The sequence variation and functional differentiation of CRDs in a scallop multiple CRDs containing lectin

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Abstract

A C-type lectin of multiple CRDs (CfLec-4) from *Chlamys farreri* was selected to investigate the sequence variation and functional differentiation of its CRDs. Its four CRDs with EPD/LSD, EPN/FAD, EPN/LND and EPN/YND key motifs were recombined separately. The recombinant proteins of CRD1 and CRD2 (designated as rCRD1 and rCRD2) could bind LPS and mannan, while the recombinant proteins of CRD3 and CRD4 (designated as rCRD3 and rCRD4) could bind LPS, PGN, mannan and glucan. Moreover, rCRD3 displayed broad microbe binding spectrum towards Gram-positive bacteria *Staphylococcus aureus* and *Micrococcus luteus*, Gram-negative bacteria *Escherichia coli* and *Vibrio anguillarum*, as well as fungi *Pichia pastoris* and *Yarrowia lipolytica*. These results indicated CRD3 contributed more to CfLec-4's nonself-recognition ability. Furthermore, CRD1, CRD3 and CRD4 functioned as opsonin participating in the clearance against invaders in scallops. The sequence variation in Ca²⁺ binding site 2 among CRDs was suspect to be associated

with such functional differentiation.

Keywords: *Chlamys farreri*; C-type lectin; carbohydrate-recognition domain; Ca^{2+} binding site 2; functional differentiation

1 Introduction

C-type lectins are a superfamily of Ca^{2+} -dependent carbohydrate-recognition proteins. They function as PRR to discriminate self and nonself by recognizing and binding the terminal sugars from various microbes (Weis et al., 1998; Zelensky and Gready, 2005). The carbohydrate recognition domains (CRDs) endow C-type lectins with activities of nonself-recognition and clearance of invaders. Recently, C-type lectins have been assorted into 17 subgroups in vertebrates according to their structural and functional similarities (Zelensky and Gready, 2005), in which collectin is one subgroup serving as PRR to discriminate self and nonself (Cambi and Figdor, 2003; Medzhitov and Janeway, 2002), while selectin mainly mediates cellular adhesion to activate encapsulation and inflammation (Patel et al., 2002). For instance, mannose-binding lectin (MBL) belonging to collectin subgroup can recognize and bind terminal mannose residues and initiate the lectin pathway of complement system (Endo et al., 2006; Fujita, 2002). And P-selectin in selectin subgroup can attract and enrich leukocytes to the site of infection to quickly eliminate the pathogenic bacteria.

There are 1-4 Ca^{2+} binding sites in each CRD, among which Ca^{2+} binding site 2 has been proved to determine the binding specificity of C-type lectins in vertebrates (Weis et al., 1991; Weis and Drickamer, 1996; Weis et al., 1998; Zelensky and Gready,

2005). Briefly, there are two motifs existing in Ca^{2+} binding site 2. The first motif is EPN (Glu-Pro-Asn) or QPD (Gln-Pro-Asp), which is experimentally verified to determine the binding specificity of C-type lectin. The second motif is WND (Trp-Asn-Asp) which is identified to cooperate with the first motif in binding process against carbohydrates (Cambi et al., 2005; Weis and Drickamer, 1996; Weis et al., 1998). Moreover, intensive researches demonstrated that C-type lectins with EPN/WND motifs bind D-mannose or similar sugar, while C-type lectins with QPD/WND motifs bind D-galactose or similar sugar (Wang et al., 2011a; Zelensky and Gready, 2005). However, the two motifs are of great diversity of characteristics in invertebrate, and various kinds of amino acid sequence have been found in the corresponding sites of two specific motifs. For instance, EPD (Glu-Pro-Asp), QPG (Gln-Pro-Gly), QPS (Gln-Pro-Ser), YPG (Tyr-Pro-Gly), and YPT (Tyr-Pro-Thr) were found in the first motif, at the same time WID (Trp-Ile-Asp), WSD (Trp-Ser-Asp), WHD (Trp-His-Asp), FSD (Phe-Ser-Asp) and LSD (Leu-Ser-Asp) were found in the second motif (Wang et al., 2011a).

The number and organization of CRDs determine the affinity and spectrum of lectins to recognize and bind nonself invaders. Some vertebrate C-type lectins contained several CRDs and single CRD containing C-type lectins usually tend to form polymers to perform functions. For instance, the mannose receptor is composed of eight CRDs, whereas MBL is inclined to form polymers to activate complement system (Zelensky and Gready, 2005). To our knowledge, most reported invertebrate C-type lectins contain one single CRD (Ao et al., 2007; Belogortseva et al., 1998;

Huang et al., 2015; Li et al., 2015; Mu et al., 2012). Very recently, however, several invertebrate C-type lectins with double or multiple CRDs have been proved to involve in immune responses including PAMPs and microbes binding (Yang et al., 2015), bacteria agglutination (Tian et al., 2009) and opsonization (Wang et al., 2011b). Fc-Lec2 from *Fenneropenaeus chinensis* with two tandem CRDs and CfLec-3 from *C. farreri* with three dissimilar CRDs have broad and strong affinity to recognize microbes and PAMPs (Yang et al., 2015; Zhang et al., 2009). Nevertheless, the relationship between the structure and functions of multidomain C-type lectins is still defectively understood, especially in invertebrates.

