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The functional relevance of shrimp C-type lectins in host-pathogen interactions

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ABSTRACT

C-type lectins (CTLs) are key recognition proteins in shrimp immunity. A few years ago we reviewed sequence information, ligand specificity, expression profiles and specific functions of the shrimp CTLs. Since then, multiple integrated studies that implemented biochemical approaches using both the native and recombinant proteins, functional genetic approaches using RNA interference, and mechanistic studies by analyzing protein-protein interactions were carried out. Results from these rigorous studies revealed the functions and mechanisms of action of selected members of the shrimp CTL family. This review focuses on this new knowledge, that includes unique structural aspects, functions, and mechanisms in host-pathogen interactions, the functional relevance of regions other than the C-type lectin domain, and the regulation of transcription of shrimp CTLs. Thus, this review aims to provide a detailed update of recent studies that have contributed to our better understanding of the shrimp immune events that involve CTL functions.

1. Introduction

C-type lectins (CTLs), initially described based on their requirement of calcium for binding to their carbohydrate ligands, were identified as a *bona fide* lectin family by the identification of a unique amino acid sequence motif in the C-type carbohydrate recognition domain (CRD) (Drickamer, 1988). This sequence motif is present, at least in part, even in those members of this family that have lost their carbohydrate recognition ability (Ewart and Fletcher, 1993). The CTL family is characterized by its structural diversity resulting from the CTL CRD forming part of "chimeric" or "mosaic" proteins in which the CTL domain (CTLD) is joined to one or more structurally and functionally diverse domains (Zelensky and Gready, 2005). The CTLD usually contains 120–150 amino acid residues, and adopts an overall loop-in-loop structure which is stabilized by two conserved disulfide bonds. Lying in the overall loop structure, the "long loop" region in involved in ligand binding (Zelensky and Gready, 2005). The flexibility of this long loop region constitutes the basis for the diversity in ligand recognition. By recognizing multiple ligands, including carbohydrates, lipids, proteins, and even inorganic matters, CTLs participate in diverse physiological processes, including immunity, development, reproduction, metabolism, and others (Zelensky and Gready, 2005, 2009).

CTLs play important roles in mammalian innate immunity. The mannose binding lectin (MBL) is mostly expressed in liver and released to plasma, where it not only recognizes potentially pathogenic microbes, but upon binding to the microbial surface can also activate the complement pathway (Thiel and Gadjeva, 2009). Therefore, MBL, considered as a prototypical soluble pattern recognition receptor (PRR), is endowed not only with anti-microbial recognition, but also effector properties. Dectin-1, a type II transmembrane mammalian protein, recognizes glucans on the surface of fungus, induces NF-κB activation, and expression and release of proinflammatory cytokines (Drummond and Brown, 2011). Similarly, CTLs are also key immune recognition receptors in invertebrates. In fact, the prevalence of the CTLD in invertebrates is much higher than in vertebrates. For example, the CTLD is the 7th most abundant domain in *C. elegans* genome, but only the 43rd most abundant domain in the human genome (Zelensky and Gready, 2005). The evolutionary expansion of CTLD in invertebrates suggests its deep involvement in innate immunity. Since invertebrates lack the typical adaptive immunity of mammals, and mostly rely on robust innate immunity to resist pathogen invasion, it may be hypothesized that the CTLD is more critical in invertebrates than in

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vertebrates, in which an adaptive immune system, tightly integrated with innate immune recognition, ensures a pathogen-specific and long-lasting protection. For example, a total of 39 CTLs are expressed in the *Aedes aegypti* mosquito genome, with some members acting as essential factors for virus infection (Waterhouse et al., 2007). A total of 34 CTLs are expressed in the *Manduca sexta* genome, among which the "immulectins" function as recognition proteins in antibacterial immunity (Rao et al., 2015; Yu et al., 1999, 2006).

Given the economical relevance of shrimp aquaculture worldwide and the catastrophic losses frequently caused by bacterial and viral diseases, research on shrimp immunity has been heavily promoted in producer/exporter countries, and has achieved significant progress in the last thirty years. A large number of CTLs have been identified in multiple species of shrimp, particularly on species of economic value, with several CTLs involved in shrimp immunity. In a previous review, we addressed the diversity of the shrimp CTL repertoire, with a focus on sequence information, ligand specificity, expression profiles, and specific functions of selected family members (Wang and Wang, 2013). Since then, substantial progress has been achieved on the identification and characterization of shrimp CTLs. Integrated multidisciplinary studies implementing biochemical approaches using the native or recombinant proteins, functional genetic approaches using RNA interference, and mechanistic studies by analyzing protein-protein interactions were performed. These rigorous studies revealed novel aspects concerning the function(s) and mechanism(s) of action of CTLs in shrimp innate immunity. In the present review, we focus on the new knowledge about both the structural and functional aspects of selected CTLs in shrimp immunity acquired in recent years. Specifically, we describe their shared and unique structural features, discuss their function(s) and mechanism(s) involved in host-pathogen interactions, examine the functional relevance of region(s) other than the C-type lectin domain, and the regulation of transcription of shrimp CTLs. Therefore, the goal of this review is to provide updated information on selected shrimp CTLs, and specifically focus on their *in vivo* participation in shrimp immunity.

