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REVIEW

Innate and intrinsic immunity in planarians

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Abstract

Planarians in the Phylum Platyhelminthes occupy a peculiar phylogenetic position and have a strong regenerative capacity of their adult tissues, which has aroused general attention. Planarians are spontaneously exposed to various pathogens (microbes and harmful chemicals), but typically survive these challenges. Therefore, these animals can provide useful insights into the evolution of the innate immune system. This review mainly focuses on immune tissues (epidermis, pharynx, and intestine), immune cells (phagocytic reticular cells) and immune genes of planarians. In addition, we provide an overview of the critical proteins in the innate immune system - for example, pattern recognition receptors, complement system proteins, anti-microbial peptides and antioxidant enzymes. In particular, the effectors of the signaling pathways activated upon planarian infection are reviewed.

Key Words: planarian; innate immune system; immune-related genes

Introduction

Animals are exposed to microbes from the environment and require defense systems for protection from infectious agents. In the process of survival and evolution, animal immune systems have been improved by the invasion and attack of various exotic pathogens and endogenous harmful substances. The immune system generally includes immune tissues and organs, immune cells and immune molecules (Franchi and Ballarin, 2017). When a pathogen invades an organism, a series of responses occur through these molecules, cells and organs to eliminate the invader. In vertebrates, immunity includes both innate and acquired immunity (Nyholm and Graf, 2012). Acquired immunity, which is stimulated by infection and vaccination, exhibits antigen specificity, diversity, immunological memory, and non-self recognition that is mediated by activated B and T cells. Conversely, the innate immune system is an evolutionarily older defense strategy, and is an ubiquitous immune system found in every species of organisms studied so far (Medzhitov and Janeway, 2000b).

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Invertebrates, which are distributed in all parts of the world, do not produce specific antibodies to recognize and cope with multiple invasive pathogens; they rely on innate immune recognition and have the capacity to recognize self and non-self (Chu and Mazmanian, 2013). Upon the invasion of non-self, the innate immune system exploits two defense mechanisms: constitutive defenses and inducible defenses (Medzhitov and Janeway, 2000a). Constitutive defenses effectively prevent the non-self pathogens from entering the body through inherent barriers, *e.g*., surface barriers, mucous membranes of the gastrointestinal tract, respiratory tract and reproductive tract, antimicrobial peptides secreted by epithelial cells, antimicrobial enzymes and lysozymes in body fluids. The vast majority of the defense responses upon infection are through inducible defenses. In these processes, the exotic pathogens are recognized by immune cells, triggering a cascade of immune responses and high expression of related proteins. Finally, pathogens are eliminated through phagocytosis, encapsulation and nodulation by phagocytes (Browne *et al.*, 2013). In addition, cytotoxicity by ROS production is another way to kill microorganisms widely distributed among invertebrates (Jiang *et al.*, 2007; Xu *et al.*, 2017). The recognition mentioned above, also referred to as pattern recognition, is mediated by pattern recognition receptors (PRRs) and pathogen-,

microbial- or damage-associated molecular patterns (PAMPs, MAMPs or DAMRs) (Wenger *et al.*, 2014). A number of PRRs have been reported in invertebrates, including Toll-like receptors, Nod-like receptors, RIG-I-like receptors, C-type lectin (CTL), scavenger receptors, mannose receptor, peptidoglycan recognition proteins (PGRPs), thioester-containing proteins, Gram-negative binding proteins and so on (Gao *et al.*, 2017a). PRRs specifically recognize the common and highly conservative molecular structures on the surface of pathogenic microorganisms to remove these pathogens.

Planarians (Platyhelminthes, free-living rhabditophorans, Tricladida) are of great interest to scientists because of the special evolutionary status and the prominent capacity of regeneration. Planarians are exposed to a wide range of pathogenic microorganisms and harmful chemicals, and sometimes they feed on detritus, fungi, and bacteria (Gonzalez-Estevez, 2009; Abnave *et al.*, 2014). What is noteworthy is that there is little wound infection in regenerating planarians, suggesting that they may possess an efficient immune system in order to prevent infections. Therefore, the study of planarian immunity is important to shed light on the relationship between the immune system and the regenerating capacity. Here, we review the limited studies in this field, encompassing all levels of immune defenses to expound the innate and intrinsic immunity of planarians, and attempt to provide support for future immune response studies.

The sites of immune responses

Planarians are naturally exposed to various pathogens within their habitat. Epidermis, pharynx, intestines and reticular cells, are the most effective weapons to defend against pathogen invasion, by detecting and removing pathogens with different defense mechanisms.

