urochordate complement system represents the primitive evolutionary stage just before the development occurred in the common ancestor of vertebrates. It lacks some components and functions of the mammalian complement system and shows no sign of acquisition of unique function. Figure 1 shows a schematic representation of the putative activation mechanism of the core part of the urochordate complement system comprising C3, Bf and MASP. Conservation of most structural motifs involved in proteolytic activation and C3 convertase formation of these complement components between urochordates and mammals strongly suggests that the activation mechanism of the urochordate complement system is the same as that of the mammalian complement system, although direct experimental evidence is still missing.

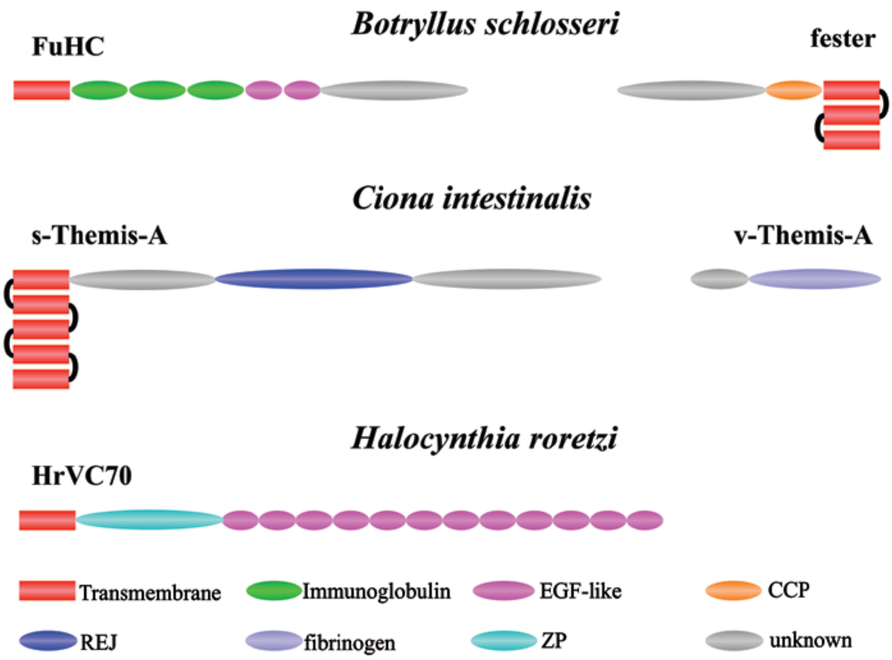

Figure 2. Domain structure of putative allorecognition molecules of three ascidian species. As shown here there is no orthologous relationship among these putative allorecognition molecules. Mutual interaction between FuHC and fester or between S-Themis and v-Themis is postulated, although it is still to be demonstrated directly. The *C. intestinalis* genome contains another set of the s-Themis and v-Themis genes containing similar domain structure as shown in this figure. Abbreviations of domain names not described in the legend to Figure 1 are: REJ, receptor for egg jelly; ZP, zona pellucida.

ALLORECOGNITION

Allorecognition is well known in vertebrates in the context of tissue transplantation where self or isogenic grafts are accepted whereas allogenic grafts are rejected. Although multiple genetic loci are involved in vertebrate allorecognition, by far the most important locus is the MHC. The evolutionary analyses indicated that the MHC was established in the common ancestor of jawed vertebrates[1] and urochordates completely lack the MHC. However, urochordates have two famous allorecognition systems working at colony fusion of the colonial ascidian[29] and fertilization of the solitary ascidian.[30] In both systems, the candidate genes for the key recognition molecules have been identified recently.

It has been known for 50 years that when two individuals of *Botryllus schlosseri*, a colonial ascidian, come into contact, they show histocompatibility reaction based on their genetic background.[29] If they share one or both alleles at a single histocompatibility locus, they will fuse. If they share no alleles, the colonies will reject each other. The candidate histocompatibility gene was isolated recently by positional cloning as described below and was termed *FuHC*.[31] About 1 Mb region identified by segregation analysis was sequenced to identify a candidate gene which showed polymorphism correlating with defined histocompatibility alleles in a fusion assay. The predicted open reading frame of this gene encoded a Type I transmembrane protein of 1007 amino acids in length. The amino terminus begins with a signal sequence, followed by an extracellular epidermal growth factor (EGF) repeats, two tandem immunoglobulin domains and the transmembrane domain and intracellular tail (Fig. 2). The high degree of polymorphism was demonstrated by identifying 18 alleles from 10 wild individuals. Most of allelic differences are single amino acid substitutions spread throughout the extracellular domains, with no obvious highly variable regions. Expression pattern analyzed by RT-PCR and in situ hybridization indicated that strong expression is observed in epithelia of ampullae and in a subset of blood cells, intimately associated with histocompatibility.