2. Shared and unique features of selected shrimp CTLs

In recent years, multiple studies were performed to obtain sequence information, and characterize expression profiles and functional significance of CTLs in shrimp immunity. The detailed structural and functional characterization of shrimp CTLs is summarized in Table 1. Besides of these studies, we have made significant progress in our recent research on the CTL family of the kuruma shrimp *Marsupenseus japonicus*. In this regard, decoding the genome of the Pacific white shrimp *Litopenaeus vannamei* provided valuable information to fully appreciate the magnitude of the shrimp CTL repertoire (Zhang et al., 2019b). Based on the published results from our lab and others, and our unpublished data, three main features of the shrimp CTL family can be highlighted.

Firstly, CTL family shows significant expansion in shrimp. Analysis of the transcriptome data of *M. japonicus* has identified more than 95 proteins containing CTLDs. This number is substantially larger than the CTLDs identified in insects (Waterhouse et al., 2007). This expansion of CTLD-containing proteins may confer host increased ability to discriminate potential infectious challenges. The shrimp homologs of Toll like receptors (TLRs), the prototypical mammalian PRRs, do not participate in immune recognition. Furthermore, the shrimp genome does not encode for peptidoglycan recognition receptors (PGRP), which are important immune recognition proteins in *Drosophila* (Kuruta, 2014; Satake and Sekiguchi, 2012). Therefore, it could be surmised that the expansion of CTL family may, at least to a certain degree, compensate for the functional deficiency or lack of other pattern recognition proteins.

Secondly, the most majority of shrimp CTLs are soluble molecules. Almost all CTLs from *M. japonicus* contain a signal sequence. Some CTLs are readily detectable and can be purified from shrimp plasma (Luo et al., 2006), and several of them are induced after stimulation by bacteria or virus challenge (Wang et al., 2014a, 2014b, 2017). Since the shrimp vascular system is open, the soluble CTLs in the circulating plasma can effectively interact with and defend the host during the very first steps of pathogen entry. However, the level of each CTL in shrimp plasma varies with a wide range, and it is possible that this may be related to the CTL specific function. For example, MjsvCL and Leulectin can be detected only after enrichment of the protein from plasma, while MjHeCL is readily detectable without additional enrichment. The high expression level of MjHeCL enables it to tightly control homeostasis of the hemolymph microbiota in healthy shrimp, while the induction of MjsvCL or Leulectin after pathogen challenge enables the host to resist invasion of exogenous pathogens.

Thirdly, many shrimp CTLs display a tandem arrangement of one or more CTLDs and other distinct domains or modules. These additional domains are essential for the function of CTLs (Sun et al., 2017; Wang et al., 2014b, 2014c, 2017). The high prevalence of these additional regions in shrimp CTLs may be related to the fact that most CTLs are soluble plasma proteins. By interacting with other soluble molecules or cell surface receptors, these domains can cross-link the plasma CTL and enable both pathogen recognition and the downstream immune effector function(s). This aspect will be specifically discussed in the latter in this review.

3. Diverse roles of CTLs in shrimp-pathogen interactions

The shrimp CTLD's ability to bind multiple ligands, and the inducible expression profiles of the CTLs upon immune challenge strongly suggest their involvement of in host-pathogen interactions. Recent studies have revealed multiple functions and mechanisms of shrimp CTLs in antibacterial and antiviral immunity. Generally, these defense functions of shrimp CTLs are based on the recognition and binding capacity of their CTLDs (Sun et al., 2017; Wang et al., 2014b, 2014c, 2017).

3.1. Host-virus interactions

During the last three decades, WSSV has become the most serious viral pathogen in shrimp aquaculture. Several studies have shown that shrimp CTLs play important roles in the antiviral immunity (Fig. 1). Several CTLs can neutralize WSSV by binding the viral envelope proteins. For example, recombinant LvCTL1 from *L. vannamei* directly binds to several structural proteins of WSSV, including VP28, VP26, VP24, VP19, and VP14. These binding properties confer LvCTL1 the ability to suppress the cytopathic effects caused by WSSV in shrimp hemocyte primary cultures, and to reduce host mortality after WSSV infection (Zhao et al., 2009). Recent studies showed that several newly identified shrimp CTL possess the capacity to bind WSSV proteins. Both CTLs containing the low-density lipoprotein receptor class A domain (LdlrA) from *M. japonicus* can interact with WSSV VP28, but not VP24 and VP26 (Xu et al., 2014). A CTL with similar domain organization from *Fenneropenaeus merguiensis* can bind VP28, VP15 and VP39A (Kwankaew et al., 2018). All three proteins can inhibit WSSV gene expression or reduce host mortality caused by WSSV infection. Another *F. merguiensis* CTL named FmLC6 can directly bind WSSV virions and the virus proteins including VP15, VP39A and VP28 (Runsaeng et al., 2018).

In addition to the antiviral activity, some CTLs are hijacked or employed by virus for viral pathogenesis. In man, the dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) functions as a receptor for HIV-1 transmission by capturing virus gp120 (Geijtenbeek et al., 2000). It also functions as cofactor for cellular entry of human cytomegalovirus and Ebola virus by capturing viral envelope glycoproteins (Alvarez et al., 2002; Halary et al., 2002). By orally delivering WSSV inoculum into the shrimp digestive tract to

Table 1
Recent identification and characterization of shrimp CTLs.