Epidermis

In planarian, as in other organisms, the epidermis not only prevents the leakage of internal molecules but also acts as the first barrier against invading pathogens and parasites in the environment. Epidermal cells are constantly replacing themselves to maintain homeostasis and normal function. The planarian epidermis is a simple and unilaminar epithelium, which mainly consists of two types of cells: true, ciliated epidermal cells and microtubule-lined necks of the rhabdite-forming gland cells, whose cellular bodies lay in the parenchymal cell layers (Skaer, 1965; Rieger, 1991; Hayes, 2017).

The body surface of planarians is covered by a layer of secreted mucus mainly derived from specialized secretory granules known as rhabdites (Hayes, 2017). The ejected slime structures are rich in sulphated glycosaminoglycans (sGAGs), and can be divided into two broad categories: a large one presenting in the outermost epidermal layer, and a small and highly striated one produced by rhabdite-forming gland cells (Hayes, 2017). The viscous slime produced by these unique rhabdites not only lubricates the interface and prevents damage to the soft body, but also contributes to adhesion to the substratum. Previous studies suggest that the slime is implicated in ciliary gliding, locomotion, predator avoidance, prey capture, innate immunity, and substrate adhesion (Bocchinfuso *et al.*, 2012; Hayes, 2017). Interestingly, some entrapped bacteria or 'outer membrane' embedded in the complex fibrous sGAG-containing nets formed by ruptured rhabdites have been observed (Hayes, 2017), suggesting that external mucous coating exerts a strong influence in response to environmental stimuli. This hypothesis has been confirmed through proteomic analysis of mucous secretions of the planarian, *Schmidtea mediterranea* (Bocchinfuso *et al.*, 2012). Proteomics data reveal that 119 planarian mucous proteins appear to be orthologs or significantly similar to mucous proteins in nasal mucus, olfactory mucus, cervical mucus, and tear fluid in human (Bocchinfuso *et al.*, 2012). Among these annotated proteins, we discovered several immune-associated proteins, including 14-3-3 protein zeta (Lu *et al.*, 2017), alpha-2 macroglobulin (Armstrong and Quigley, 1999; Ponprateep *et al.*, 2017), DJ-1 (Tsushima *et al.*, 2012), syntenin-1 (Gordon-Alonso *et al.*, 2012; Liu *et al.*, 2015), peroxiredoxin-6 and superoxide dismutase (Bocchinfuso *et al.*, 2012).

Pharynx

Planarian pharynx is the exclusive food entrance towards the internal cavity, referred to as pharyngeal pouch or pharynx cavity, in the middle portion of the body. The highly extensible pharynx is a complex muscular organ consisting of multiple cell types, *e.g.,* epithelial cells, muscle cells, neuronal cells and secretory cells (Kobayashi *et al.*, 1999; Shimoyama *et al.*, 2016). This cylindrical organ is also a channel that connects the intestine duct through the esophagus at the proximal end of the pharyngeal cavity (Scimone *et al.*, 2016) and the outside world rich in pathogenic microorganisms and harmful chemicals. Pharynx epithelia had been recognized as a site of microbiota colonization and the first line of defense against pathogens in food and environment. Therefore we speculate that it houses a large number of symbiotic species. Recently, Adler *et al.* discovered many genes required for pharynx regeneration through microarray technologies. Among these genes, 356 are significantly up-regulated after amputation (Adler *et al.*, 2014). Our follow-up study shows that many of these genes are immune-associated genes, *e.g.,* placenta specific protein 8 (PLAC8), low-density lipoprotein receptor-related protein (LRP), and beta-defensin 114. Our previous study found that PLAC8 of *Dugesia japonica* inhibits the growth of the Gram-negative bacteria in pharynx (Pang *et al.*, 2017). Similarly, *D. japonica* LRP was found to be expressed in pharynx using an *in-situ* hybridization technique (unpublished data). In addition, in amphioxus, BjLRP, a novel pattern recognition protein, is capable of identifying and interacting with invading bacteria (Gao *et al.*, 2017c). Beta-defensin, a member of AMPs, also possesses antimicrobial activities by resisting a broad spectrum of microorganisms, including Gram-positive bacteria, Gram-negative bacteria and fungi (Pero *et al.*, 2017).

It is not difficult to find that all of these immune-associated genes, located in pharynx, can identify and restrain exogenous microbes, indicating that pharynx, as the early immune defense organ, plays a pivotal role in immune responses.