Another candidate for possible histocompatibility components is *fester* encoded near the *FuHC* locus.[32] The *fester* locus is highly polymorphic although this polymorphism does not contribute to histocompatibility, since it is not correlated with defined histocompatibility alleles. The *fester* is a Type I membrane protein having a signal peptide and several extracellular domains containing a single SCR domain. Alternative splicing generates several forms both membrane bound and secreted, all expressed in tissues intimately associated with histocompatibility. SiRNA-mediated knockdown of fester resulted in no histocompatibility reaction in both compatible and incompatible pairs, suggesting that *fester* is a receptor involved in histocompatibility. These data suggest that *FuHC* and *fester* are involved in allorecognition of *B. schlosseri*, although the underlying molecular mechanism including the possibility that *FuHC* and *fester* bind to each other is still to be clarified.

Ascidians are hermaphroditic and exhibit self-incompatibility (SI) at fertilization, self-sterility. Two species, *Ciona intestinalis* and *Halocynthia roretzi*, have been studied in detail for their SI system.[30] The SI system of *C. intestinalis* is genetically determined by multiple loci and takes place in the interaction between sperm and vitelline coat (VC), since removal of VC by acid treatment results in the loss of self-sterility. Recently, positional cloning of the SI loci was carried out using acid-induced self-fertilized siblings, the draft genome sequences and the detailed physical map.[33] Two loci A and B were identified in chromosome 2q and 7q, respectively. At both loci, a pair of genes termed s-Themis and v-Themis are present with a curious configuration that the v-Themis gene is located in the first intron of the s-Themis gene in opposite transcriptional direction. s-Themis is a polycystin-1 receptors and is expressed in testis. On the other hand, v-Themis is fibrinogen-like molecule and is a component of VC. Both s-Themis and V-Themis are highly polymorphic and autologous interaction between them is believed to reduce the binding ability of sperms. *H. roretzi* has much more strict SI system than *C. intestinalis*. In addition, *H. roretzi* has another allorecognition system termed “contact reaction”, in which allogenic hemocytes show cytotoxic reactions. Although the common underlying mechanism is suggested for the SI system and contact reaction, their molecular basis is still to be clarified. Recently, a candidate for the VC molecule responsible for the SI system was reported.[34] A 70 kDa VC protein termed HrVC70 consists of 12 EGF-like repeats and show a high degree of polymorphism. HrVC70-agarose beads binds more nonself-sperms than self-sperms and pretreatment of sperm with nonself-HrVC70 more strongly inhibited fertilization than the pretreatment of sperm with self-HrVC70. These results suggest that HrVC70 is involved in the SI system of *H. roretzi*. Therefore, at least two different SI systems seem to be present in ascidians, although detailed molecular mechanism and evolution are still to be clarified.

CONCLUSION

Two major arms of innate immunity, TLRs and complement, show clear orthologous relationship between urochordates and vertebrates. Although functional information is still missing, Cnidaria also has the orthologous genes of vertebrate TLRs and complement, suggesting that their evolutionary origin can be traced back to the common ancestor of eumetazoa. In contrast, allorecognition systems of ascidians seem to be innovated in the urochordate lineage and use totally different genes from vertebrate MHC. Although vertebrate MHC has evolved as the antigen presentation system of adaptive immunity and its involvement in allorecognition is an accidental side effect, allorecognition seems to be the original purpose of the urochordate allorecognition systems.

