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Characterization of the immune defense related tissues, cells, and genes in amphioxus

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Amphioxus is an important animal model for phylogenetic analysis, including comparative immunology. Exploring the immune system in amphioxus contributes to our understanding of the origin and evolution of the vertebrate immune system. We investigated the amphioxus immune system using ultrastructural examination and *in situ* hybridization. The expression patterns of *TLR1* (*toll-like receptor 1*), *C1Q* (*complement component 1*, *q subcomponent*), *ECSIT* (*evolutionarily conserved signaling intermediate in Toll pathways*), *SoxC*, *DDAHa* (*Dimethylarginine dimethylaminohydrolase* a), and *NOS* (*nitric oxide synthase*) show that these genes play key roles in amphioxus immunity. Our results suggest that the epidermis and alimentary canal epithelium may play important roles in immune defense, while macrophages located in the coelom and so-called lymph spaces may also be crucial immune cells.

amphioxus, epidermis, alimentary canal, macrophages, immunity, evolution

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Amphioxus, a cephalochordate representing the invertebrate-to-vertebrate transition, is an important experimental animal model for phylogenetic analysis, including comparative immunology. However, the amphioxus immune system remains largely unexplored.

Amphioxus possesses the basic vertebrate structures, including a notochord, dorsal neural cord, intramuscular somites, and pharynx region with gill slits. Similar to other chordates, the exterior covering of the amphioxus body is skin, which can provide protection against the invasion of microorganisms and play an important role in innate immune responses. Amphioxus skin consists of a single layer of columnar epithelium or pseudostratified epithelium covered by a thin film of keratose cuticles. Distinct ultrastructural characteristics of endocytosis in amphioxus epidermal cells at the 24-h larva stage have been observed [1]. A key amphioxus immune organ is the alimentary canal just after the mouth, which can be divided into the pharynx region and the intestine. The intestinal epithelium in amphioxus plays an essential role in amphioxus immune defense against LPS challenge [2].

The amphioxus body possesses many cavities, including the coelom and so-called lymph spaces. The coelom consists of the following sections, the suprapharyngeal, subchordal, branchial, perienteric, perigenital, endostyle, and

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metapleure. Lymph spaces are considered to be discontinuous with the coelom [3]. Important immune defense related cells include macrophages in the coelom and lymph spaces, and a small number of granulocytes in blood vessels, which have been confirmed by transmission and scanning electron microscopy [4].

Amphioxus has always been considered to lack an adaptive immune system. However, using molecular methods, Cannon *et al.* [5] discovered the V region-containing chitin binding proteins (VCBPs), which represent a core feature of the adaptive immune receptors in amphioxus. Genome sequencing of *Branchiostoma floridae* revealed a series of homologous chordate genes and gene families involved in immune defense [6–10]. Although some studies have been published, more research is required to better understand the origin of immune recognition and immune defense in amphioxus. TLRs play an important function in triggering the innate immune response and ECSIT is an evolutionarily conserved intermediate in the Toll/IL-1 signal transduction pathway. C1Q is the recognition protein of the classical complement pathway and is an important link between the innate and acquired immune system. Sox4, a member of the SoxC family, is expressed in T and pre-B lymphocytes in the murine thymus [11]. DDAH and NOS regulate the synthesis of NO, which serves as a primary immune activator and a signaling molecule with multiple roles in vertebrates [12–14]. Therefore, we first analyzed the expression pattern of genes involved in vertebrate immunity, such as *TLR1*, *ECSIT*, *C1Q*, *SoxC*, *DDAHa*, and *NOS* in amphioxus. At the same time, we investigated the ultrastructural characteristics of the epidermis and epithelium of the gill and intestine. This expression analysis and the ultrastructural analysis may provide more clues and a better understanding of the evolution of the vertebrate adaptive immune system.

1 Materials and methods

1.1 Amphioxus collection

Adult amphioxus (*Branchiostoma belcheri*) were caught in the Yellow Sea near Qingdao, China and the South Sea near Beihai, Guangxi, and spawned in the laboratory [15]. Synchronously developing embryos and larvae were cultured and collected at different development stages.