Intestines

The planarian intestinal duct is the organ managing nutrient digestion and metabolite distribution, it can be easily visualized by whole-mount *in-situ* hybridization with the innexin1 riboprobe (Nogi and Levin, 2005). Anatomically, the gut can be divided into three main branches; one anterior, located in the head and prepharyngeal regions, which is linked together with the anterior end of the pharynx, and two posterior branches located in the pharyngeal and tail regions (Forsthoefel *et al.*, 2011). Virtually, the branches can extend to the lateral margins of the body, and the gut can reach almost all positions of the body except the anterior region of the head. The intestinal epithelium, or gastrodermis, is composed of a single columnar layer surrounded by a thick basement membrane and muscle fibers (Bueno *et al.*, 1997), and consists mainly of two cell types, namely, absorptive phagocytes and secretory goblet cells (Forsthoefel *et al.*, 2011).

Phagocytes play a crucial role in the elimination of apoptotic cells and necrotic tissues deriving from injury and immune responses. In addition, phagocytes also ingest and breakdown invading pathogens and parasites (Peiris *et al.*, 2014; Franchi and Ballarin, 2017). Phagocytosis is one of the main processes of bacterial elimination in planarians. One study demonstrates that the labeled bacteria, *Legionella pneumophila* and *Staphylococcus aureus*, in intestinal tissues shows a descending trend on day 6 post-feeding (Abnave *et al.*, 2014). In addition, whole-mount *in situ* hybridization reveals that 18 genes, which are highly involved in the elimination of *L. pneumophila* and *S. aureus*, are primarily expressed in the intestinal tissues in response to infection of bacteria (Abnave *et al.*, 2014). In summary, these findings underscore the remarkable ability of planarian intestine in the elimination of a broad range of bacterial strains.

Reticular cells

All animals must maintain the bodies functional by eliminating wastes, repairing tissues, and resisting pathogens and foreign invaders. In these events, phagocytes play a pivotal role. Phagocytes are common to all invertebrates; they can move freely in the circulating body fluids, where foreign materials and their own cells need to be recognized and obliterated, either actively or passively (Morita, 1991). Morita *et al.* have demonstrated that a special type of cells, which is similar to the fixed parenchymal cells, migrates to the wounded tissues (Morita and Best, 1974). They named this type of cells the reticular cell. Planarian reticular cells play a significant role in nutrient transportation, homeostatic regulation of cells, and surveillance systems (Morit, 1995). But beyond that, planarian reticular cells can be considered a sort of primitive mobile cells, equivalent to circulating cells involved in immune responses (immunocytes) in other invertebrates (Kounatidis and Ligoxygakis, 2012; Taffoni and Pujol, 2015; Franchi and Ballarin, 2017).

Structural and specific functional characteristics of planarian reticular cells have been investigated by light and electron microscopy; numerous glycogen granules, lipid droplets, some lysosomes and a nucleus with an irregular shape have been discovered in the cytoplasm (Morit, 1995). The defense mechanism of reticular cells against invading pathogenic microorganisms was explored by injecting heat-killed bacteria into an incision and then examining the tissues around the incision (Morita, 1991). As early as 10 h after the injection, foreign intruders are found in the phagosomes of reticular cells. By 12 h, a large amount of bacteria is encapsulated by the cytoplasmic processes of reticular cells, and by 24 h, the encapsulated bacteria are eliminated in the intestine (Morit, 1995; Morita, 1991). Subsequent investigation in *D. japonica* and *S. mediterranea* has also confirmed the strong capacity of planarians to cope with infection of any of the 16 bacterial strains that are pathogenic to humans, *Caenorhabditis elegans* and/or *Drosophila melanogaster* (Abnave *et al.*, 2014). These findings indicate that foreign invaders can be recognized, phagocytized and encapsulated by reticular cells and expelled into the intestinal cavity to be digested and degraded. It is thus conceivable that reticular cells act as the main immunocytes involved in the defense against pathogens.

In addition to participating in the immune response, reticular cells also take part in the process of tissue regeneration. Immediately after the amputation, the cut surface lacking the epidermis and basement membrane is totally exposed to the environment which contains various pathogenic bacteria, fungi and harmful chemicals (Morita and Best, 1974). One hour after the amputation, the pre-existing epidermal cells around the wound surface stretch out their own bodies and maintain a close connection with each other to make a thin basement membrane (Morita and Best, 1974). Six to eight h after the amputation, the reticular cells appear on the entire cut surface underneath the epidermal film, and then, some of them appear to extend their cytoplasmic processes and contact with one another to form the regeneration blastema or a specific meshwork (Morita and Best, 1974; Morit, 1995). Approximately 16 h after the amputation, the entire wound surface is covered by the basement membrane, after which, the thinly stretched epidermal film begins to thicken and the neoblasts begin to differentiate (Morita and Best, 1974). In the late stages of the wound regeneration, the epidermal film is broken down, and differentiating cells infiltrate the interstices of the meshwork of reticular cells. During this period, reticular cells phagocytize the debris of the damaged or degenerating cells. The reticular cells, which appear early in the cut surface, play a very important role in both its own cell clearance and pathogen scavenging during the regeneration process.