In our previous study, CfLec-4 with four CRDs was demonstrated to be involved in PAMPs recognition, microbe binding and phagocytosis enhancement against bacteria. In the current study, the four CRDs of CfLec-4 were expressed in *Escherichia coli* separately, and their PAMP binding specificities, microbe binding spectrums as well as the phagocytosis enhancement activities were examined to comprehensively explore the role of each CRD played in immune response against invaders.

2 Materials and methods

2.1. Scallop and microbes

Adults of scallop *C. farreri* with an average 55 mm of shell length were collected from a farm in Qingdao, Shandong Province, China, and maintained in the aerated seawater at 18°C for a week before processing.

Bacteria *Micrococcus luteus*, *Staphylococcus aureus* and *Escherichia coli* was

purchased from Microbial Culture Collection Center (Beijing, China). *Vibrio anguillarum* was kindly provided by Dr. Zhaolan Mo. *Yarrowia lipolytica* was kindly provided by Dr. Zhenming Chi. *Pichia pastoris* GS115 was purchased from Invitrogen.

2.2. Sequence analysis

The ClustalW Multiple Alignment program (<http://www.ebi.ac.uk/clustalw/>) was used to create the multiple sequence alignment. An unrooted phylogenetic tree was constructed based on the sequence alignment by the neighbor-joining (NJ) algorithm using the Mega4.0 program (Wang et al., 2015). Bootstrap re-sampling (1000 pseudo-replicates) was employed to test the reliability of the branching.

2.3. Preparation of recombinant proteins

The four CRDs of CfLec-4 were recombined to plasmid pET-32a and were transformed into the strains *E. coli* BL21(DE3)-pLysS respectively. The recombinant protein rCRD1, rCRD2, rCRD3, rCRD4 and the recombinant protein Trx (designated as rTrx) were purified and quantified as described before (Huang et al., 2012).

2.4. Preparation of antibodies and Western-blot analysis

The renatured protein rCfLec-4 and rTrx were immunized to 6 weeks old mice to acquire polyclonal antibody. The specificity of antibodies were detected as described previously (Huang et al., 2012).

2.5. Microbe binding assay

Gram-positive bacteria (*M. luteus*, and *S. aureus*), Gram-negative bacteria (*E. coli* and *V. anguillarum*) and fungi (*P. pastoris* and *Y. lipolytica*) were used to determine the microbe binding spectrum of rCRD1, rCRD2, rCRD3 and rCRD4 according to the method described by Lee (Lee and Söderhäll, 2001). One hundred microliters of rCRD1, rCRD2, rCRD3 and rCRD4 were incubated with microbes under rotations for 30 min at room temperature. Then the microbes were washed with TBS and finally, subjected to elution with 8% SDS. The microbe binding assay was detected by Western-blot analysis. rTrx group was employed as negative control.

2.6. The PAMPs binding assay

The PAMP binding activities of rCRD1, rCRD2, rCRD3, and rCRD4 were measured according to previous report (Huang et al., 2012). After incubated with primary antibody and second antibody, one hundred microliters of 0.1% (w/v) p-nitrophenyl phosphate (pNPP) containing 0.5 mM MgCl₂ was added to each well for chromogenic reaction. The same concentration of rTrx was used as control. The wells filled with 100 µL of TB were used as blank. The absorbance was measured with an automatic ELISA reader at 405 nm. Each experiment was repeated in triplicates and the results were given in terms of the mean of three individual measurements $\pm$ S.E. ($N = 3$). Samples with P (sample)-B (blank)/ N (negative)-B (blank) > 2.1 were considered as positive.

2.7. Phagocytosis assay

Phagocytosis assay was performed according to previous method (Huang et al., 2012). The phagocytic activity of hemocyte attached to the slide was measured using a light microscope. Two hundred hemocytes on each slide were counted, and the

phagocytic rate (PR) representing the phagocytic activities was expressed as following: $PR = (\text{phagocytic hemocytes}) / (\text{total hemocytes}) \times 100\%$. For each treatment, assay was performed in three different slides for statistical analysis.