Species	Name	mRNA distribution	Protein distribution	Stimulants	Domain architecture	Specific function	Reference
M. japonicus	hFcLec4	gills, stomach	gills, stomach		SP, N-terminal region, CTLD	Interact with β-integrin to promote phagocytosis	Wang et al. (2014c)
	MjHeCL	hemocytes	plasma		SP, CTLD	Modulate AMPs expression and maintains hemolymph microbiota homeostasis	Wang et al. (2014a)
	MjCC-CL	widely distributed	widely distributed	*V. anguillarum*	SP, CCD, CTLD	Interact with Dome and activate JAK/STAT pathway	Sun et al. (2017)
	Leulectin	hepatopancreas	plasma	*Vibrio spp.*	SP, LRRs, CTLD	Prevent bacteria colonization and promote phagocytosis	Wang et al. (2017)
	MjsvCL	stomach, muscle	stomach, muscle, plasma	WSSV	SP, collagen-like, CTLD	Bind VP28 and calreticulin, facilitate WSSV infection	Wang et al. (2014b)
	LdlrLec1	widely distributed		WSSV	SP, LdlrA, CTLD	Bind VP28, inhibit WSSV infection	Xu et al. (2014)
	LdlrLec2	widely distributed		WSSV	SP, LdlrA, CTLD	Bind VP28, inhibit WSSV infection	Xu et al. (2014)
	MjGCTL	gills, stomach			SP, LdlrA, CTLD	Facilitate encapsulation	Alenton et al. (2017)
L. vannamei	LvCTLD	gills, nerve		WSSV	SP, LdlrA, CTLD	Facilitate encapsulation, activate PO, bind YHV	Junkunlo et al. (2012)
	LvCTL3	widely distributed		LPS, polyI:C, *V. parahaemolyticus* WSSV	SP, CTLD	Protect host from *V. parahaemolyticus* infection	Li et al. (2014)
	LvCTL4	widely distributed		*V. parahaemolyticus*	SP, CTLD		Li et al. (2015)
	LvAV	hepatopancreas		*V. harveyi*, WSSV	SP, CTLD	inhibit WSSV infection at early stage, promote WSSV infection at late stage	He et al. (2015)
	LvLdlrCTL	hemoctes		*Vibrio spp*, WSSV, LPS, poly I:C	SP, LdlrA, CTLD	Inhibit bacterial infection, facilitate WSSV infection, promote phagocytosis	Liang et al. (2019)
	LvLec					stimulate the exocytosis, activate PO. promote phagocytosis	Zhang et al. (2019a)
P. monodon	PmCL1	hepatopancreas		*Vibrio*, ammonia nitrogen stress	SP, CTLD	promote bacterial clearance	Qin et al. (2019)
	PmCLec	widely distributed			SP, CTLD	inhibit *V. harveyi* infection	Wongpanya et al. (2017)
F. chinensis	FcCTL	hepatopancreas		WSSV	SP, CTLD	inhibit bacteria growth directly	Lai et al. (2013)
F. merguiensis	FmLC1	hepatopancreas		*V. harveyi*, WSSV	SP, CTLD		Thepnarong et al. (2015)
	FmLC2	stomach, gills		*V. harveyi*, WSSV	SP, CTLD	inhibit *V. harveyi* and WSSV infection	Runsaeng et al. (2015)
	FmLC3	hepatopancreas		*V. harveyi*, WSSV	SP, CTLD	inhibit bacteria growth directly, inhibit *V. harveyi* and WSSV infection	Runsaeng et al. (2017)
	FmLC4	hepatopancreas		*V. harveyi*, WSSV	SP, CTLD	promote bacterial clearance	Utarabhand et al. (2017)
	FmLC5	hemocytes		*V. harveyi*, WSSV	SP, CTLD, CTLD	inhibit *V. harveyi* and WSSV infection	Senghoi et al. (2017)
	FmLC6	hepatopancreas			SP, CTLD, CTLD	inhibit bacteria growth directly, inhibit *V. harveyi* and WSSV infection	Runsaeng et al. (2018)
	FLdlr	hemocytes		*V. parahaemolyticus, WSSV*	SP, LdlrA, CTLD	bind VP28, 15, 39A and inhibit WSSV infection, inhibit bacteria growth directly, promote phagocytosis	Kwankaew et al. (2018)

CTLD, C-type lectin domain; LrlrA: low density lipoprotein receptor domain A; LPS, lipopolysaccharides; PO, phenoloxidase,; SP, signal peptide; WSSV, white spot syndrome virus; YHV, yellow head virus; VP, Viral protein.