Immune-related genes

In the innate immune responses, PRRs have an essential role in identifying specific components of foreign pathogens and triggering a series of cascade

Table 1 Expression of innate immune candidate genes of planarian upon challenge with bacteria. The table is adopted from the whole-transcriptome analysis performed by Abnave *et al.* (2014)

Protein name	Pfam domain	Sequence numbers
Trafficking protein particle complex subunit 1 (TRAPPC1)	Sybindin domain (PF04099)	Seq51130
Progestin and adipoQ receptor family member 3 (PAQR3)	Hemolysin-III-related domain (Hly III) (PF03006)	Seq53353
Trafficking protein particle complex subunit 2-like (TRAPPC2L)	Sedlin N domain (PF04628)	Seq74013
Membrane Occupation and Recognition Nexus (MORN) repeat-containing-2 (MORN2)	COG4642 superfamily domain (PF02493)	Seq74746
ATOX1	Heavy-metal-associated (HMA) domain (PF00403)	Seq75036
Hydroxyacylglutathione hydrolase (HAGH)	Lactamase B domain (PF00753)	Seq78560
CutA	CutA1 domain (PF03091)	Seq76275
DUSP19	Dual-specificity phosphatase (DUSP) domain (PF00782)	Seq31603
Hypothetical protein	Dynamin N domain (PF00350)	Seq39446
Dynein light chain roadblock-type (DYNLRB)	Roadblock/LC7 domain (PF03259)	Seq75092

reactions through the downstream adaptor proteins and effector molecules to eliminate the invading microbes. Planarians are exposed to a wide range of pathogens and harmful chemicals. However, infections which lead to death rarely occur, suggesting that they possess an effective immune system. Abnave *et al.* (2014) discovered that planarians can resist infections by multiple bacterial species pathogenic to humans, *Drosophila* and nematode. They identified 18 genes expressed in the intestine of the infected planarians that actively contribute to the elimination of *L. pneumophila* and *S. aureus*. Table 1 provides the detailed information of the ten genes that contain clear domains recorded by Pfam. Other studies have also reported that immune-related genes play an essential role in different aspects of the immune responses. It should be noted that planarian immune-related genes and immune response pathways are also involved in other physiological processes. In addition, Peiris *et al.* (2014) revealed that planarians contain many potential homologs of the mammalian innate immune system that are activated during injury and repair of adult tissues. Furthermore, Tsoumtsa *et al.* (2017) uncovered that Smed-Tim, a homolog of the mammalian clock gene Tim, is an indispensable protein for anti-microbial activities against *S. aureus* infection during the light/dark cycle. Table 2 includes a collection of candidate genes that possess potential immune defense functions. Details about these candidate genes are provided below.

Pattern recognition receptors (PRRs)

In animals, especially invertebrates, the innate immune system plays a crucial role in the defense against causative agents. The innate immune responses rely on the specific pattern recognition, in which, PRRs identify PAMPs, such as bacterial cell wall composition, dsRNA, and some obsolete components released by damaged and dead cells. In the absence of the adaptive immunity, the number and diversity of PRRs may provide an advantage for animals living in pathogen-rich environments. Our analysis of previous reports of planarians identified some PRR genes, including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), CTL/Selectins, mannan binding lectin, peptidoglycan recognition protein-1 (PGRP1) and G-Protein-coupled receptor (GPCR).

Table 2 Summary of the innate immune candidate genes reported so far in planarians. A brief description of the domain, data sources and reference is also reported