3 Results

3.1. The sequence features and phylogeny of CRD1, CRD2, CRD3 and CRD4

Multiple sequence alignment of CRDs was developed by ClustalW to identify the signature sequences of the four CRDs (Fig. 1). Each CRD contained four cysteine residues involving in the formation of the conserved internal disulfide bridges. Besides, the two N-terminus cysteine residues in all CRDs indicated that they were of long-form (Zelensky and Gready, 2005). The key motifs of Ca^{2+} binding site 2 in four CRDs were EPD/LSD, EPN/FAD, EPN/LND and EPN/YND, respectively. A phylogenetic tree was constructed by NJ method with 1000 bootstrap-test based on the multiple alignments of CRDs from bivalve lectins and other C-type lectins from *Botryllus schlosseri*, *Danio rerio*, *Gallus gallus*, *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Salmo salar* and *Taeniopygia guttata* (Fig. 2). Three distinct groups were separated in the phylogenetic tree. The CRDs of collectins were clustered into a branch and the CRDs of selectins formed as another branch. The four CRDs of CfLec-4, CfLec-1 and AiCTL-7 from scallops clustered into a branch.

3.2. The recombinant proteins and antibodies of CRD1, CRD2, CRD3 and CRD4

The whole cell lysate of *E. coli* BL21 (DE3)-pLysS with pET-32a-CRD1, -CRD2,

-CRD3 and -CRD4 induced by IPTG were analyzed by SDS-PAGE. A distinct band with molecular weight of 25 kDa was revealed and was consistent with the predicted molecular mass (Fig. 3A, lane 3, 7, 11 and 15). Four clear bands were revealed in the Western-blot analysis with anti-CfLec-4 polyclonal antibody (Fig. 3A, lane 4, 8, 12 and 16). In the control of *E. coli* BL21 (DE3)-pLysS with pET-32a, there was clear band corresponding to rTrx with 18 kDa molecular weight (Fig. 3B).

3.3. PAMPs binding activities of rCRD1, rCRD2, rCRD3 and rCRD4

All the rCRDs of CfLec-4 exhibited a dose-dependent binding capability towards the different tested PAMPs. rCRD1 and rCRD2 bound LPS and mannan while rCRD3 and rCRD4 bound four kinds of PAMPs including LPS, PGN, mannan and glucan. Furthermore, the binding affinity of four rCRDs was different from each other. rCRD3 and rCRD4 exhibited the stronger binding affinity for LPS, PGN and glucan, indicating that rCRD3 and rCRD4 were the major recognition elements of CfLec-4. As negative control, rTrx could not bind any tested PAMPs (data not shown).

3.4. Microbe binding spectrum of rCRD1, rCRD2, rCRD3 and rCRD4

In order to further confirm the interaction between CRDs and microorganism, a direct binding assay was carried out to determine the binding activity of the four rCRDs against Gram-negative bacteria, Gram-positive bacteria and fungi. The binding spectrums of rCRD1 and rCRD2 were the same and they could bind *V. anguillarum* and *S. aureus*. rCRD3 exhibited the widest microbe binding spectrum and the clear bands were detected in all microbes including *E. coli*, *V. anguillarum*, *S.*

aureus, *M.luteus*, *P. pastoris* and *Y. lipolytica* (Fig. 5). As for rCRD4, there was only a clear band detected in *S. aureus* (Fig. 5).

3.5. The phagocytosis enhancement of rCRD1, rCRD2, rCRD3 and rCRD4

The phagocytosis enhancement assay of rCRD1, rCRD2, rCRD3 and rCRD4 was performed with scallop hemocytes. The phagocytic rate of rCRD1, rCRD2, rCRD3 and rCRD4 groups was 28.7%, 22.1%, 32.7% and 25.3%, respectively. While the phagocytic rate of TBS and rTrx group was 17.0% and 18.1%, respectively (Fig. 6). Compared with TBS and rTrx groups, rCRD1, rCRD3 and rCRD4 could significantly enhance ($P < 0.01$) the phagocytic activity of scallop hemocytes against *E. coli*.

4 Discussion

C-type lectins play key roles in defense against pathogen infection by serving as PRR in innate immunity (Akira et al., 2006; Cambi and Figdor, 2003; Weis et al., 1998). The immune functions of C-type lectin mainly rely on its characteristic CRDs, which can bind various carbohydrates through Ca^{2+} binding site 2. Some multidomain C-type lectins have been reported to involve in pathogen recognition (Akira et al., 2006; Cambi et al., 2005; Geijtenbeek et al., 2004; Vasta et al., 2007; Zelensky and Gready, 2005), cellular adhesion (Kerrigan and Brown, 2009), antigen uptake (Kang et al., 2004) and complement pathway activation in vertebrates (Endo et al., 2006; Fujita, 2002). Recently, C-type lectins with multidomain have also been identified and characterized in invertebrates, such as BmMBP (Watanabe et al., 2006), CfLec-3

(Yang et al., 2015) and Fc-Lec2 (Zhang et al., 2009). But their immune functions and the detailed mechanism were still not well understood. In the present study, the four CRDs of CfLec-4 were recombined separately, and their immune functions were thoroughly studied to comprehensively understand the immune response mechanism of multidomain C-type lectin in invertebrate.