Embryos and larvae were fixed for whole-mount *in situ* hybridization in 4% paraformaldehyde in 0.1 mol L^{-1} 3-(N-morpholino)-propanesulfuric acid buffer with 1 mmol L^{-1} ethyleneglycotetraacetic acid, and 0.5 mol L^{-1} NaCl (pH 7.5) at room temperature for 1 h and stored in 70% ethanol at −20°C.

1.2 Ultrastructural studies

Adult and larval specimens for transmission electron microscopy (TEM) were cut into 1–2 mm blocks and fixed in 4% glutaraldehyde in 0.2 mol L^{-1} sodium cacodylate, pH 7.4, for 2 h at room temperature. Larvae were cultured for 30 min in sea water, with a drop of Chinese ink, before fixation to detect endocytosis using carbon particles of cells under TEM.

Specimens were postfixed in 1% osmium tetroxide for 1 h, followed by dehydration in a graded ethanol series and embedded in Epon 812. Semi-thin-sections (1 μm) were prepared for primary observation, followed by preparation of a series of ultrathin sections (0.05 μm). After staining with uranyl acetate and lead citrate, ultrathin sections were observed and photos taken under a JEM-200CX electron microscopy.

1.3 Cloning of amphioxus *TLR*, *ECSIT*, *C1Q*, *SoxC*, *DDAHa,* and *NOS* genes

To generate *in situ* hybridization probes, PCR was performed with the following primers using sequences from NCBI or JGI: *TLR1* (DQ400125: F, 5′-CACAGCATCGCAACAATCCC-3'; R, 5′-CGCAGCCTCCTCCAGAACAG-3'); *ECSIT* (XM_002595374: F, 5′-GAGGCCATGTGGAGTTTATCTA-3′; R, 5′-GGGGAACCAGTACAGGATACG-3′); *C1Q* (XM_002586916: F, 5′-TACAGGTCCACAAGGCCCA-3′; R, 5′-AGAAGAAACCCGGTGAAG-3′); *DDAHa* (DQ860841: F, 5′-ACTGGTGGAGGTTTGATGTC-3′; R, 5′-CCGCAAGGAACTCGGAATGT-3′); *NOS* (AF396968: F, 5′-AGGAGGACGCCTGGTTGGTA-3′; R, 5′-CGGGTGCCTCATGTGAAGTAA-3′) and *SoxC* (FJ176301: F, 5′-CGTTTATGGTCTGGTCTCAGATC-3′; R, 5′-GCTTTCTTCACCTTTTTCC-3′). All PCR products were cloned, sequenced, and used as templates for making digoxigenin (DIG) labeled probes.

1.4 Section *in situ* hybridizations

DIG-labeled sense and antisense RNA probes were prepared according to manufacturer's instructions (Dig RNA Labeling Mix, Roche, USA; SP6 or T7 RNA Polymerases, Promega, USA). Adult transverse sections (8 μm) were mounted onto slides (ThermoFisher, USA). After deparaffinization, sections were rehydrated in gradient ethanol, immersed in 0.3% Triton X-100 solution for 15 min at room temperature and in proteinase K solution for 20 min at 37°C. Sections were then incubated with 4% paraformaldehyde/PBS for 5 min at 4°C. After washing with PBS and 0.1 mol L^{-1} triethanolamine buffer containing 0.25% acetic anhydride, the slides were incubated with prehybridization solution for 2 h at 37°C. Sense and antisense probes were added at a concentration of 1 μg mL^{-1} and hybridized overnight at 50°C. After high stringency washing, sections were incubated with an alkaline phosphatase-conjugated-anti-digoxigenin antibody, and NBT/BCIP substrate, and monitored for a color reaction.