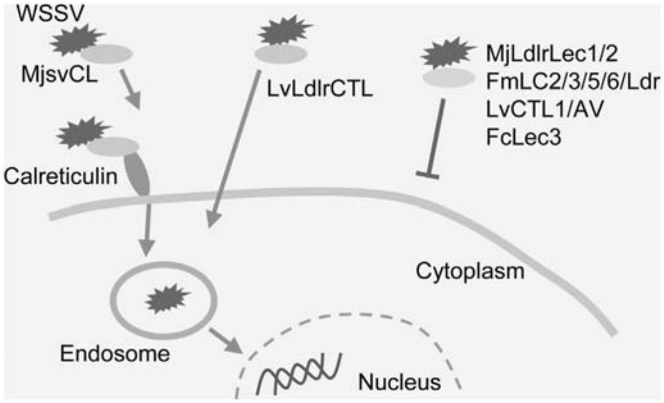

Fig. 1. The function and mechanism of shrimp CTLs in host-WSSV interactions. Shrimp CTLs, such as MjLdlrLec1 and MjLdlrLec2 from *M. japonicus*, FmLC2, FmLC3, FmLc5, FmLC6, and FLdr from *F. merguiensis*, LvCTL1and LvAV from *L. vannamei*, and FcLec3 from *F. chinensis* can bind to the WSSV structural proteins and inhibit virus entry. MjsvCL from *M. japonicus* binds WSSV VP28 through the CTLD, and interacts with cell surface calreticulin through the Q/N rich region. By serving as a bridge factor between virions and a cell surface receptor, MjsvCL facilitate virus entry through endocytosis. LvLdrCTL from *L. vannamei* also facilitates WSSV infection. The green arrow indicates "promoting" while red lines indicates "preventing". (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

model the natural infection, a CTL from *M. japonicus* was found to be critical for WSSV entry into the stomach, the primary target tissue of WSSV. MjsvCL expression was significantly induced by WSSV infection. Importantly, this protein is soluble in shrimp hemolymph, and the titer in hemolymph increases with the WSSV infection. By introducing the exogenous double strand RNA or recombinant protein into shrimp to either silence or mimic overexpression, respectively, MjsvCL was identified as a necessary factor for WSSV infection. This observation suggests a possible WSSV strategy of hijacking a host CTL for viral entry into the shrimp. MjsvCL functions as a direct recognition protein and binds WSSV envelope protein VP28 through its lectin domain. Subsequently, through its collagen like region interacts with calreticulin, which functions as a cell surface receptor. The formation of VP28-MjsvCL-calreticlun complex suggests that MjsvCL establishes as a bridge between virions and the host cell by cross-linking the virions to the cell surface receptor (Wang et al., 2014b). The hijacking host CTLs by multiple viruses for cell entry in both vertebrates and invertebrates suggests a conserved or prevalent strategy of viruses for adhesion and entry into the host cell. In contrast, silencing the expression of LvAV, a CTL from *L. vannamei*, leads to increased WSSV load in shrimp tissues at the initial stage of viral infection, but decreased viral load in the later stages. Therefore, it is possible that LvAV is also employed by WSSV for its infection but perhaps by a different mechanism (He et al., 2015). These apparently opposite functions of CTLs during WSSV infection reflect the functional diversity and complexity of shrimp CTLs (Table 1).

Yellow head virus (YHV) is the cause of yellow head disease, which is a serious viral epizootic of penaeid shrimp. YHV is an enveloped virus containing three major structural proteins. Two proteins, named gp116 and gp64, are transmembrane envelope glycoproteins. Another protein named p20 is the major nucleocapsid protein. Among the three proteins, gp16 is mostly related to the attachment to cell surface receptors (Jitrapakdee et al., 2003). A CTL named LvCTLD from *L. vannamei* can bind YHV particles, suggesting that it is involved in YHV pathogenesis or host defense against YHV. The binding of LvCTLD to YHV virions occurred only in the presence of shrimp hemolymph, suggesting that some additional components in hemolymph may function as a co-factor (s) for LvCTLD-YHV interaction (Junkunlo et al., 2012).

3.2. Antibacterial activity

Vibriosis is a long-term, serious disease that threatens shrimp aquaculture. Multiple *Vibrio* species have been shown as shrimp pathogens, including *V. harveyi*, *V. anguillarum*, *V. alginolyticus*, *V. parahaemolyticus*, and others (Thompson et al., 2004). In recent years, the "early mortality syndrome (EMS)/acute hepatopancreatic necrosis" disease (AHPND) is a newly emergent disease that causes significant losses to the shrimp aquaculture industry. It firstly appeared in China in 2009 and spread through all major shrimp culture regions in Southeast Asia, including Vietnam, Malaysia and Thailand, and as far as Mexico (Nunan et al., 2014). The global loss to the industry is estimated at no less than 1 billion US dollars per year (De Schryver et al., 2014). The characteristic symptom of AHPND includes atrophied hepatopancreas, empty stomach and midgut, and sloughing of the hepatopancreas tubule epithelial cells. The causative pathogen of AHPND is a specific strain of *V. parahaemolyticus*. This strain becomes virulent upon acquisition of a plasmid expressing deadly toxins (Lee et al., 2015; Tran et al., 2013). Multiple shrimp CTLs have been proven to play important roles in antibacterial immunity, by agglutinating, killing, or promoting bacterial clearance in shrimp hemolymph (Lai et al., 2013; Li et al., 2014; Qin et al., 2019; Runsaeng et al., 2018; Wang and Wang, 2013; Wongpanya et al., 2017).