So far, many C-type lectins identified from invertebrates exhibited nonself recognizing and binding activity against certain PAMPs and microbes (Hatakeyama et al., 2011; Wang et al., 2011a). For instance, AmphiCTL1 from *Branchiostoma belcheri* could bind PGN and glucan (Yu et al., 2007), and BmMBP could interact with LTA, PGN and mannan (Watanabe et al., 2006). Interestingly, the C-type lectins with multidomain were found to possess extensive nonself-recognition and binding spectrum. For example, CfLec-3 with three CRDs from *C. farreri* possessed wide PAMPs and microbes binding spectrum (Yang et al., 2015), and Fc-Lec2 with two CRDs from *Litopenaeus vannamei* displayed strong binding affinity against microbes and polysaccharide (Zhang et al., 2009). However, the wide nonself-reconition machanism of multidomain C-type lectins were still not well documented. In the current study, the binding ability and binding spectrum towards PAMPs and microbe of the four CRDs in CfLec-4 were examined to better understand the possible binding mechanism. rCRD3 and rCRD4 could bind all the tested PAMPs, while rCRD1 and rCRD2 only bound LPS and mannan. Moreover, rCRD3 and rCRD4 exhibited stronger binding affinity to LPS, PGN and glucan compared with other rCRDs. It could be inferred that CRD3 and CRD4 were the dominant recognition CRDs of

CfLec-4 and there was function differentiation among the four CRDs of CfLec-4. Meanwhile, rCRD3 had the widest microbe binding spectrum among the four rCRDs of CfLec-4. It was consistent with its PAMPs binding spectrum. rCRD1 and rCRD2 had the same microbe binding spectrum to *V. anguillarum* and *S. aureus* while rCRD4 could only bind to *S. aureus*, which was not corresponding with their PAMPs binding spectrum. This phenomenon was also found in AiCTL-3 from *A. irradians* (Huang et al., 2013). AiCTL-3 could bind to PGN, so it was inferred to bind *S. aureus* and *M. luteus*. However, it could only bind to *S. aureus* other than *M. luteus*. The mechanism behind this phenomenon needs further research.

It is accepted that C-type lectins act not only as PRR to bind PAMPs but also as opsonin to promote the phagocytosis of hemocytes against bacteria (Yang et al., 2010; Yang et al., 2011). Recently, several vertebrate C-type lectins, such as DC-SIGN, Dectin-1 and mannose receptor, were found to trigger the opsonization after interacting with microbes (Geijtenbeek et al., 2000; Yang et al., 2015), and some invertebrate C-type lectins were also found to initiate opsonization against bacteria and parasites (Wang et al., 2011a; Wang et al., 2011b). In our previous report, CfLec-4 was found to enhance the phagocytosis ability of hemocytes after binding PAMPs and adhering to the cell surface of certain bacteria (Huang et al., 2012). In the present study, rCRD1, rCRD3 and rCRD4 exhibited the activity to trigger the phagocytosis of hemocytes, indicating that not all the CRDs of CfLec-4 were necessary in the process of triggering opsonization against invaders. CRD1, CRD3 and CRD4 were the main contributor of the opsonization triggered by CfLec-4.

250 The Ca^{2+} binding site 2 in CRD is the binding site of carbohydrates and determine
251 the binding specificity of CRD, which is composed of two conserved characteristic
252 motifs. The first one is EPN or QPD, which is the carbohydrate binding site and also
253 the key motif determining the carbohydrate binding specificity. For example, C-type
254 lectin with EPN motif could bind D-mannose or similar sugar, whereas those with
255 QPD motif bind D-galactose or similar sugar in vertebrates. The second conserved
256 motif in Ca^{2+} binding site 2 is WND which has been proved to assist the binding
257 between CRD and certain carbohydrates. The position of hydrogen bond donors and
258 acceptors in the two motifs of Ca^{2+} -binding site 2 determined C-type lectins' binding
259 specificity. However, accumulating evidences have demonstrated that the Ca^{2+}
260 binding site 2 in invertebrate C-type lectins are of diverse structure and multifunction.
261 The first key motif in Ca^{2+} binding site 2 seems to be more various in invertebrates.
262 For example, there were EPN, EPD, QPG, QPS, YPT and YPG in bivalve C-type
263 lectins, which were supposed to bind diversified carbohydrates (Wang et al., 2011a).
264 Analogously, the second key motif of Ca^{2+} binding site 2 was also diverse as the first
265 one. There were WID, WSD, WHD, LND, LSD and FSD motifs, which were
266 supposed to affect the immune recognition activity against carbohydrates (Wang et al.,
267 2011a). In the present study, four CRDs of CfLec-4 with motifs of EPD/LSD,
268 EPN/FAD, EPN/LND and EPN/YND in their key motifs of Ca^{2+} binding site 2 were
269 recombined to expression vector and the recombinant proteins were purified. Their
270 PAMPs binding spectrums were examined in order to find the possible PAMPs
271 binding mechanism of multidomain C-type lectins. It was found that the CRD2,

CRD3 and CRD4 with EPN motif displayed different PAMPs binding ability. In detail, rCRD2 could bind LPS and mannan while rCRD3 and rCRD4 could bind all PAMPs tested. It was a new discovery in bivalve C-type lectin which was not corresponding with the binding rules in that of vertebrates. It was noticeable that the second key motif of Ca^{2+} binding site 2 in CRD2, CRD3 and CRD4, which were FAD, LND and YND respectively, which were quite different from the WND motif in vertebrates. The asparagine and aspartate were involved in hydrogen bond formation with carbohydrates. Thus, the replacement of amino acids in two key motifs might change the hydrogen bond formation involved in carbohydrates binding, and thus affect the binding specificity of Ca^{2+} binding site 2 which worth further investigation.