1.5 Injection of *Staphylococcus aureus Rosenbach* induces different expression patterns of *TLR*, *ECSIT*, *C1Q*, *SoxC*, *DDAHa* and *NOS* in amphioxus

Amphioxus subjected to celiac injections with *Staphylococcus aureus* Rosenbach in phosphate buffered solution (PBS) (experimental group) and germ-free PBS (control group) were processed as follows. After culturing the injected amphioxus in sea water for 3 d, RNA was isolated from the alimentary canal, RT-PCR performed with the above primers (in materials and methods 1.3) and β-actin primers (F, 5′-GGAAGAGAGACTCGGGGC-3′; R, 5′-CTCACAGAGCGTGGCTACAG-3′), to determine expression patterns of *TLR*, *ECSIT*, *C1Q*, *SoxC*, *DDAHa*, and *NOS*.

2 Results

2.1 Ultrastructural studies on epidermis, gill epithelium and alimentary canal as well as macrophages in larval and adult amphioxus

Epidermal cells of 24 to 72-h amphioxus larvae were examined by TEM after culturing for 30 min in seawater containing ink. As shown in Figure 1, the monolayer epidermal cells possessed numerous vesicles containing different electron-dense materials in the cytoplasm. Several vesicles with tracer carbon particles were in apical cytoplasm and the endocytosing vesicles on the cytoplasmic membrane could be observed in epidermal cells (Figure 1A and B). The nucleus is located close to the basal surface of the cell (Figure 1A and B). Between adjacent epidermal cells, typical septate junctions were detected in the apical area, with no tight junction (Figure 1C). Tracing carbon particles were also found in the intercellular spaces (Figure 1C), suggesting that larval epidermal cells are capable of endocytosis.

The ultrastructural observations were also performed on the differentiating branchial cells and epithelial cells of the alimentary canal, from 48 to 72-h amphioxus larva. Prominent characteristics of larval branchial epithelial cells were numerous cilia orientated to pharyngeal lumen on the apical surface and developed Golgi apparatus in the cytoplasm (Figure 1D). The epithelial cells of a nascent alimentary canal appeared as short columns. Their primary distinguishing feature was the presence of numerous vesicles with low electron-dense substances. The cytoplasm usually contained numerous rounded or elliptic mitochondria, endoplasmic reticulum, and abundant free ribosomes (Figure 1E). Strikingly, only zonula adherens rather than tight or septate junctions were found between adjacent epithelial cells of the alimentary canal. A migrating cell was occasionally observed in the epithelium of the alimentary canal of amphioxus larva with variable shape and more electron-dense nucleus in cytoplasm, and the shape of the cell and nucleus might suggest the migrating direction (Figure 1F). The migrating cell might be the precursor of mononuclear free cells, granulocytes, or macrophages. The macrophages in the perigenital coelom of larvae and adults exhibited irregular shapes and many cellular protrusions. Numerous vesicles, multivesicular bodies, lysosomal granules and phagosomes with different electron-dense substance were observed in the cytoplasm of macrophages (Figure 1G).

2.2 Expression patterns of immune defense related genes in epidermis, gill epithelium, intestinal epithelium, and macrophages

Several homologs of vertebrate immune system genes were detected in the epidermis, gill and intestine epithelium, as

