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Review

Protochordate amphioxus is an emerging model organism for comparative immunology

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Abstract

Protochordate amphioxus is an extant invertebrate regarded quite recently as a basal chordate. It has a vertebrate-like body plan including a circulation system with an organization similar to that of vertebrates. However, amphioxus is less complex than vertebrates for having a genome uncomplicated by extensive genomic duplication, and lacking lymphoid organs and free circulating blood cells. Recent studies on immunity have demonstrated the presence in amphioxus of both the constituent elements of key molecules involved in adaptive immunity such as proto-major histocompatibility complex (proto-MHC), V region-containing chitin-binding protein (VCBP) and V and C domain-bearing protein (VCP), and the complement system operating via the alternative and lectin pathways resembling those seen in vertebrates. In addition, the acute phase response profile in amphioxus has been shown to be similar to that observed in vertebrates. These findings together with the relative structural and genomic simplicity make amphioxus an ideal organism for gaining insights into the origin and evolution of the vertebrate immune system, especially adaptive immunity, and the composition and mechanisms of the vertebrate innate immunity.

Keywords: Amphioxus; *Branchiostoma*; Immunity; Evolution

1. Introduction

All metazoan organisms can defend themselves against foreign substances or antigens, including microbes and macromolecules such as proteins and polysaccharides, and protect themselves from diseases. These abilities are collectively called immunity. There exist two types of immunity, innate immunity and adaptive immunity, both of which can be further divided into humoral and cellular defense responses. The innate immunity, also called natural or native immunity, refers to the basic resistance to infection that an organism is born with, while adaptive immunity, also known as specific or acquired immunity, is the responses that are stimulated by exposure to infectious agents, and it increases in magnitude and defensive capabilities with each successive exposure to a particular foreign substance. The innate immune system relies on germline-encoded factors for recognition and killing of invading agents, but the adaptive immune system produces receptors by somatic gene rearrangements that recognize specific antigens and allow hosts to develop an immunological memory [1]. Innate immunity is the first line of the host's defense against antigen invasion and interprets the biological context of antigens, and instructs the adaptive immune system to make appropriate antibody or T-cell responses [2]. It is believed that innate immunity is an ancient form of protective mechanism that appeared early in the evolution of multicellular organisms, whereas adaptive immunity is younger in origin and appeared some 500 million years ago in the ancestors of cartilaginous fish like sharks [3].

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Of the approximately 30 bilaterian animal phyla that are recognized, only chordates, echinoderms, mollusks, nematodes and anthropods have been the subjects of extensive molecular immune research. The overwhelming majority of functional and genetic data regarding immune systems comes from basically two phyla: Chordata (mainly from mammals, especially *Homo sapiens*) and Arthropoda (fruit fly *Drosophila melanogaster*). Comparative approaches have revealed the presence of common features of innate immunity between the fruit fly and mammals [4,5]. Comprehensive molecular analyses of immunity also have been conducted in four other deuterostome species, e.g. sea urchin (*Strongylocentrotus purpuratus*) [6], amphioxus (*Branchiostoma belcheri* and *B. flaoridae*) [7,8], sea squirt (*Ciona intestinalis*) [9] and lamprey (*Petromyzon marinus*) [10]. They all turned out to be useful models for elucidating the origin and evolution of vertebrate adaptive immunity although a novel adaptive immune system to generate highly variable lymphocyte receptors (VLRs) through rearrangement of leucine-rich repeats (LRR) has been found in the jawless fish lamprey.

Protochordate amphioxus or lancelet, a species of the phylum Chordata discovered by Pallas in 1774, is generally considered as a sister group of the vertebrates (Fig. 1). It has been recently repositioned at the base of the chordate phylum [11,12]. Its appearance, appears to be related anatomically and developmentally to that of the ancestor of all vertebrates, possessing a notochord, a hollow dorsal neural tube, a post-anal tail, segmented muscle blocks, gill slits, and posterior direction of blood flow in the dorsal vessels and anterior direction of blood flow in the ventral vessels (although no lymphoid organs and free circulating blood cells have been found). Amphioxus also retains a genome (17% that of the human genome) uncomplicated by extensive genomic duplication [13], bearing strong patterns of conserved "synteny" with vertebrate genomes, including the human genome [12]. All these make it an excellent model organism for gaining understanding of the origin and evolution of vertebrates [14–18]. Besides, the results achieved regarding immunity study during the past years have made amphioxus an emerging model organism for research on comparative immunology.