FcLec4 is a CTL widely distributed in multiple tissues in Chinese white shrimp *F. chinensis*. It can bind bacterial peptidoglycan, and promote the clearance of exogenous bacteria experimentally injected into the shrimp hemocel (Wang et al., 2009b). Bacteria coated by FcLec4 are more rapidly phagocytosed by shrimp hemocytes, suggesting that this CTL functions as an opsonin. By panning a T7 phage display library, a β-integrin subunit was identified as the receptor of FcLec4. The CTL-integrin interaction was dependent on the region next to the CTLD. By interacting with integrin, FcLec4 induces the rearrangement of the hemocyte cytoskeleton and promotes bacterial phagocytosis. Moreover, a homolog of FcLec4 was identified in *M. japonicus*, suggesting that the CTL-integrin-mediated phagocytosis is a general defense strategy among different shrimp species (Wang et al., 2014c). The newly identified shrimp CTLs, LvLdlrCTL and FLdlr, can also promote microbial phagocytosis (Kwankaew et al., 2018; Liang et al., 2019). In addition, treatment of shrimp hemocytes with recombinant LvLec confers higher ability to phagocytose infectious agents (Zhang et al., 2019a). Thus, the opsonic activity of shrimp CTLs is key for the rapid and effective clearance of the invading bacteria.

In addition to promoting phagocytosis of invading microorganisms, a Leulectin, a CTL from *M. japonicus* protects the shrimp host from potentially pathogenic bacteria by inhibiting the flagellin-mediated bacterial attachment to shrimp cells and tissues. Leulectin is named due to its unique domain architecture with several leucine rich repeats (LRR) and a CTLD. The LRR and CTLD modules show distinct roles in the host defense against bacterial infection. The Leulectin LRRs can recognize flagellins A-E of *V. anguillarum*, and flagellins A of *V. alginolyticus* and *Aeromonas hydrophila*. By sensing the flagellin, LRRs inhibit the flagellin-mediated bacterial attachment to shrimp hemocytes and hepatopancreas tubes, and suppress the *in vivo* colonization of several *Vibrio* species. The Leulectin CTLD is responsible for the recognition of LPS exposed on the surface of Gram-negative bacteria, and binds to bacteria by interacting with the carbohydrate moieties of LPS. Once the bacteria are opsonized by the CTLD module, they would be more easily phagocytosed by shrimp hemocytes. Mutation of the ligand binding region in both LRR and CTLD modules abolished the CTL's protecting role, suggesting that the protecting ability of Leulectin is based on its ability to recognize potential pathogens (Wang et al., 2017).

Shrimp CTLs are also important regulators of the expression of downstream immune effectors, by recognizing bacterial pathogens and activating specific immune signaling pathways. MjCC-CL, a CTL from *M. japonicus*, contains a coiled-coil region with structural similarity to

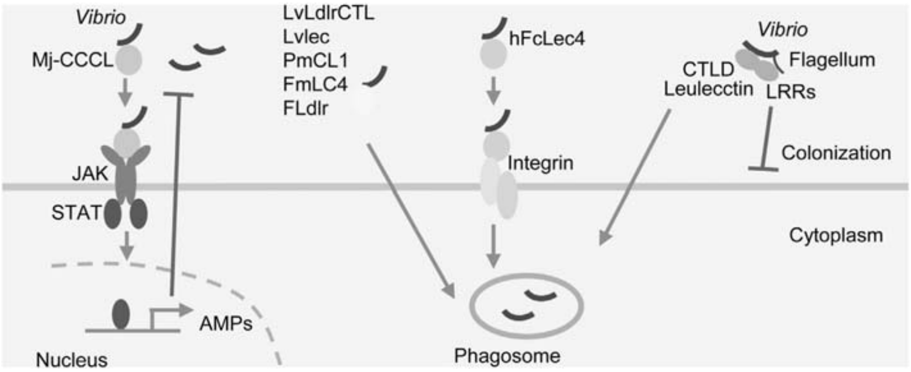

Fig. 2. The function and mechanism of shrimp CTLs in antibacterial immunity. Mj-CCCL from *M. japonicus* senses a *Vibrio* infection by recognizing the bacterial glycans through the CTLD, and initiates JAK/STAT signaling by interacting with the Domeless through the coiled-coil region. The AMPs induced by JAK/STAT signaling would target the invading bacteria. hFcLec4 from *M. japonicus* binds bacteria through its CTLD, and interacts with β-integrin through its N-terminal region. The interaction between hFcLec4 and β-integrin leads to the cytoskeleton rearrangement and hemocytic phagocytosis. Leulectin from *M. japonicus* senses a *Vibrio* infection by recognizing the glycans through the CTLD and recognizing flagellin through the LRRs region. Recognition of glycans by CTLD promotes hemocytic phagocytosis, while recognition of flagellin by LRRs inhibits the attachment and colonization of bacteria. Some other CTLs, including LvLrlrCTL and LvLec from *L. vannamei*, PmCL1 from *P. monodon*, FmLC4 and FLdlr from *F. merguiensis*, also function in antibacterial immunity possibly by promoting hemocytic phagocytosis. Green arrow Indicates “promoting” while red lines indicates “preventing”. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the mammalian interleukin 10, and a CTLD that recognizes the bacterial surface carbohydrates. Upon this initial CTLD-bacteria interaction, the coiled coil region, interacts with Domeless, a cell surface receptor of JAK/STAT signaling. Thus, after recognition of bacterial pathogens by the CTLD, MjCC-CL would activate the JAK/STAT pathway and induce the expression of several antimicrobial peptides (AMPs). By regulating the expression of the AMPs, MjCC-CL protects the shrimp host from bacterial infection (Sun et al., 2017). In this regard, it is noteworthy that silencing of LdlrLec1 and LdlrLec1 leads to the decreased expression of crustin which is an important AMP in shrimp (Xu et al., 2014) (Fig. 2).