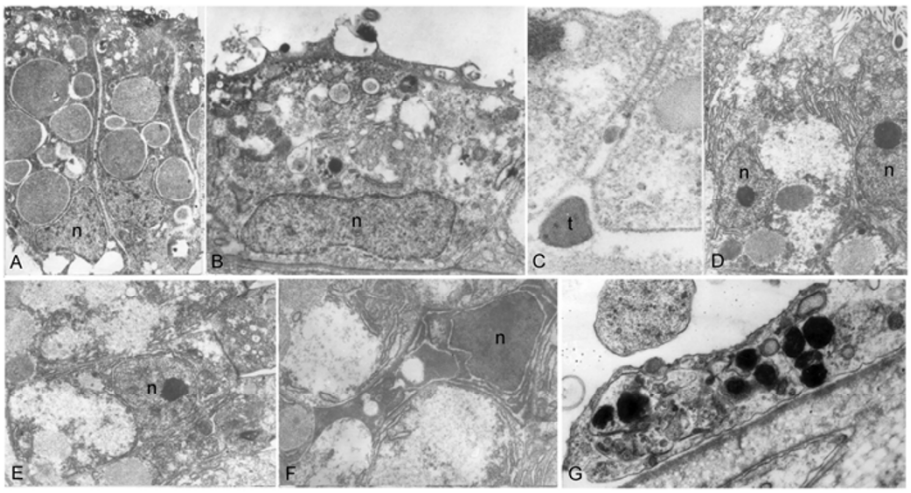

Figure 1 Transmission electron micrographs of epidermal cells, branchial cells, epithelium of the alimentary canal and macrophages in amphioxus. A and B, Epidermal cells in 24-h and 48-h larva, respectively. The vesicles with tracer carbon particles in the apical cytoplasm and endocytosing vesicles on the cytoplasmic membrane can be observed. C, At the 36 to 48-h larva stage, carbon tracer particles supplied in cultured seawater are observed in the intercellular space. D, The branchial epithelial cells of 48-h larva. E, The epithelial cells in the differentiating alimentary canal of 48-h larva. F, A migrating cell in the epithelium of alimentary canal in 72-h larva. G, A macrophage is observed in the perigenital coelom of adult amphioxus. n, nucleus; t, tracer carbon particles. Magnification: A, ×12300; B, ×155000; C–E, ×11000; F, ×8700; G, ×8500.

well as macrophages. These genes were *TLR1* [16], *SoxC* [8], and *DDAHa* [17], respectively. Moreover, we also detected expression of *ECSIT*, *C1Q* and *NOS* homologs, whose amino-acid sequences were over 40% identical to human.

TLR1 was clearly expressed in the epidermal cells covering the body (Figure 2A), in the cytoplasm of intestinal epithelial cells (Figure 2B), and in all ciliary epithelial cells of the branchial lamellas (Figure 2C). The expression of *TLR1* was limited to macrophages located in the coelom and lymphoid cavities (Figure 2B–E).

The expression patterns of *C1Q*, *ECSIT*, and *SoxC* were similar to *TLR1* in the pharyngeal region, which were observed in all ciliary epithelial cells of the branchial lamella, the wall of the septal coelom, as well as in coelomic macrophages (Figure 3A–C). Expression of *DDAHa* and *NOS* was also detected in the branchial cells except for the lateral ciliary epithelial cells (Figure 3D and E). Similar to *TLR1*, all genes were expressed in all epithelial cells of the intestine and the macrophages in the perienteric coelom (Figure 3F–J). Similar to *TLR1*, *DDAHa* is expressed in all epidermal cells while expression of the other genes appeared mainly in the epidermal cells at the metapleural fold region (Figure 3K–O).

2.3 *Staphylococcus aureus* Rosenbach alters expression of *TLR*, *ECSIT*, *C1Q*, *SoxC*, *DDAHa*, and *NOS* in amphioxus

RT-PCR results show that after amphioxus was injected with *Staphylococcus aureus* Rosenbach, the *ECSIT*, *TLR*, and *C1Q* were up-regulated. The *DDAHa* was up-regulated slightly in amphioxus. The expression of *NOS* did not change. It was interesting to find that *SoxC* was down-regulated after injection of *Staphylococcus aureus* Rosenbach (Table 1).