Protein types	Protein name	Domain	Data sources	References
Receptor	Toll-like receptors	Leucine rich repeat domain (LRR); Toll/Interleukin-1 receptor domain (TIR)	SmedGD	Peiris *et al.,* 2014
	RIG-I-like receptors	HELIC domain; DEXD domain	Dj-BSTD	Pang *et al.,* 2016
	C-type lectin/ Selectins	C-type lectin like domain	Dj-BSTD	Pang *et al.,* 2016; Peiris *et al.,* 2014
	Mannan binding lectin (MBL)	C-type lectin like domain	Dj-BSTD	Pang *et al.*, 2016
	Peptidoglycan recognition protein-1	PGRP superfamily domain	AFJ24867.1	Wenemoser *et al.*, 2012
	G-Protein-Coupled Receptor	7tm_GPCRs superfamily domain	AB495373	Nishimura *et al.*, 2009
Anti-microbial peptides/ Defense	Antimicrobial peptide resistance and lipid A acylation protein PagP	PagP superfamilu domain; OM_channels superfamily domain	SmedGD	Peiris *et al.*, 2014
	Beta-defensin 114	Defensin_beta_2 superfamily domain	Sm-PRTD	Adler *et al.,* 2014
	Lysozyme	Lysozyme_like superfamily domain	Dj-BSTD	Pang *et al.,* 2016
	Membrane attack complex component /perforin/C9	MACPF superfamily domain	SmedGD	Altincicek *et al.*, 2008
Complement system	Alpha 2-macroglobulin	A2M domain	Dj-BSTD	Pang *et al.,* 2016; Peiris *et al.*, 2014
	Complement	CUB domain; C1q domain	SmedGD	Peiris *et al.*, 2014
	Properdin	thrombospondin type 1 domain	SmedGD	No reference
	Complement factors or integrins	Von Willebrand factor type A domain	SmedGD	Peiris et al., 2014
Antioxidant enzyme	Catalase (CAT)	catalase_clade_3 domain	Sm-MPD	Bocchinfuso *et al.*, 2012; Zhang *et al.,* 2014; Zhang *et al.,* 2016
	Superoxide dismutase (SOD)	SodA domain	Sm-MPD	Bocchinfuso *et al.*, 2012; Zhang *et al.,* 2014; Zhang *et al.,* 2016
	Glutathione peroxidase (GPx)	GRX_GRXh_1_2_like domain	SmedGD	Zhang *et al.*, 2014; Zhang *et al.*, 2016
	Peroxiredoxin	PRX5_like domain	Sm-MPD	Bocchinfuso *et al.*, 2012
	Thioredoxin peroxidase (TPx)	PRX_Typ2cys domain	SmedGD	No reference
Various immune associated proteins	Phenoloxidase (PO)	Tyrosinase domain	Dj-BSTD	Pang *et al.*, 2010; Lapan *et al.*, 2011
	LPS-induced TNF-alpha factor	LITAF-like zinc ribbon domain	SmedGD	Peiris *et al.,* 2014
	TRAF family proteins	TRAF-type zinc finger; MATH domain	Dj-BSTD	Peiris *et al.,* 2014; Pang *et al.,* 2016
	Low-density lipoprotein receptor-related protein (LRP)	LDLa; EGF_CA; LY domain	Sm-PRTD	Adler *et al.,* 2014
	Placenta specific protein 8 (PLAC8)	PLAC8 superfamily domain	ALJ10579	Pang *et al.,* 2017
	Phospholipid scramblase	LOR superfamily domain	APL96720.1	Han *et al.*, 2017
	Syntenin	PDZ domain	Sm-MPD	Bocchinfuso *et al.*, 2012

	c-Jun N-terminal Kinase (JNK)	STKc_JNK domain	Dj-BSTD	Pang *et al.,* 2016
	Mitogen-activated protein kinase (MAPK)	PB1 domain; Catalytic domain; Serine/Threonine Kinase	Dj-BSTD	Pang *et al.,* 2016
	P38	Serine/Threonine Kinase; Catalytic domain	Dj-BSTD	Pang *et al.,* 2016
	TRAF family member-associated NF-κB activator-binding kinase 1 (TBK1)	PKc_like superfamily; S_TKc domain	Dj-BSTD	No reference
	Interferon regulatory factor (IRF)	IRF-3 domain; IRF superfamily domain	Dj-BSTD	No reference
	Mx	P-loop_NTPase; Dynanin_M domain	Dj-BSTD	No reference
	γ-IFN-inducible lysosomal thiol reductase	GILT superfamily domain	Dj-BSTD	No reference
	Timeless (Tim)	TIMELESS domain	Sm-PMTD	Tsoumtsa *et al.,* 2017
	Toll-interacting protein (TOLLIP)	C2 domain; CUE domain	Dj-BSTD	Pang *et al.,* 2016

TDj-BSTD: Dj-BS transcriptome database (https://www.ncbi.nlm.nih.gov/sra/?term=SRX1479355)
Sm-PRTD: *S. mediterranea* pharynx regeneration transcriptome database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE56181)
Sm-MPD: *S. mediterranea* mucous proteomic database (http://www.mcponline.org/content/11/9/681/suppl/DC1)
SmedGD: *S. mediterranea* genome database (http://smedgd.neuro.utah.edu/)
DDBJ: DNA data bank of Japan (http://www.ddbj.nig.ac.jp/)
Sm-PMTD: *S. mediterranea* transcriptome database PlanMine (http://planmine.mpicbg.de/planmine/begin.do)

The transmembrane TLRs belong to type I transmembrane receptors, composed of the C-terminal Toll/Interleukin-1 receptor (TIR) domain and N-terminal leucine rich repeat (LRR) domain. Peiris *et al.* identified several gene segments that contain the LRR domain and TIR-like domain, respectively (Peiris *et al.*, 2014). In the Dj-BS (clonal strain *D. japonica* collected from Boshan district of China) transcriptome database, we also found many sequences that contain different combinations of LRR domains, but no TIR domain was found in these sequences. It is unknown whether these LRR repeats have TLR-like activities. Interestingly, Toll-interacting protein (TOLLIP) and IFN-stimulated genes (ISGs), such as Mx and γ-IFN-inducible lysosomal thiol reductase (GILT), were identified in the databases. Subsequently, we need to do more research on the TLR signaling pathway of planarians.