This review focuses on the recent progress of immunity study in amphioxus. We present the examples to demonstrate the presence in amphioxus of the constituent elements of key molecules involved in adaptive immunity,

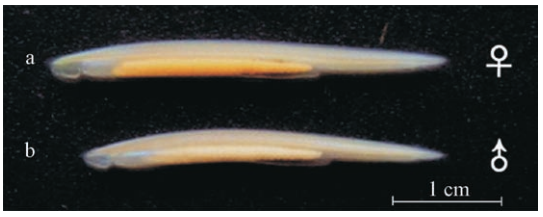

Fig. 1. Photomicrographs of a female (a) and a male (b) living amphioxus adults (*Branchiostoma belcheri*). Both are fully matured. Both individuals are about 4 cm long.

and of the complement system operating via the alternative and lectin pathways resembling those seen in vertebrates. We also discuss the acute phase response in amphioxus, and provide some evidence that amphioxus hepatic caecum is similar to the vertebrate liver, an organ involved in the acute phase response.

2. Amphioxus constituent elements of key molecules involved in adaptive immunity: insights into the origin of vertebrate adaptive immunity

The hallmark of adaptive immunity is the occurrence of cells and their products (molecules) participating in immune recognition of foreign antigens and memory of this recognition. The cells for adaptive immunity primarily are B lymphocytes, T lymphocytes and antigen-presenting cells (APCs). B lymphocytes take part in the humoral immune response by secreting immunoglobulin (Ig) recognizing foreign antigens. APCs interact with T lymphocytes to perform cellular immune response by presenting foreign antigens in the context of major histocompatibility complex (MHC) to T-cell receptors (TCRs) on the surface of T lymphocytes. The variable portions of Ig and TCR genes are composed of separate V (variable), D (diversity), and J (joining) segments, which are represented by fewer than a few hundred copies each. In a B- and T-cell site-specific recombination reaction, commonly known as V(D)J recombination, one V, one D, and one J segment are joined together into a single exon encoding the variable antigen-binding region of the receptor. In V(D)J recombination, DNA cleavage is catalyzed by two proteins encoded by the recombination-activating genes, RAG1 and RAG2 [19,20].

Previous attempts to identify key components of adaptive immunity including TCRs, Ig, MHC, and RAG have been unsuccessful in invertebrates and even in the jawless fish; it is therefore believed that adaptive immunity emerged abruptly in a manner often referred to as the immunological "Big Bang" in jawed vertebrates. The studies for the origin of adaptive immunity focus on mainly three aspects, i.e. the origin of the antigen receptor, the antigen presenting and processing system, and effector cells [21,22]. In seeking evidence for the existence of adaptive immunity in ancient chordates, Yu et al. [7] have sequenced cDNA clones of six libraries from the amphioxus *B. belcheri*, and presented extensive molecular evidence for the presence of genes homologous to many of those that are involved in adaptive immunity directly or indirectly, including some which may represent the constituent elements of vertebrate adaptive immunity-related genes such as genes of the Ig superfamily like the V and C domain-bearing protein (VCP) gene, genes involved in rearrangement like the RAG1 gene, genes controlling lymphocyte development and signaling like the lymphoid transcription factor LyF gene, the tumor necrosis factor receptor (TNFR) gene, the CD63 gene, and the CD53 gene, and genes involved in antigen presenting like the interferon-γ-

inducible lysosomal thiol reductase gene. On this basis, they have suggested that adaptive immunity is in its twilight in amphioxus and the evolution of the constituent elements of adaptive immunity-related genes in amphioxus is waiting for recruitment by the emergence of adaptive immunity [7]. This also appears to gain additional support from a series of recent studies. A multigene family containing Ig V region-containing chitin-binding proteins (VCBPs), and an Ig superfamily gene homologous to CD47 have been identified in amphioxus [23,24]. The amphioxus VCBP molecules are encoded by five or more multigene families that are polymorphic within the population. VCBP gene products are secreted into the intestine, where they may play a role in preventing microbial invasion [25]. These exhibit that amphioxus and jawed vertebrates are more homologous than jawless vertebrates in selecting Ig domains as the recombinatorial units of antigen receptors (Fig. 2). In addition, a single MHC region referred to as proto-MHC, which lacks key genes for adaptive immunity but includes the anchor genes as in vertebrates, is identified in amphioxus [26,27]. Comparative analysis provides clear evidence that the four jawed vertebrate MHC genes emerged from the proto-MHC region as a result of two rounds of *en bloc* duplication taking place after the split of cephalochordates and vertebrates. Moreover, an unexpected feature of the evolution of the duplicated regions is uncovered: one region appears to have largely retained the organization and gene substitution patterns comparable to that in amphioxus (Fig. 3), and the other three have faster substitution rates and massive gene losses [27].