3 Discussion

One main function of skin is to protect an organism against

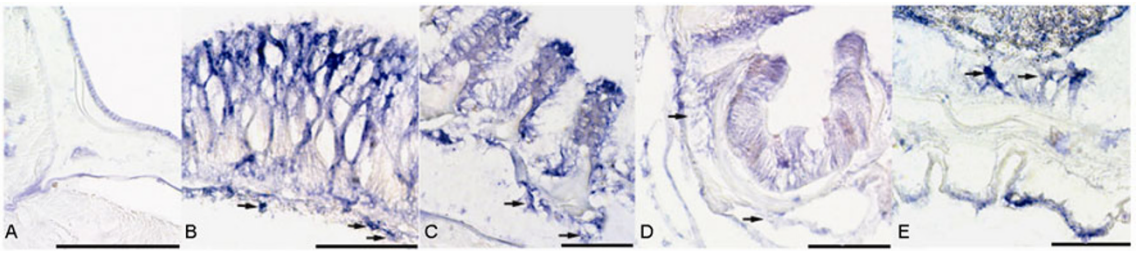

Figure 2 The expression of *TLR1* is detected in epidermis, gill epithelium and the intestinal epithelium as well as macrophages by *in situ* hybridization. A, Expression of *TLR1* in epidermal cells near the notochord. B, Expression of *TLR1* in intestinal epithelial cells and macrophages (arrows) in perienteric coelom. C, *TLR1* expression in gill epithelium and macrophages (arrows) in the branchial coelom. D, The expression of *TLR1* is detected in endostyle epithelial cells and macrophages (arrows) in lymphoid cavities. E, Expression of *TLR1* is also observed in the epithelial cells of metapleure and perigenital coelomic macrophages (arrows). Scale bars, 50 μm.

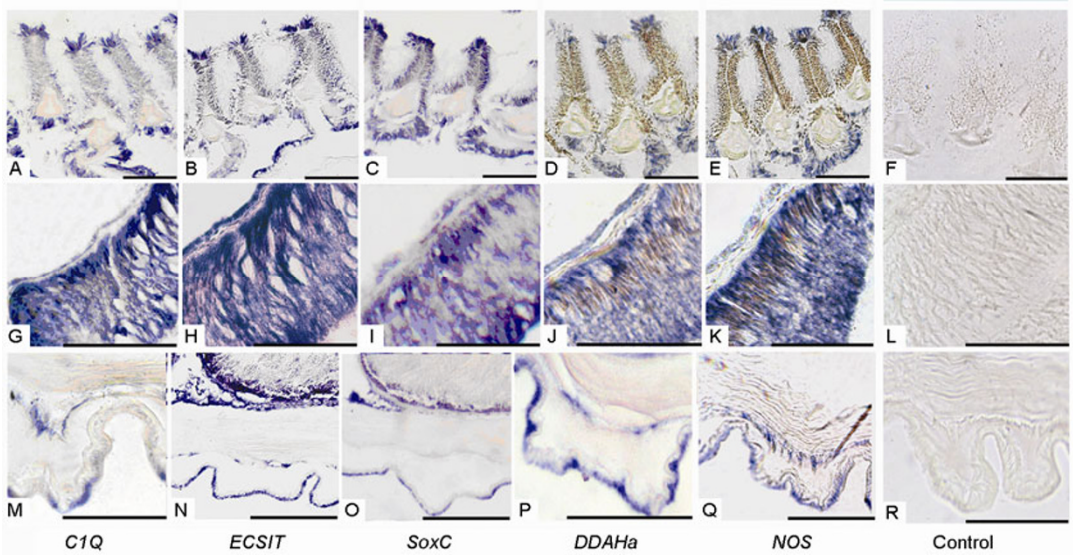

Figure 3 The expression pattern of *C1Q*, *ECSIT*, *SoxC*, *DDAHa*, and *NOS* of amphioxus gill epithelium and branchial coelom macrophages (A–E); the intestinal epithelium and perienteric coelom macrophages (G–K); the metapleural fold epidermis and metapleure coelom macrophages (M–Q) by *in situ* hybridization. F, L, and R are controls using sense *DDAHa* RNA probes. Scale bars, 50 μm for A–L and 25 μm for M–R.

external pathogens. Early studies found that the amphioxus epidermis could secret lectins and other antibacterial peptides [18]. Our previous report [1] and the TEM data presented here show that epidermal cells of amphioxus larva older than 24 h possess typical morphologic characteristics, such as endocytic vesicles on the cell membrane and endocytosis particles in the apical cytoplasm. Furthermore, tracing carbon particles were observed in the intercellular spaces of epidermal cells. These data indicate that amphioxus larval epidermal cells are capable of endocytosis. The less electron-dense vesicles in the cytoplasm were more likely involved in lectin and antibacterial peptide secretion. Additionally, the *in situ* hybridization results suggest that expression of *TLR1* in epidermis is similar to *TLRs* expression in human keratinocytes, where a functional TLR is expressed and that contains an antimicrobial defense barrier [19]. We also demonstrate the expression of other potential immune defense vertebrate homologs, including *C1Q*, *ECSIT*, *SoxC*, *DDAHa*, and *NOS*. This is consistent with the suggestion that epidermis performs an important role in the immune defense of amphioxus.