Acknowledgements

The authors would thank all of the colleagues in our lab for helpful discussion and technical advice. This research was supported by National High Technology Research and Development Program (863 Program, No. 2014AA093501) from the Chinese Ministry of Science and Technology, grants (No. 31530069 to L.S., No. 41006096 to H.Z.) from National Science Foundation of China, and Dalian high level talent innovation support program (No. 2015R020). Thanks for Dr. Zhenming Chi for kindly providing *Yarrowia lipolytica*.

References

- Akira, S., Uematsu, S., Takeuchi, O., 2006. Pathogen recognition and innate immunity. *Cell* 124, 783-801.
- Ao, J., Ling, E., Yu, X., 2007. *Drosophila* C-type lectins enhance cellular encapsulation. *Mol.*

296 Immunol. 44, 2541-2548.
 297 Belogortseva, N.I., Molchanova, V.I., Kurika, A.V., Skobun, A.S., Glazkova, V.E., 1998. Isolation
 298 and characterization of new GalNAc/Gal-specific lectin from the sea mussel *Crenomytilus*
 299 *grayanus*. Comp. Biochem. Physiol., C: Toxicol. Pharmacol. 119C, 45-50.
 300 Cambi, A., Figdor, C.G., 2003. Dual function of C-type lectin-like receptors in the immune system.
 301 Curr. Opin. Cell Biol. 15, 539-546.
 302 Cambi, A., Koopman, M., Figdor, C.G., 2005. How C-type lectins detect pathogens. Cell.
 303 Microbiol. 7, 481-488.
 304 Endo, Y., Takahashi, M., Fujita, T., 2006. Lectin complement system and pattern recognition.
 305 Immunobiology 211, 283-293.
 306 Fujita, T., 2002. Evolution of the lectin-complement pathway and its role in innate immunity. Nat.
 307 Rev. Immunol. 2, 346-353.
 308 Geijtenbeek, T.B., Kwon, D.S., Torensma, R., van Vliet, S.J., van Duijnhoven, G.C., Middel, J.,
 309 Cornelissen, I.L., Nottet, H.S., KewalRamani, V.N., Littman, D.R., 2000. DC-SIGN, a
 310 dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. cell 100,
 311 587-597.
 312 Geijtenbeek, T.B.H., van Vliet, S.J., Engering, A., 't Hart, B.a., van Kooyk, Y., 2004. Self- and
 313 nonself-recognition by C-type lectins on dendritic cells. Annu. Rev. Immunol. 22, 33-54.
 314 Hatakeyama, T., Kamiya, T., Kusunoki, M., Nakamura-Tsuruta, S., Hirabayashi, J., Goda, S.,
 315 Unno, H., 2011. Galactose recognition by a tetrameric C-type lectin, CEL-IV, containing the
 316 EPN carbohydrate recognition motif. J. Biol. Chem. 286, 10305-10315.
 317 Huang, M., Song, X., Zhao, J., Mu, C., Wang, L., Zhang, H., Zhou, Z., Liu, X., Song, L., 2013. A
 318 C-type lectin (AiCTL-3) from bay scallop *Argopecten irradians* with mannose/galactose
 319 binding ability to bind various bacteria. Gene 531, 31-38.
 320 Huang, M., Wang, L., Yang, J., Zhang, H., Wang, L., Song, L., 2012. A four-CRD C-type lectin
 321 from *Chlamys farreri* mediating nonself-recognition with broader spectrum and opsonization.
 322 Dev. Comp. Immunol. 39, 363-369.
 323 Huang, M., Zhang, H., Jiang, S., Wang, L., Liu, R., Yi, Q., Song, L., 2015. An EPD/WSD motifs
 324 containing C-type lectin from *Argopectens irradians* recognizes and binds microbes with broad
 325 spectrum. Fish Shellfish Immunol. 43, 287-293.