Until recently, the consensus view has been that the precursors of the genes involved in adaptive immunity including RAG and TCRs entered the ancestral gnathostome genome through one or two lateral genome transfer events and were derived from a transposase. Interestingly, a gene encoding the protein related to deuterostome RAG1 has recently been found to be present in the cnidarian genomes [28], pushing the evolutionary acquisition of this gene back to at least Ureumetazoa (the common ancestor of all true animals). On the other hand, recent reports have demonstrated that the jawless fish have VLR with somatically rearranged LRR ectodomains, suggesting that the BCR/TCR system is not the only form of adaptive immunity [10,29]. All these present a challenge to the "Big Bang" theory, making the searching of the key components of adaptive immunity in the ancient chordates seemingly astray.

3. Amphioxus complement: an image of the ancestral status of the vertebrate complement system before the emergence of adaptive immunity

Complement, a central component of innate immunity, plays multiple roles in immunity such as immune cell activation, chemotaxis, opsonization, and lysis of antigens, protecting the host from infection. There are three pathways by which the complement system can be activated: the classical pathway, which is initiated by an antigen-antibody complex; the alternative pathway, which is an antibody-independent route triggered by C3 as well as certain structures on the surface of microbes; and the lectin pathway, which is activated by the binding of mannose-binding lectin to carbohydrates on microbial surfaces. These three pathways merge at a common amplification step involving C3 and proceed through a terminal pathway that leads to the formation of a membrane attack complex, which can directly lyse bacterial cells [1]. Notably, the origin of C3 can be dated even before the split of cnidaria and bilateria, whereas the absence of the corresponding gene in the fly, worm and mosquito may reflect secondary losses [28,30].

Recently, the humoral fluids of amphioxus *B. belcheri* were examined for the presence of complement-like activity by the standard analysis method of Yano [31]. It was found that the humoral fluids exhibit hemolytic activity for erythrocytes of rabbit and of those species representing mammals, birds, amphibians and fish, but not for sensitized sheep erythrocytes [32]. The hemolytic activity is Mg^{2+}-dependent and heat-sensitive, and can be abrogated by treatment with rabbit anti-human C3 serum, zymosan, methylamine, hydrazine, and phenylmethylenesulfonyl fluoride. Moreover, both Western blotting and titration by the turbidimetric immunoassay as well as molecular cloning have demonstrated the existence of the C3 component [33,34]. These suggest that the hemolytic activity displayed by the humoral fluids is similar to the vertebrate complement system operating via the alternative pathway [35]. In agreement, we have recently cloned the gene of factor B (Bf), a key molecule in the alternative complement pathway from *B. belcheri* [36]. The lectin pathway also appears to be present in amphioxus as the key enzymes involved in complement activation, and mannose-binding lectin-associated serine proteases have been identified in *B. belcheri* [34]. The recent genomic analysis of the immune gene repertoire of amphioxus *B. floridae* revealed a great expansion of the complement system in this primitive animal, as evidenced by the presence of 50 C1q-domain-containing genes (compared to 29 in humans) and many CCP-, CUB- and FBG-containing genes [37,38]. Because no evidence has shown so far the existence of adaptive immunity in amphioxus, and amphioxus appears as a basal chordate species, the complement system in amphioxus may represent a relatively pure form, bearing a strong resemblance to the ancestral status of the complement system existing in vertebrates.