The immune system is composed of the innate and adaptive immune systems. TLRs play a crucial role in the recognition and induction of the immune response. Immunocytes, such as macrophages, dendritic cells, and B lymphocytes recognize pathogens via TLRs. The stimulation of TLRs via microbial invasion activates the innate immune response. In some cases, activation of antigen-presenting cells (APCs) via TLRs up-regulates nitric oxide and induces the adaptive immune response [20]. ECSIT is a regulator involved in both BMP and TLR signaling. C1Q, a recognition protein in the classical complement system, is involved in both innate and acquired immunity. *SoxC*, which plays a role in lymphocyte maturation, is expressed in tissues where macrophages exist [11]. DDAHa and NOS are involved in the regulation of endogenous NO synthesis [21], and NO is active in both acquired and innate immunity. Following celiac injection with *S. aureus*, we observed up-regulation of *ECSIT*, *TLR*, and *C1Q*, which suggests these genes play an important role in immune defense. The little changed expression of *DDAHa* implies that stimulation by *S. aureus* may have no direct influence on *NOS* expression. The down-regulation of *SoxC* indicates that *SoxC* might have a different role in immune defense. We found that similar to *TLR1*, expression of *C1Q*, *ECSIT*, *SoxC*, *DDAHa*, and *NOS* appear in gill and intestinal epithelial, suggesting that in addition to the epidermis, the entire alimentary canal has an immune related role in amphioxus. Previous studies have indicated that one of VCBP genes, *VCBP1*, is expressed selectively in the alimentary canal [5]. *TLR1* is also expressed in amphioxus gut and gill, which implies that the alimentary canal in amphioxus is likely to be the major immune system tissue. It is interesting to find potential precursors to free mononuclear cells, granulocytes or macrophages, in the differentiating epithelium of the alimentary canal in larva. It is worth noting that potential homologs of *TLR1*, *C1Q*, *ECSIT*, *SoxC*, *DDAHa*, and *NOS* are also expressed in amphioxus macrophages, suggesting a crucial immune function for macrophages in this animal.

In amphioxus, some lymphoid related genes, such as the *Ikaros-like* gene and the *Bam32* gene (B lymphocyte adaptor molecule of 32 kD) [22] are expressed in the metapleural fold and intestine (Table 2). Other immune defense related genes show similar expression patterns in immune related tissues (epidermis and the alimentary canal) and cells (macrophage), which suggests that all these genes play a role in immune defense.

Table 1 RT-PCR detection of *ECSIT*, *TLR*, *C1Q*, *DDAHa*, *SoxC*, and *NOS* expression[a)]

	ECSIT	*TLR1*	*C1Q*	*DDAHa*	*NOS*	*SoxC*
Expression pattern changes after injection	↑ ↑ ↑	↑ ↑	↑ ↑	↑	–	↓

a) The number of ' ↑ ' indicates the level of up-regulation; '–' indicates no significant change in expression; ' ↓ ' indicates the level of down-regulation.

Table 2 Comparison of the expression patterns of homologous immune defense genes

	Macrophages in coelom	Epidermal cells of metapleural fold	Pharynx epithelium	Gut epithelium
TLR	+	+	+	+
ECSIT	+	+	+	+
C1Q	+	+	+	+
SoxC	+	+	+	+
DDAHa	+	+	+	+
NOS	+	+	+	+
Thymosin β4 [23]			+	+
Ikaros-like gene [22]			+	
Bam32 [22]	+	+		
VCBP [5]				+

In summary, we report the ultrastructural characteristics of amphioxus larvae epidermis, gill, and intestine epithelium. Our results indicate that the epidermis and the alimentary canal play important roles in immune defense, while macrophages in the coelom and so-called lymph spaces may be crucial immune cells. Moreover, *in situ* expression patterns of *TLR1*, *C1Q*, *ECSIT*, *SoxC*, *DDAHa*, and *NOS* suggest that they play a role in an immune response. Our studies should facilitate understanding of the amphioxus immune system and evolution of the primordial adaptive immune system.

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