RLRs play an important role in viral infection by recognizing and binding the double stranded RNAs and 5'-triphosphate single stranded RNAs in the invading virus (Xu *et al.*, 2005). After binding with viral RNAs, RLRs interact with MAVs/VISA/Cardif/IPS-1 and form a protein complex to recruit tumor necrosis factor receptor associated factor 3 (TRAF3) and TRAF6. Then, a number of transcription factors, including nuclear factor-κB (NF-κB) and interferon-regulated-factor (IRF), are activated, and type I interferons (IFN-α/β) are successfully expressed (Kawai *et al.*, 2005; Xu *et al.*, 2005). In our previous study, we revealed that the mRNAs of RIG-I, TRAF3, TRAF6, and P38 involved in the RIG-I-like receptor signaling pathway are significantly up-regulated after being induced by LPS, peptidoglycan, and Poly (I:C) as well as Gram-negative and Gram-positive bacteria; the results show that these factors may be involved in planarian immune responses (Pang *et al.*, 2016). The other two proteins, TRAF family member-associated NF-κB activator-binding kinase 1 (TBK1) and IRF in this signaling pathway, have also been identified. But the adaptor protein MAVs/VISA/Cardif/IPS-1 was not found in the annotated proteins of the databases (Fig. 1). Additional experiments are required to investigate whether there is a novel adaptor protein in planarians.

CTL, selectins and MBL have the same conserved C-type lectin (CTL)/C-type lectin-like (CTLD) domain (CLECT domain), homologous to the carbohydrate-recognition domains (CRDs) of the CTL. The CLECT of these lectins can recognize specific carbohydrates on the surface of pathogens, allergens, necrotic or apoptotic cells, and then mediate the functions associated with phagocytosis and elimination. Recently, we identified a novel C-type lectin-like protein, which participates in the immune response to bacteria infection (Gao *et al.*, 2017a) (Fig. 1). In the Dj-BS transcriptome database, we identified several gene segments that contain different amounts of the CRD domain. This phenomenon has also been reported in scallops (*Chlamys farreri*), where three CRD-containing genes, CfLec-1, CfLec-2 and CfLec-4, have been identified, and their relative immune functions and binding mechanism have been elucidated (Yang *et al.*, 2010; Yang *et al.*, 2011; Huang *et al.*, 2013).

GPCRs, a large family of seven transmembrane domain receptors, are involved in multiple signaling pathways. They are directly or indirectly related to many important physiological processes, such as