326 Kang, Y.-S., Kim, J.Y., Bruening, S.a., Pack, M., Charalambous, A., Pritsker, A., Moran, T.M.,
 327 Loeffler, J.M., Steinman, R.M., Park, C.G., 2004. The C-type lectin SIGN-R1 mediates uptake
 328 of the capsular polysaccharide of *Streptococcus pneumoniae* in the marginal zone of mouse
 329 spleen. Proc. Natl. Acad. Sci. USA. 101, 215-220.
 330 Kerrigan, A.M., Brown, G.D., 2009. C-type lectins and phagocytosis. Immunobiology 214,
 331 562-575.
 332 Lee, S.Y., Söderhäll, K., 2001. Characterization of a pattern recognition protein, a masquerade-like
 333 protein, in the freshwater crayfish *Pacifastacus leniusculus*. J. Immunol. 166, 7319-7326.
 334 Li, H., Zhang, H., Jiang, S., Wang, W., Xin, L., Wang, H., Wang, L., Song, L., 2015. A single-CRD
 335 C-type lectin from oyster *Crassostrea gigas* mediates immune recognition and pathogen
 336 elimination with a potential role in the activation of complement system. Fish Shellfish
 337 Immunol. 44, 566-575.
 338 Medzhitov, R., Janeway, C.a., 2002. Decoding the patterns of self and nonself by the innate
 339 immune system. Science 296, 298-300.
 340 Mu, C., Song, X., Zhao, J., Wang, L., Qiu, L., Zhang, H., Zhou, Z., Wang, M., Song, L., Wang, C.,
 341 2012. A scallop C-type lectin from *Argopecten irradians* (AiCTL5) with activities of
 342 lipopolysaccharide binding and Gram-negative bacteria agglutination. Fish Shellfish Immunol.
 343 32, 716-723.
 344 Patel, K.D., Cuvelier, S.L., Wiehler, S., 2002. Selectins: critical mediators of leukocyte
 345 recruitment. Semin. Immunol. 14, 73-81.
 346 Tian, Y.Y., Liu, Y., Zhao, X.F., Wang, J.X., 2009. Characterization of a C-type lectin from the
 347 cotton bollworm, *Helicoverpa armigera*. Dev. Comp. Immunol. 33, 772-779.
 348 Vasta, G., Ahmed, H., Tasumi, S., Odom, E., Saito, K., 2007. Biological roles of lectins in innate
 349 immunity: molecular and structural basis for diversity in self/non-self recognition. Current
 350 topics in innate immunity, 389-406.
 351 Wang, L., Huang, M., Zhang, H., Song, L., 2011a. The immune role of C-type lectins in molluscs.
 352 ISJ 8, 241-246.
 353 Wang, L., Wang, L., Zhang, D., Jiang, Q., Sun, R., Wang, H., Zhang, H., Song, L., 2015. A novel
 354 multi-domain C1qDC protein from Zhikong scallop *Chlamys farreri* provides new insights into
 355 the function of invertebrate C1qDC proteins. Dev. Comp. Immunol. 52, 202-214.

356 Wang, L., Yang, J., Zhang, H., Huang, M., Kong, P., Zhou, Z., Song, L., 2011b. A multi-CRD
 357 C-type lectin with broad recognition spectrum and cellular adhesion from *Argopecten irradians*.
 358 Dev. Comp. Immunol. 36 (3), 591-601.

359 Watanabe, A., Miyazawa, S., Kitami, M., Tabunoki, H., Ueda, K., Sato, R., 2006. Characterization
 360 of a novel C-type lectin, *Bombyx mori* multibinding protein, from the *B. mori* hemolymph:
 361 mechanism of wide-range microorganism recognition and role in immunity. J. Immunol. 177,
 362 4594-4604.

363 Weis, W., Kahn, R., Fourme, R., Drickamer, K., Hendrickson, W., 1991. Structure of the
 364 calcium-dependent lectin domain from a rat mannose-binding protein determined by MAD
 365 phasing. Science 254, 1608-1615.

366 Weis, W.I., Drickamer, K., 1996. Structural basis of lectin-carbohydrate recognition. Annu. Rev.
 367 Biochem. 65, 441-473.

368 Weis, W.I., Taylor, M.E., Drickamer, K., 1998. The C-type lectin superfamily in the immune
 369 system. Immunol. Rev. 163, 19-34.

370 Yang, J., Huang, M., Zhang, H., Wang, L., Wang, H., Wang, L., Qiu, L., Song, L., 2015. CfLec-3
 371 from scallop: an entrance to non-self recognition mechanism of invertebrate C-type lectin. Sci.
 372 Rep. 5, e10068.

373 Yang, J., Qiu, L., Wei, X., Wang, L., Zhou, Z., Zhang, H., Liu, L., Song, L., 2010. An ancient
 374 C-type lectin in *Chlamys farreri* (CfLec-2) that mediate pathogen recognition and cellular
 375 adhesion. Dev. Comp. Immunol. 34, 1274-1282.

376 Yang, J., Wang, L., Zhang, H., Qiu, L., Wang, H., Song, L., 2011. C-type lectin in *Chlamys farreri*
 377 (CfLec-1) mediating immune recognition and opsonization. PLoS One 6, e17089.

378 Yu, Y., Huang, H., Feng, K., Pan, M., Yuan, S., Huang, S., Wu, T., Guo, L., Dong, M., 2007. A
 379 short-form C-type lectin from amphioxus acts as a direct microbial killing protein via
 380 interaction with peptidoglycan and glucan. J. Immunol. 179, 8425-8434.