4. Amphioxus acute phase response: insights into the origin of the acute phase response profile in vertebrates

Acute phase response is a pervasive physiological response occurring soon after the onset of infection, trauma, and inflammatory processes, permitting survival of the host during the period immediately following the insult. It involves a large number of acute phase proteins (APPs) functioning in a variety of defense-related activities

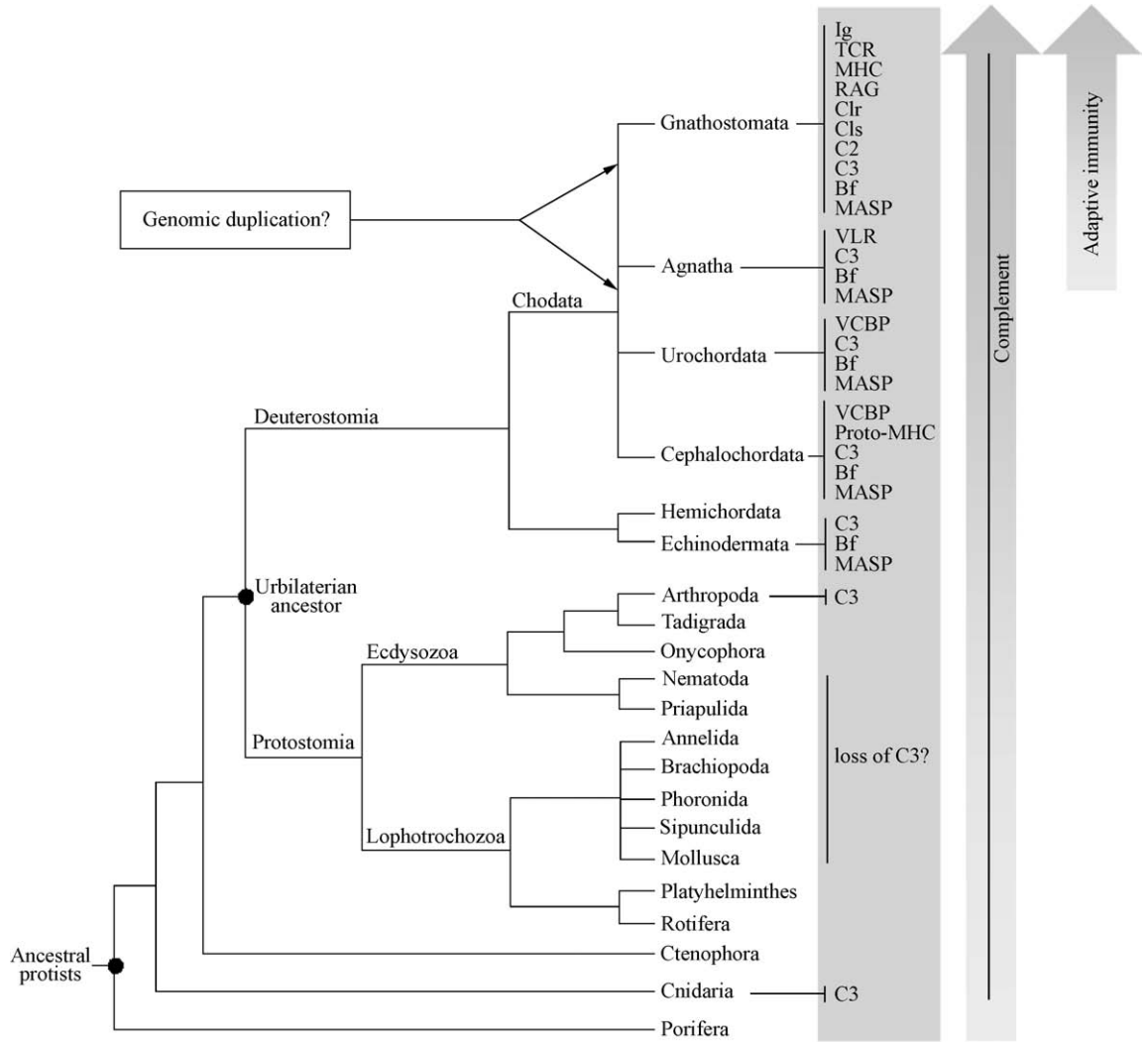

Fig. 2. Phylogeny of the metazoan organisms and the major events in the evolution of innate and adaptive immunities. A simplified phylogeny of innate and adaptive immunities is shown. All jawed vertebrates examined so far possess the classical, alternative, and lectin complement pathways, whereas only components of the alternative and lectin pathways have been identified in echinoderm, urochordate, cephalochordate and gnathostome. Recently, a prototypic complement-effector pathway involving C3 is also found to be present in arthropod and cnidaria. Two rounds of genomic duplication are thought to have taken place in a lineage leading to vertebrates, after its separation from the ancestor leading to cephalochordate.

such as limiting the dispersal of infectious agents, repairing of tissue damage, killing of microbes, and restoration of a healthy state [39]. One of the most studied aspects of the acute phase response has been the change in concentrations of APPs in serum. There are two groups of APPs, positive APPs and negative APPs [40], and their concentrations increase and decrease accordingly during an acute phase response, while the total protein contents remain constant in serum [39,41,42].