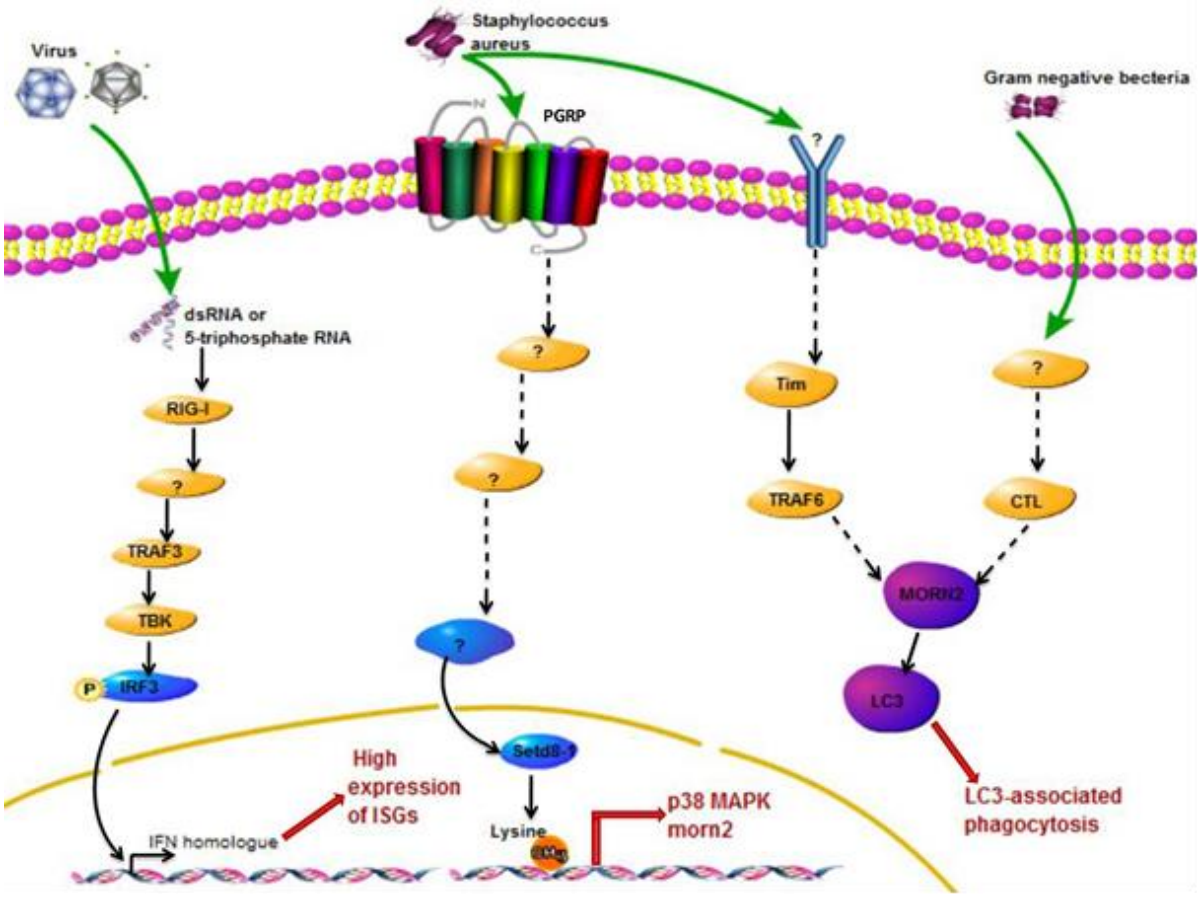

Fig. 1 Overview of the supposed intracellular immune signaling pathway activated by PAMP binding. Only parts of the identified components are shown. Proteins not yet identified are marked by a question mark. The proposed but yet to be verified interaction between proteins is indicated using a dashed arrow.

nerve impulse transmission, cell metabolism, immune defense, and cell differentiation (Hollenstein *et al.*, 2013). In invertebrates, numerous pieces of evidence point to an important role of GPCRs in host defense through different signaling pathways (Reboul and Ewbank, 2016). In *D. japonica*, a novel GPCR (DjSER-7) has been identified, but its specific immunological functions have yet to be identified (Nishimura *et al.*, 2009). Similarly, the preliminary study of PGRP of planarians is also limited. Four PGRP-like receptors, namely, Smed-PGRP-1, -2, -3, and -4, have been identified in *S. mediterranea* (Arnold *et al.*, 2016). Recently, Torre *et al.* discovered that primo-infection of *S. aureus* leads to the expression of Smed-PGRP-2, which, in turn, promotes Smed-setd8-1 methyltransferase signaling and increases the lysine methylation level in neoblasts to sensitize anti-bacterial gene responses during re-infection (Torre *et al.*, 2017) (Fig. 1). In future, more members of GPCRs and PGRPs of planarians and immune response mechanisms need to be explored.

Antimicrobial peptides

Peptides with a variety of broad-spectrum antimicrobial activities are important components of non-specific defense systems. These small peptides are characterized by the evolutionarily conserved clusters of positively charged and hydrophobic amino acids (Pasupuleti *et al.*, 2012). The antibacterial mechanism of AMPs is mainly through dissolving bacterial cell membranes or altering the permeability of cell walls. Meanwhile, a few AMPs can kill bacteria by inhibiting the synthesis of bacterial DNA, RNA and proteins (Pasupuleti *et al.*, 2012). Besides AMPs, in vertebrates, membrane attack complex component/perforin/C9 also contributes to the elimination of pathogens, which is one of the important effector molecules in immune responses and immune defenses. Undeniably, antibacterial peptides and perforin play an indispensable role in the innate immune system of animals. We describe four proteins, beta-defensin 114, lysozyme, antimicrobial peptide resistance and lipid A acylation protein PagP and membrane attack complex component/perforin/C9, which were discovered in different databases of planarians. These proteins are all functional proteins at the bottom of the signaling pathway activated after external pathogen invasion. How these critical proteins are activated during infection remains to be ascertained.