REFERENCES

1. Flajnik MF, Kasahara M. Origin and evolution of the adaptive immune system: genetic events and selective pressures. Nat Rev Genet 2010; 11:47-59.
2. Delsuc F, Brinkmann H, Chourrout D et al. Tunicates and not cephalochordates are the closest living relatives of vertebrates. Nature 2006; 439:965-968.
3. Azumi K, De Santis R, De Tomaso A et al. Genomic analysis of immunity in a Urochordate and the emergence of the vertebrate immune system: "waiting for Godot". Immunogenetics 2003; 55:570-581.
4. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004; 4:4499-4511.
5. Dunne A, O'Neill LA. Adaptor usage and Toll-like receptor signaling specificity. FEBS Lett 2005; 579:3330-3335.
6. Shimazu R, Akashi S, Ogata H et al. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J Exp Med 1999; 189:1777-1782.
7. Matsuo A, Oshiumi H, Tsujita T et al. Teleost TLR22 recognizes RNA duplex to induce IFN and protect cells from birnaviruses. J Immunol 2008; 181:3474-3485.
8. Takano T, Kondo H, Hirono I et al. Molecular cloning and characterization of Toll-like receptor 9 in Japanese flounder, Paralichthys olivaceus. Mol Immunol 2007; 44:1845-1853.
9. Yu Y, Zhong Q, Li C et al. Isolation and characterization of Toll-like receptor 9 in half-smooth tongue sole Cynoglossus semilaevis. Fish Shellfish Immunol 2009; 26:492-499.
10. Ishii A, Matsuo A, Sawa H et al. Lamprey TLRs with properties distinct from those of the variable lymphocyte receptors. J Immunol 2007; 178:397-406.
11. Huang S, Yuan S, Guo L et al. Genomic analysis of the immune gene repertoire of amphioxus reveals extraordinary innate complexity and diversity. Genome Res 2008; 18:1112-1126.
12. Hibino T, Loza-Coll M, Messier C et al. The immune gene repertoire encoded in the purple sea urchin genome. Dev Biol 2006; 300:349-365.
13. Davidson CR, Best NM, Francis JW et al. Toll-like receptor genes (TLRs) from Capitella capitata and Helobdella robusta (Annelida). Dev Comp Immunol 2008; 32:608-612.
14. Bosch TC, Augustin R, Anton-Erxleben F et al. Uncovering the evolutionary history of innate immunity: the simple metazoan Hydra uses epithelial cells for host defence. Dev Comp Immunol 2009; 33:559-569.
15. Sasaki N, Ogasawara M, Sekiguchi T et al. Toll-like receptors of the ascidian Ciona intestinalis: prototypes with hybrid functionalities of vertebrate Toll-like receptors. J Biol Chem 2009; 284:27336-27343.
16. Volanakis JE. Overview of the complement system. In: Volanakis JE, Frank MM, eds. The Human Complement System in Health and Disease. New York: Marcel Dekker, Inc., 1998:9-32.
17. Kimura A, Sakaguchi E, Nonaka M. Multi-component complement system of Cnidaria: C3, Bf and MASP genes expressed in the endodermal tissues of a sea anemone, Nematostella vectensis. Immunobiology 2009; 214:165-178.
18. Nonaka M, Kimura A. Genomic view of the evolution of the complement system. Immunogenetics 2006; 58:701-713.
19. Nonaka M, Azumi K, Ji X et al. Opsonic complement component C3 in the solitary ascidian, Halocynthia roretzi. J Immunol 1999; 162:387-391.
20. Marino R, Kimura Y, De Santis R et al. Complement in urochordates: cloning and characterization of two C3-like genes in the ascidian Ciona intestinalis. Immunogenetics 2002; 53:1055-1064.

21. Yoshizaki FY, Ikawa S, Satake M et al. Structure and the evolutionary implication of the triplicated complement factor B genes of a urochordate ascidian, Ciona intestinalis. Immunogenetics 2005; 56:930-942.
22. Ji X, Azumi K, Sasaki M et al. Ancient origin of the complement lectin pathway revealed by molecular cloning of mannan binding protein-associated serine protease from a urochordate, the Japanese ascidian, Halocynthia roretzi. Proc Natl Acad Sci USA 1997; 94:6340-6345.
23. Bonura A, Vizzini A, Salerno G et al. Isolation and expression of a novel MBL-like collectin cDNA enhanced by LPS injection in the body wall of the ascidian Ciona intestinalis. Mol Immunol 2009; 46:2389-2394.
24. Kenjo A, Takahashi M, Matsushita M et al. Cloning and characterization of novel ficolins from the solitary ascidian, Halocynthia roretzi. J Biol Chem 2001; 276:19959-19965.
25. Miyazawa S, Azumi K, Nonaka M. Cloning and characterization of integrin alpha subunits from the solitary ascidian, Halocynthia roretzi. J Immunol 2001; 166:1710-1715.
26. Miyazawa S, Nonaka M. Characterization of novel ascidian beta integrins as primitive complement receptor subunits. Immunogenetics 2004; 55:836-844.
27. Sekine H, Kenjo A, Azumi K et al. An ancient lectin-dependent complement system in an ascidian: novel lectin isolated from the plasma of the solitary ascidian, Halocynthia roretzi. J Immunol 2001; 167:4504-4510.
28. Pinto MR, Chinnici CM, Kimura Y et al. CiC3-1a-mediated chemotaxis in the deuterostome invertebrate Ciona intestinalis (Urochordata). J Immunol 2003; 171:5521-5528.
29. McKitrick TR, De Tomaso AW. Molecular mechanisms of allorecognition in a basal chordate. Semin Immunol 2009; 22:34-38.
30. Harada Y, Sawada H. Allorecognition mechanisms during ascidian fertilization. Int J Dev Biol 2008; 52:637-645.
31. De Tomaso AW, Nyholm SV, Palmeri KJ et al. Isolation and characterization of a protochordate histocompatibility locus. Nature 2005; 438:454-459.
32. Nyholm SV, Passegue E, Ludington WB et al fester, A candidate allorecognition receptor from a primitive chordate. Immunity 2006; 25:163-173.
33. Harada Y, Takagaki Y, Sunagawa M et al. Mechanism of self-sterility in a hermaphroditic chordate. Science 2008; 320:548-550.
34. Sawada H, Tanaka E, Ban S et al. Self/nonself recognition in ascidian fertilization: vitelline coat protein HrVC70 is a candidate allorecognition molecule. Proc Natl Acad Sci USA 2004; 101:15615-15620.