Liang et al. [16] and Lun et al. [43] have demonstrated the presence of antithrombin (AT) and alanine aminotransferase (AAT) in amphioxus. Interestingly, both AT and AAT are up-regulated after the acute challenge with lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria capable of inducing acute phase reaction, while the total protein levels are unchanged in the humoral fluids, resembling that seen in some mammalian species. More recently, we have shown that transthyretin (TTR), a typical negative APP, is also present in amphioxus *B. belcheri*, and it is markedly down-regulated by the acute challenge with LPS, again resembling that observed in some eutherian mammals (Fig. 4). These suggest the existence of the mammalian-like acute phase response profile in amphioxus, pushing its evolutionary origin back to the cephalochordate.

Liver is the principal organ synthesizing APPs [39]. Amphioxus has just a hepatic caecum, which has long been considered to be the precursor of vertebrate liver [44]. It has been noted that both AT and AAT as well as TTR are localized primarily in the hepatic caecum in amphioxus *B. belcheri* (Fig. 4) [16,43]. Furthermore, the acute phase action-relevant molecules including Bf and fibrinogen-related protein genes that are primarily expressed in the vertebrate liver are also predominantly expressed in the

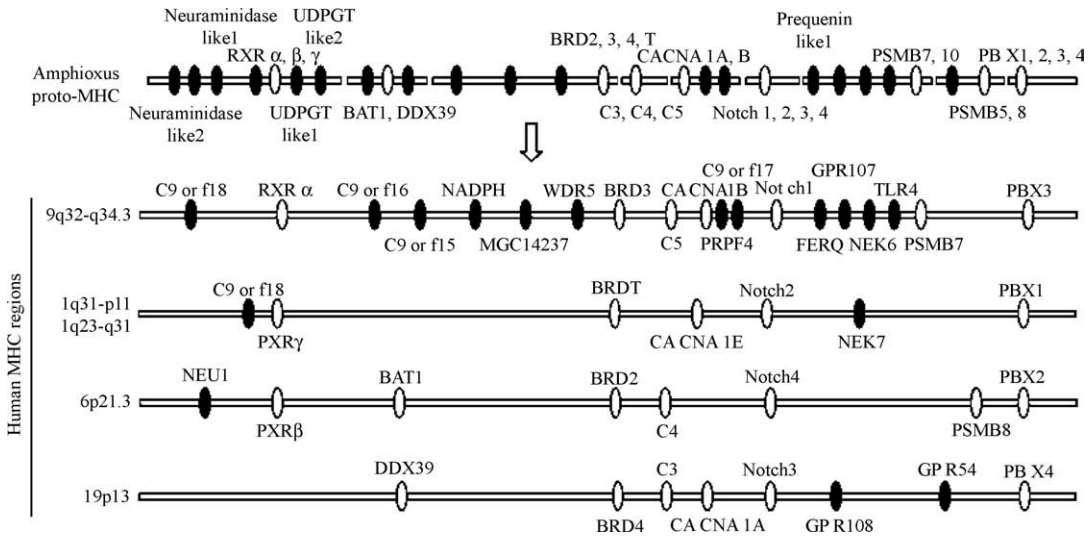

Fig. 3. Scheme for evolution of the major histocompatibility (MHC) genomic region. Amphioxus genomic MHC region and the four human MHC paralogous regions are shown. The anchor genes are indicated in white ellipse, while genes surrounding the anchor are indicated in black ellipse.