In contratst with the indirect antibacterial mechanism of MjCC-CL, other shrimp CTLs exert direct antimicrobial activity. For example, Fc-hsL, a hepatopancreas-specific CTL from *F. chinensis* can inhibit the growth of multiple bacteria and two fungal species (Sun et al., 2008). Additionally, FmLC6 and FmLdl, two CTLs from *F. merguiensis*, and FcCTL, a CTL from *F. chinensis*, strongly inhibit the growth of both Gram-positive bacteria and Gram-negative bacteria (Kwankaew et al., 2018; Lai et al., 2013; Runsaeng et al., 2018). Further, FmLC3, a mannose-specific CTL from *F. merguiensis* exhibits direct antimicrobial activity against several *Vibrio* species and some Gram-positive bacteria. Knockdown of FmLC3 expression leads to increased mortality by *V. harveyi*, suggesting that it can successfully protect shrimp from this pathogen. In addition, the protective role of FmLC3 appears to be also effective against WSSV, as silencing FmLC3 expression leads to a lower survival rate upon WSSV experimental infection (Runsaeng et al., 2017). The observation that a single CTL plays dual functions against both bacterial pathogens and viral pathogens have been found for several shrimp CTLs, underscores the significance of CTLs in shrimp immunity (Wang et al., 2009a). Furthermore, the direct antibacterial activity of these CTLs raises possibility for application of recombinant CTLs as therapeutic agents for disease control in aquaculture settings.

3.3. Regulation of microbiota homeostasis

The commensal microbiota regulates multiple physiological processes of the host, including nutrition, metabolism, development, immunity, and others (Backhed et al., 2005). The bulk of the microbiota primarily resides in the gut, and multiple immune mechanisms regulate the homeostasis of the microbial populations and suppress unwanted proliferation of any particular component (Buchon et al., 2013). CTLs are active participants in these regulatory mechanisms. In the mouse, for example, a secreted CTL (RegIIIγ) shows significant antibacterial activity *via* interaction with peptidoglycan (Cash et al., 2006). Its human equivalent, RegIIIα can bind phospholipids of the bacterial membrane and induce the formation of membrane-permeabilizing pores (Mukherjee et al., 2014). In addition to direct antimicrobial activity, RegIIIγ is essential in the regulation of the spatial relationship between the microbiota and host intestinal epithelium by maintaining a 50 µm zone of physical separation (Vaishnava et al., 2011). RegIIIγ can also inhibit the colonization of bacteria on mucosal surfaces, and suppress the translocation of bacteria from the gut to the mesenteric lymph nodes and liver (Wang et al., 2016). In contrast, the commensal microbiota can utilize host CTLs to evade the host immunity, and to maintain the host-bacterial mutualism. For example, the coating of mosquito gut microbiota by CTLs impairs the deposition of antimicrobial peptides onto the surface of bacterial cell, and thus contributes to homeostasis of the microbiome in the mosquito gut (Pang et al., 2016). There is no direct evidence that a shrimp CTL functions in gut microbiota homeostasis. In *L. vannamei*, however, changes of gut microbiota composition are accompanied by changes in expression levels of the C-type lectin 3, suggesting a possible involvement of this CTL in regulating the gut microbiota (Suo et al., 2017).

In addition to the gut commensal microbiota, a unique microbiota is present in the hemolymph of healthy aquatic invertebrates (Wang and Wang, 2015; Zhang et al., 2018b). This microbiota possibly originates from translocation of gut microbiota into hemolymph, or by acquision of external bacteria during molting. The opportunistic pathogenic bacteria of this microbiota must have evolved effective strategies to adapt the hostile hemolymph environment which contains a diverse array of anti-microbial factors. Similarly, the host must have also co-evolved strategies to suppress any uncontrolled proliferation of hemolymph microbiota. The shrimp MjHeCL, a CTL from *M. japonicus* is critical in the homeostasis of hemolymph microbiota. MjHeCL is specifically expressed in shrimp hemocytes and released into circulating plasma. Although expression MjHeCL is not modulated by external bacterial challenge, experimentally silencing its expression leads to uncontrolled proliferation of bacteria in hemolymph, followed by death of the shrimp. MjHeCL exhibits a strong and broad-spectrum recognizing activity towards components of the hemolymph microbiota. More importantly, MjHeCL can modulate the expression of several AMPs which are highly expressed in healthy shrimp, including penaeidin, anti-lipopolysaccharides factor, and lysozyme. By modulating the expression of these immune factors, MjHeCL inhibits excessive proliferation of any bacterial population and successfully maintains homeostasis of the hemolymph microbiota (Wang et al., 2014a) (Fig. 3).