381 Zelensky, A.N., Gready, J.E., 2005. The C-type lectin-like domain superfamily. FEBS J. 272,
 382 6179-6217.

383 Zhang, X.W., Xu, W.T., Wang, X.W., Mu, Y., Zhao, X.F., Yu, X.Q., Wang, J.X., 2009. A novel
 384 C-type lectin with two CRD domains from Chinese shrimp *Fenneropenaeus chinensis*
 385 functions as a pattern recognition protein. Mol. Immunol. 46, 1626-1637.

Figure legends

Fig. 1

Multiple sequence alignment by ClustalW of four CRDs from CfLec-4 with other genes' CRDs. These gene include CfLec-1 (GenBank accession no. DQ209290) and CfLec-2 (DQ209289) of *C. farreri*, AiCTL-7 from *A. irradians*. Amino acid residues that are conserved in at least 50% sequences are shaded in dark, and similar amino acids are shaded in grey. Conserved cysteine residues involved in the formation of the CRD internal disulfide bridges were marked with black square, whereas two extra cysteine residues in long-form CRD were marked with black triangle. The letters in box were Ca^{2+} binding site 2 of CRDs which determined the ligand binding specificity.

Fig. 2

Unrooted phylogenetic tree of the CRDs in CfLec-4 (CRD1, CRD2, CRD3 and CRD4) with those in other C-type lectins. The tree is constructed by the neighbor-joining (NJ) algorithm using the Mega4.0 program based on the multiple sequence alignment by ClustalW. Bootstrap values of 1000 replicates (%) are indicated for the branches. The scale bar corresponds to 0.2 estimated amino-acid substitutions per site. The CRDs sequences used for phylogenetic analysis are as follows, 1: NP_002996 (*H. sapiens* P-selectin); 2: AAI09160 (P-selectin *M. musculus*); 3: NP_035476 (L-selectin *M. musculus*); 4: CAI19356.1 (L-selectin *H. sapiens*); 5: CAX13223.1 (L-selectin *D. rerio*); 6: XP_002190413 (L-selectin *T. guttata*); 7: AAI31552 (E-selectin *H. sapiens*); 8: AAA37577 (E-selectin *M. musculus*); 9: CAA62217 (MBP *B. schlosseri*); 10: NP_001134969 (MBP *S. sala*); 11: AAB94071 (MBP *G. gallus*); 12: Q8IWL2 (SPA *H.*

412 *sapiens*); 13: NP_075623 (SP-A *M. musculus*); 14: NP_989937 (SP-A *G. gallus*); 15:
413 CAA34079 (MBP-A *H. sapiens*); 16: P19999 (MBP-A *R. norvegicus*); 17:
414 NP_033186 (SP-D *M. musculus*); 18: AAB59450 (SP-D *H. sapiens*); 19: HM149769
415 (AiCTL-7 *A. irradians*); 20: DQ209290 (CfLec-1 *C. farreri*); 21: CRD1 of CfLec-4;
416 22: CRD2 of CfLec-4; 23: CRD3 of CfLec-4; 24: CRD4 of CfLec-4.

417

418 Fig. 3

419 SDS-PAGE and Western-blot analysis of rCRD1, rCRD2, rCRD3, rCRD4 and rTrx.

420 (A) Lane M: protein molecular standard; lane 1: negative control for rCRD1 (without

421 induction); lane 2: induced rCRD1; lane 3: purified rCRD1; lane 4: Western-blot

422 based on the sample of lane 3; lane 5: negative control for rCRD2; lane 6: induced

423 rCRD2; lane 7: purified rCRD2; lane 8: Western-blot based on the sample of lane 7;

424 lane 9: negative control for rCRD3; lane 10: induced rCRD3; lane 11: purified rCRD3;

425 lane 12: Western-blot based on the sample of lane 11; lane 13: negative control of

426 rCRD4; lane 14: induced rCRD4; lane 15: purified rCRD4; lane 16: Western-blot

427 based on the samples of lane 15. (B) Lane M: protein molecular standard; lane 1:

428 induced rTrx; lane 2: negative control for rTrx (without induction); lane 3: purified

429 rTrx; lane 4: Western-blot based on the sample of lane 3.

430

431 Fig. 4

432 ELISA analysis of the interaction between four rCRDs and the PAMPs. Plates were

433 coated with four kinds of PAMPs including glucan, LPS, PGN and mannan. Then

434 incubated with different amounts of rCRDs and rTrx at 18°C for 3h. After incubated

435 with rat polyclonal antiserum, the interaction was detected with goat-anti-rat

436 Ig-alkaline phosphatase conjugate at 405 nm. Samples with P/N>2.1 were considered

positive. Results are representative of the mean of three replicates $\pm$ SE. The significant difference between CRDs were marked with letters, and different letters were used for significant difference ($P < 0.05$) between CRDs.