Complement system

In vertebrates, the complement system, as one of the original innate immune systems, is a complex protein reaction system composed of serum proteins, regulatory proteins and membrane receptors (Ghebrehiwet, 2016). When responding to pathogen and inflammation, the host employs the activation pathways of complements, including the classical pathway, the activating pathway and the lectin pathway, to form a membrane attack complex and finally give rise to immune cytolysis and immune haemolysis. In this process, complement component 3 (C3) is the central node of these pathways and locates in a position not to be ignored in immune surveillance and immune responses (Ricklin *et al.*, 2016). As an important and highly conserved component of the innate immune system, the complement system plays a significant role in the process of host defense against infections. Planarians appear to have some conservative complement system associated domains that may resist invading pathogens, but their exact functions need to be confirmed and further studied.

Only a few homologous domains of complement proteins and factors have been discovered in the *S. mediterranea* genome database - for example CUB domain, C1q domain and von Willebrand factor type A domain (Peiris *et al.*, 2014). These domains are associated with more than one protein family, so, without knowing the full length of a gene, we could not assign it to a complement protein. Similarly, in the Dj-BS transcriptome database and many other databases, we did not find any annotated complement protein either. Although planarian C3 is absent, we cloned the full length of A2M which possesses almost exactly the same structural domain as C3 from *D. japonica*. A2M is an evolutionary conservative element of the innate immune system which is also considered as a broad-spectrum protease inhibitor (Armstrong and Quigley, 1999). In recent years, a large number of A2M in invertebrates have been identified, which are involved in a variety of immune responses in the case of pathogen invasion (Pathirana *et al.*, 2016; Ponprateep *et al.*, 2017). Properdin, also known as P factor, has also been discovered in planarians. As a serum glycoprotein, properdin can initiate and positively regulate the alternative pathway activity (Blatt *et al.*, 2016). However, planarians lack the necessary complement system proteins, so we hypothesize that properdin may engage in immune responses by other mechanisms. In amphioxus, recombinant BjfP (factor P of *Branchiostoma japonicum*) is capable of interacting with both Gram-negative and Gram-positive bacteria as well as LPS and lipoteichoic acid (Gao *et al.*, 2017b).

Antioxidant enzymes

Organisms have evolved a variety of mechanisms to protect themselves from the toxic effects of heavy metals, harmful chemicals and microorganisms that cause oxidative stress. Reactive oxygen species (ROS) is derived from one-electron reduction of oxygen molecule mainly in mitochondria (Bernard *et al.*, 2015). ROS production can be associated with phagocytosis and lead to killing of phagocytes, but abnormal ROS levels can lead to damage to the animal body (Tan *et al.*, 2016). In order to minimize oxidative damage, organisms have developed antioxidant defenses composed of antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), thioredoxin peroxidase (TPx) and other peroxiredoxin (Bernard *et al.*, 2015). An induction of the antioxidant defenses can be considered as an adaptation of the organisms to overcome insecure environments and to prevent toxicity (Zhang *et al.*, 2014). Coincidentally, these antioxidant enzymes are also present in planarians.

Bocchinfuso *et al.* (2012) identified several antioxidant enzymes, including peroxiredoxins, CAT and SOD, in the mucus secreted by *S. mediterranea*. In addition, studies have shown that copper (Zhang *et al.*, 2014) and 1-octyl-3methylimidazolium bromide ($[C_8mim]Br$) (Zhang *et al.*, 2016) can induce oxidative stress in planarians, which, in turn, activates the corresponding antioxidant enzymes. In these studies, the activity changes of the enzymes also indicate that they participate in the corresponding immune responses in facing the external environment stresses. How these antioxidant enzymes are activated, when animals land in harsh environments, remains to be ascertained.

Concluding remarks

Planarian is traditionally a favored animal model in regeneration and development, while recently, its immune system has also attracted wide attention from biologists. The complex living environment, containing various pathogens, and high survival rates of planarians suggest that they have an effective innate immune system to prevent infections. Surface barriers and mucous membranes from epidermis, pharynx and intestine of planarian effectively prevent pathogens from entering the internal body by constitutive defense mechanisms. In addition, some of the pathogens that evade the constitutive defense system and enter the planarian body are eliminated by inducing defense mechanisms, in which, PRRs, *e.g.*, RLRs, CTL, GPCR and PGRP1, recognize the PAMPs on the surface of those non-self foreign pathogens, leading to a high expression of related proteins and a series of immune responses.

In conclusion, the reports on the planarian immune system indicate that more studies need to be carried out to expound the strong survivability of planarians. Moreover, although numerous transcriptome and proteome studies on planarians have been reported, a large number of sequences are not spliced or annotated. Hence, one important step in the study of the planarian immune system is to acquire the complete genome information. Finally, it is worth mentioning that cell culture techniques are yet to be established to explore the elaborate molecular mechanisms in specific cell lines as well as to ascertain how many immune cell types are present in planarians.

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