4. Significance of CTLD-associated domains in shrimp CTLs

Like CTLs from most vertebrate and invertebrate species, shrimp CTLs show considerable sequence diversity, in most part due to the

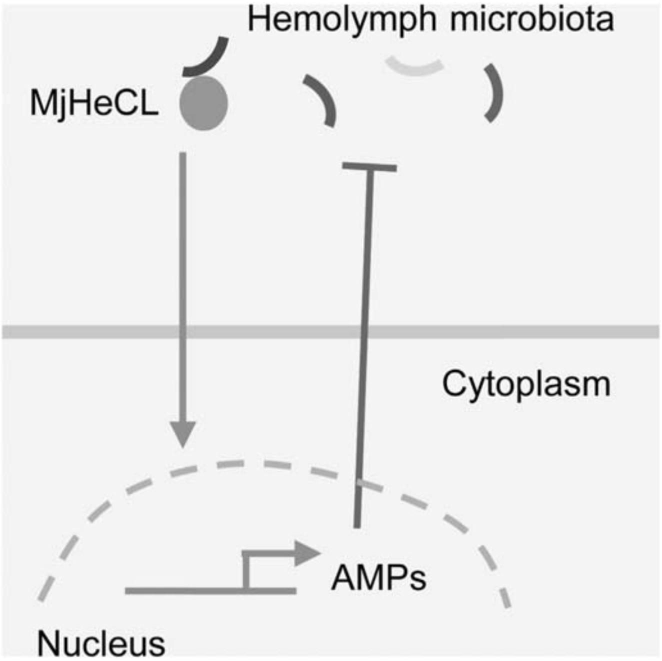

Fig. 3. The significance of MjHeCL in the homeostasis of hemolymph microbiota. MjHeCL from *M. japonicus* possesses broad spectrum recognition capacity to recognize the components in hemolymph microbiota. By targeting a receptor on the hemocyte surface, it modulates expression of important AMPs. These AMPs are critical to the suppression of proliferation of hemolymph bacteria, and thus are key to maintain homeostasis of the hemolymph microbiota.

chimeric nature of these proteins, in which the CTLD, is associated with additional structurally and functionally distinct domains. Some of the additional domains are immune recognition modules, while others can interact with diverse immune components or adaptors. In recent years, the relevance of some of these additional domains for CTLs' function has been elucidated. Thus both CTLDs and the associated domains contribute to the functional diversity and effectiveness of CTLs in shrimp immunity.

4.1. Additional domains expand the recognition spectrum of shrimp CTLs

Among the multiple CTLs described in the shrimp *M. japonicus*, Leulectin is unique in that in addition to the CTLD, it harbors a series of LRR modules (Wang et al., 2017). The LRR motif is found in many proteins, and mediates protein-protein interactions (Kobe and Kajava, 2001). It usually contains less than 30 amino acid residues, with a majority of leucine residues, and forms a structure of β strand–α helix (Kobe and Kajava, 2001). Multiple tandemly arrayed LRR modules fold into a solenoid curve structure, which can accommodate the target ligands (Kobe and Kajava, 2001). The proteins containing LRR modules are important in development, reproduction, immunity, and other functions (Bella et al., 2008). Some typical recognition receptors, including mammalian Toll like receptors and NOD-like receptors, contain LRR as the basic recognition module (Kang and Lee, 2011; Ting et al., 2008). By arranging multiple LRRs, jawless vertebrates variable lymphocyte receptors acquire significant diversity in ligand recognition (Pancer et al., 2005). As an immune recognition module, LRRs can sense several kinds of surface ligands from pathogens.

The CTLD of Leulectin can bind LPS and attach to the surface of bacteria. It functions in antibacterial responses by promoting phagocytosis of bacteria by the hemocyte. By a systematic screening of molecules that Leulectin may encounter on the surface of a pathogen, it was discovered that the LRR modules recognize *Vibrio* flagellins, the first bacterial ligand identified for invertebrate LRRs. Flagellin is the primary component of the flagellum, a structure that is necessary for both motility and pathogenesis of flagellated bacteria by facilitating bacteria spreading and attachment (O'Toole and Kolter, 1998; Shapiro, 1995). Leulectin LRRs inhibited the colonization of several *Vibrio* bacteria in shrimp hepatopancreas, and the mutant losing the flagellin binding ability also loses the colonization inhibitory activity (Wang et al., 2017). Further, deletion of Leulectin LRR modules abolished the inhibitory effect on bacterial attachment (Wang et al., 2017). Thus, recognition of flagellins by the Leulectin LRR domains may be an effective mechanism for binding to pathogenic bacteria such as *Vibrio* species, and inhibit their attachment to shrimp hemocytes and hepatopancreas cell surfaces.

From the evidence discussed above, it can be concluded that the tandem arrangement of two typical immune recognizing modules, CTLD and LRR, expands the recognizing spectrum and function of Leulectin. The two modules show coordination in the antibacterial immunity, by inhibiting the bacteria attachment and colonization and promoting phagocytosis through recognizing flagellin and LPS, respectively. The synergy between two recognition modules enhances the recognizing ability and protective role of Leulectin.