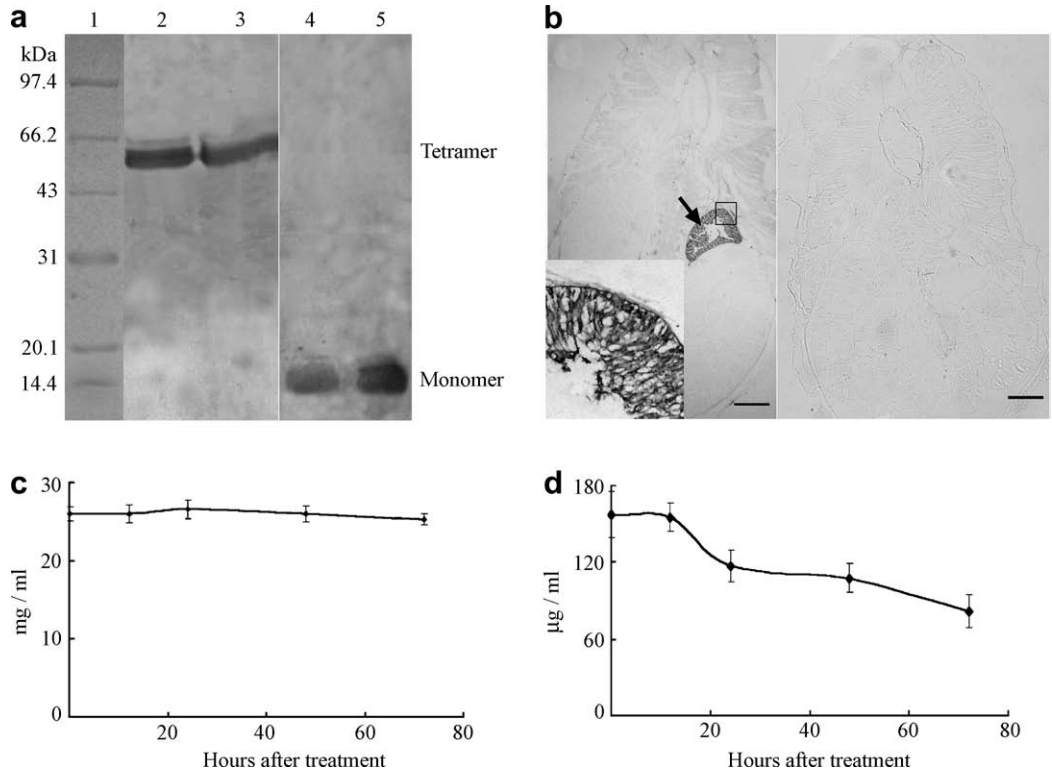

Fig. 4. Demonstration of transthyretin in amphioxus. (a) Western blotting of transthyretin. Both amphioxus humoral fluid and human serum were run on a 12% SDS–PAGE and subjected to Western blotting analysis. Amphioxus humoral fluids were found to be reactive with goat anti-human transthyretin antibody, forming a single positive band with an apparent molecular mass of ~56 kDa in the absence of urea or ~14 kDa in the presence of urea, that correspond to the tetramer and monomer of human transthyretin, respectively. Lane 1, molecular marker; lanes 2 and 4, human serum; lanes 3 and 5, amphioxus humoral fluid. (b) Immunohistochemical localization of transthyretin. Amphioxus *B. belcheri* was each severed into three to four pieces, and fixed in freshly prepared 4% paraformaldehyde in 100 mM PBS (pH 7.4) at 4 °C for 24 h, and sectioned at 5 μm. Immunohistochemical staining was carried out by the method of Liang et al. [13]. Transthyretin was observed in the digestive diverticulum (arrow) in amphioxus (left), while no positive signal was detected in the negative control (right). The insert in the figure is magnification of the marked box. Scale bars represent 100 μm. (c and d) The changes in the concentrations of total proteins (c) and transthyretin (d) in the humoral fluids of amphioxus *B. belcheri* challenged with LPS. The contents of total proteins and transthyretin were determined by the biuret method and the immunoturbidimetric assay method, respectively. Data were obtained from three experiments and expressed as the mean values ± SD. The concentration of transthyretin in the humoral fluids decreased after the acute challenge with LPS, while the level of total proteins remains constant.

hepatic caecum in *B. belcheri* [36,38]. All these not only support the hypothesis that the vertebrate liver evolved from the hepatic caecum of an amphioxus-like ancestor during early chordate evolution but also suggest that like the liver, the hepatic caecum in amphioxus is the primary tissue involved in acute phase response. Detailed studies on the expression and function of APPs will further shed light on this issue.

In summary, the recent findings regarding the constituent elements of key molecules involved in adaptive immunity, the complement and acute phase response profile in amphioxus, together with the relative structural and genomic simplicity, make amphioxus an ideal model organism for comparative immunology. With the completion of the genomic sequencing of the amphioxus *B. floridae* genome, which allows for the rapid and better identification of immune-relevant gene homologs, we are sure that the study of immunity in amphioxus and the comparative analysis of immunity across different phyla of animals are culminating.

Acknowledgements

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