Fig. 5

The microbe binding spectrum of rCRD1, rCRD2, rCRD3 and rCRD4 revealed by Western-blot. One hundred microlitres of purified rCRD1, rCRD2, rCRD3 and rCRD4 in TBS ($100 \mu\text{g mL}^{-1}$) was incubated with microorganisms under rotation for 30 min at room temperature. Microorganisms were pelleted, washed three times with TBS, resuspended in $50 \mu\text{L}$ of TBS, and then subjected to elution with 8% SDS. The supernatant was collected. The binding spectrum of rCRD1, rCRD2, rCRD3 and rCRD4 was detected by Western-blot.

Fig. 6

Phagocytosis enhanced by rCRD1, rCRD2, rCRD3 and rCRD4. The hemocytes, rCRDs and rTrx were resuspended with TBS buffer, respectively. The hemocytes were incubated with TBS, rTrx and rCRDs for 30 min, then *E. coli* was added into each suspension for another 1 h. The mixture was mounted onto a glass slide and stained with Giemsa. The phagocytic activity of hemocyte was measured using a light microscope. Two hundred hemocytes on each slide were counted. Phagocytic rate (PR) = (phagocytic hemocytes)/(total hemocytes) $\times 100\%$. For each treatment, assay was performed in three different slides for statistic analysis. The columns represent the mean of three individual counts $\pm$ S.E.M, student's t test, error-bars represent biological repeats (**: $P < 0.01$).

Figure 1

CRD1	CPV	GKWI	LIQGS	----	CFL	FSS	QGLT	----	WDQ	AATD	CRQ	FG	----	ATL	LQF	EGAD	DRE	47
CRD2	CPL	NW	IVTTN	----	CYY	VSD	LTDE	YNI	VS	WSD	ASKK	CKTL	HPG	TS	AKL	LKLE	TANE	55
CRD3	CSNG	-W	VRAGHK	----	CYF	FHI	QRPQ	----	NHRT	AAS	TCSE	MA	----	GRL	IQI	QTKD	EED	47
CRD4	CPNG	-W	ISRAANGMLT		CYL	ISNT	TTDA	-DM	TTW	QGARD	KCV	QISE	PLD	GYLL	AIN	TKDE	AA	58
CfLec-2	CPEG	-W	VSNKNA	----	CYH	ISRE	TEE	----	WVA	AEAM	CKI	YG	----	ATL	VHI	ETAE	DN	46
AiCTL-7	CEPG	-W	TQYKED	----	CLW	FSNT	AKT	----	WLA	SEND	CKNK	G	----	GWL	MTD	DNEG	KHE	46
CfLec-1	CDSD	-W	FSSEEK	----	CYY	VSS	RSSEK	----	TDW	STAV	TRC	QTKG	----	ANL	VEI	QTD	EAM	48
CRD1	LFTR	TIVN	----	MTS	ERW	WTD	LT	DYN	HPG	WSW	GED	DS	EL	AN	PGAV	VWN	-VEP	102
CRD2	YIRA	QLAE	--	LQM	TDQ	LYW	IGMS	DQA	HEG	HTW	DD	--	GSP	VNQ	S	NIEW	T-AEP	109
CRD3	WLRV	QTLR	----	YDS	YAF	WTGL	IFQP	SSSS	SWV	WNT	--	DTK	ANM	SLIN	WN	-QEP	NN	98
CRD4	FIAH	ALKN	--	ISQ	IAT	GWW	TGLN	DKK	VEGY	WEY	DTAF	NNP	VQNN	-VIP	WD	-GEP	NN	114
CfLec-2	FLSE	YLRN	NSIV	YND	HQY	WIG	LS	DWE	FEG	TFI	WVP	--	EGV	TPG	-YTN	WGP	GEP	102
AiCTL-7	FLST	IMYA	-FKN	FHF	NKFF	IGGS	DTAF	ENV	VRW	LE	--	TGI	NVG	PFS	KGW	TGEP	DG	102
CfLec-1	IIMR	NLPS	--	RVS	RADI	IYTG	-RKR	NDN	RDW	VFL	TN	--	EKL	VD	TSV	RSW	SGEP	103
CRD1	ENC	-G	AINIQ	--	GT	--	LSD	QEC	----	SEKH	G	YICE						128
CRD2	EQC	-A	LIYDN	--	GR	--	FAD	ADC	----	KRL	E	KYICQ						135
CRD3	EDC	-G	TISQD	--	GI	--	LND	LSC	----	NAN	Q	GYICE						124
CRD4	DYC	-T	VLYG	--	GR	--	YND	VNC	----	NNI	A	YICE						139
CfLec-2	QHC	-T	IHTQ	--	EHY	Q	WFDR	MC	----	NEQ	Y	S	YICE					130
AiCTL-7	N-CL	ALKW	END	--	RDL	VW	SDE	SCGH	VST	SHHH	HGHG	LLN	YICE					143
CfLec-1	CGC	-TR	KSDD	----	LEML	D	-C	-FCT	----	GYN	LF	-YICE						130

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6