4.2. Additional domains are essential to the functional relevance of shrimp CTLs

As discussed above, CTLs can harbor additional domains which carry out effector functions. For example, the mannose binding lectin (MBL) displays a collagenous domain that can interact with the MBL-associated serine protease (Masp) to activate the complement cascade (Thiel and Gadjeva, 2009). In addition to recognizing the carbohydrate ligands on the surface of bacteria or virus through the CTLD, these chimeric CTLs activate downstream immune responses by interacting with immune adaptors through these additional regions or domains.

In the shrimp *M. japonicus*, in addition to the canonical CTLD which can recognize bacterial glycans, the CTL MjCC-CL houses a coiled-coil domain (CCD) that interacts with the Domeless receptor on the surface of hemocytes, and directly activates the JAK/STAT signaling. It is noteworthy that the CCD displays an α-helix rich structure with overall similarity to mammalian interleukin 10, a cytokine which activates JAK/STAT signaling in vertebrates (Riley et al., 1999). This finding revealed the significance of the additional region for the full CTL (Sun et al., 2017).

As discussed above, MjsvCL is important for WSSV entry by acting as a bridge that links the virions to cell surface receptor calreticulin. The binding of MjsvCL to WSSV is accomplished by the CTLD, while a region rich in glutamine and asparagine residues that is located between the signal peptide and the CTLD, is responsible for the interaction with calreticulin. The recruitment of virions by the CTLD, together with the presentation of the virions to the cell surface by glutamine/asparagine-rich region, make MjsvCL a key factor for virus entry (Wang et al., 2014b).

Several CTLs can promote the clearance of exogenous bacteria experimentally injected into the shrimp hemolymph. The clearance is generally through phagocytosis by hemocytes (Kwankaew et al., 2018; Liang et al., 2019; Qin et al., 2019; Utarabhand et al., 2017; Zhang et al., 2019a). However, how these CTLs induce phagocytosis is not fully understood. By screening the possible interacting targets, (h)FcLec4 was found to interact with β-integrin, which is an important membrane receptor. The interaction is dependent on the N-terminal region of (h)FcLec4. Upon recognition of bacteria via the (h)FcLec4 CTLD, the interaction with hemocyte surface β-integrin induces the rearrangement of cytoskeleton and formation of phagosome (Wang et al., 2014c).

There are many shrimp CTLs that in addition to the CTLD contain other distinct regions or domains. Except for the few examples discussed above, however, their specific role(s) and overall relevance for CTL function remains unknown. Nevertheless, based on the available information, it can be hypothesized that the presence of these additional regions may be essential for a fully functional CTL.

5. Expression regulation of shrimp CTLs

The mechanism(s) for regulation of transcription of several shrimp CTLs has been elucidated in recent years. The promoter of LvCTL3 contains a TATA box motif and a NF-κB binding motif. The major NF-κB transcription factor, Dorsal, can activate the promoter activity of LvCTL3 (Li et al., 2014). The promoter of LvCTL4 also contained both a TATA box motif and an NF-κB binding motif. Two NF-κB transcription factors, Dorsal and Relish, induce the promoter activity. Although the activating capacity of Relish is higher than that of Dorsal, knockdown of the expression of either Relish or Dorsal leads to the decreased expression of LvCTL4 (Li et al., 2015), thereby demonstrating the NF-κB-mediated regulation of transcription of shrimp CTLs. In addition, regulation of transcription of shrimp CTLs under stressful conditions has also been examined. The FcCTL promoter contains a heat shock protein binding element, which is responsible for the upregulation of expression of FcCTL by heat shock stress and WSSV (Lai et al., 2013).

6. Conclusions and future perspectives

Significant progress have been achieved in recent years about structural and functional aspects of the CTL family in shrimp immunity, benefiting from the successful *in vivo* application of RNAi gene silencing approaches, and the protein-protein interaction analysis by using specific antibodies. The recent results provided information that has significantly contributed to a better understanding of the role(s) of selected CTLs in shrimp-pathogen interactions. The shrimp genome encodes a large number of CTLs, however, and to date, the detailed functions of a majority of the shrimp's diverse CTL family members remain largely unknown. Thus, additional efforts and innovative experimental approaches will be needed to reveal the diverse roles and mechanisms of action of this extensive lectin family. In particular, the information about regulation of expression of the CTL family members is far from complete. Several studies have provided a preliminary understanding of the transcription regulation, but more detailed analysis regarding how CTLs expression is modulated upon pathogen challenge and which transcription factors and co-factors are involved in the regulation of expression are still needed. In addition, although RNAi knockdown constitutes an effective experimental approach that has greatly facilitated the functional studies of shrimp CTLs, it is expected that incomplete silencing of expression of any given CTL will require further analysis by current gene knockout approaches. In this regard, it is noteworthy that CRISPR/Cas9 technology has been successfully applied for site-specific genome editing in the ridgetail white prawn *Exopalaemon carinicauda,* a close relative of peneid shrimp (Gui et al., 2016; Zhang et al., 2018a). It is expected that further progress in genome editing approach will greatly advance the functional studies of shrimp CTLs.

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