A single von Willebrand factor C-domain protein acts as an extracellular pattern-recognition receptor in the river prawn *Macrobrachium nipponense*

The single von Willebrand factor C-domain proteins (SVWCs) are mainly found in arthropods. Their expression may be regulated by several environmental stresses, including nutritional status and bacterial and viral infections. However, the underlying regulatory mechanism is unclear. In the present study, we identified a member of the SVWC family from the river prawn *Macrobrachium nipponense* as a soluble and bacteria-inducible pattern-recognition receptor (designated MnSVWC). In vitro, recombinant MnSVWC exhibited pronounced binding and Ca\(^{2+}\)-dependent agglutinating abilities against diverse microbes, including Gram-negative bacteria (*i.e.*, *Escherichia coli* and *Aeromonas victoria*), Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*), and yeast (*Pichia pastoris*). ELISA assays revealed that recombinant MnSVWC recognizes a broad range of various pathogen-associated molecular patterns (PAMPs) and has high affinity to lipopolysaccharide and lysine-type and diaminopimelic acid–type peptidylglycan and \( \alpha \)-galactose and low affinity to \( \beta \)-1,3-mannan and \( \beta \)-1,3-glucan. Mutant MnSVWC\(^{197A} \) with an impaired Glu-Pro-Asn (EPN) motif displayed reduced affinity to all these PAMPs to varying extent. Moreover, MnSVWC bound to the surface of hemocytes and promoted their phagocytic activity and clearance of invasive bacteria. RNAi-mediated MnSVWC knockdown in prawn reduced the ability to clear invading bacteria, but did not block the activities of the Toll pathway or the arthropod immune deficiency (IMD) pathway, or the expression of antimicrobial peptide genes. These results indicate that MnSVWC functions as an extracellular pattern-recognition receptor in *M. nipponense* that mediates cellular immune responses by recognizing PAMPs, agglutinating invasive microbes, and promoting phagocytosis in hemocytes.
bacteria recognition and resistance as an extracellular soluble PRR. To our knowledge, this is the first study to link the PRR’s function to a SVWC, and thus will enhance our understanding on the innate immunity of invertebrates.

Results

**cDNA cloning and sequence analysis**

By searching the transcriptome data of *M. nipponense*, we identified a transcript of SVWC homologous gene (named MnSVWC), whose full-length cDNA sequence was cloned and verified via RACE and PCR methods. This gene encoded a protein of 138 amino acids. SignalP analysis revealed that the deduced peptide contained a putative signal peptide of 22 amino acids and a mature peptide of 116 amino acids (**Fig. S1**). The molecular mass of the deduced mature peptide was 12.92 kDa and the theoretical isoelectric point (pI) was 4.79. An EPN motif existed in the deduced mature peptide (**Fig. S1**), which is predicted to bind to mannose. Protein sequences of SVWCs from diverse arthropods were gained from GenBank, and the Blastp analysis revealed that MnSVWC shared the highest similarity with the putative SVWC domain (from Cys-39 to Cys-106) with a theoretical pI of 8.49. An EPN motif existed in the deduced mature peptide of MnSVWC (**Fig. S1**), which is predicted to bind to mannose. Protein sequences of SVWCs from diverse arthropods were gained from GenBank, and the Blastp analysis revealed that MnSVWC shared the highest similarity with the single VWC domain protein 4 from *Penaeus japonicus* (56% identity). However, a run of Blastp against the SwissProt database using the amino acid sequence of MnSVWC revealed no close similarity to any known sequence. A multiple sequence alignment of representative SVWCs from various taxa indicated that eight highly conserved cysteine residues were highlighted in all detected arthropod SVWC proteins (**Fig. S2A**). The number of residues between each cysteine in these sequences was counted and the schematic of this consensus pattern was shown in **Fig. S2A**, where the digits in the box represented the range of amino acids. Strikingly, we found that the cysteine distribution pattern was inconsistent with the consensus pattern of *Drosophila* SVWCs reported by Sheldon *et al.* (10). An unrooted phylogenetic tree was constructed using the maximum likelihood method based on the multiple sequence alignment, and eight main distinct groups in the tree could be identified, which corresponded well to the orders or classes of the system classification (**Fig. S2B**). In the tree, MnSVWC had the closest relationship with SVWCs of other crustaceans, such as *Eriocheir sinensis*, followed by those of insects. The SVWCs from Arachnida and Merostomata gathered together to form the outer group of this tree.

**Tissue distribution and temporal expression profiles of MnSVWC**

MnSVWC mRNA was primarily expressed in gill and hemocytes, intermediate expressed in muscle, with much lower levels in hepatopancreas and intestine (**Fig. 1A**). To understand whether MnSVWC was involved in the immune response, its expression in gill was detected after challenge with *Aeromonas victoria*, an important pathogenic bacterial of aquatic animals. The results showed that the expression of MnSVWC could be enhanced remarkably by bacterial infection. Two peaks in gene expression were recorded respectively at 6 and 48 h post bacterial injection (**Fig. 1B**).

**Expression and purification of rMnSVWC and rMnSVWC<sup>P57A</sup>**

After isopropyl-β-D-thiogalactopyranoside (IPTG) induction, rMnSVWC and rMnSVWC<sup>P57A</sup> were expressed in *E. coli* BL21 (DE3). The molecular mass of the recombinant proteins was consistent with the predicted molecular mass (**Fig. 2A**). The His-tagged recombination proteins were purified and desalted, then subjected to further activity detection.

**Microbial and polysaccharide binding activities of rMnSVWC**

To gain further insight into the role of MnSVWC in antimicrobial immunity, we analyzed its potential binding ability to both bacteria and fungi *in vitro*. Western blotting assay revealed that rMnSVWC could bind significantly to all the tested microbial strains, including G<sup>−</sup> bacteria (*E. coli* and *A. victoria*), G<sup>+</sup> bacteria (*S. aureus* and *B. subtilis*) and yeast *P. pastoris*, with the strongest binding ability to *S. aureus*, and a weak bond to *P. pastoris* (**Fig. 2B**). To clarify whether the microbial binding ability of rMnSVWC was mediated by cell surface polysaccharides, ELISAs were performed to detect the binding capacity of rMnSVWC toward lipopolysaccharide (LPS), peptidylglycan (Dap-type PGN and Lys-type PGN), d-galactose, d-mannan and β-1,3-glucan. The results indicated that rMnSVWC could bind all these ligands in a dose-dependent manner, rather than soluble starch and d-trehalose (**Fig. 2C**). The apparent dissociation constant (*K<sub>d</sub>*<sub>1</sub>) of rMnSVWC toward LPS, Dap-type PGN, Lys-type PGN, d-galactose, d-mannan and β-1,3-glucan, calculated from the saturation curve, were 3.3 × 10<sup>-8</sup>, 4.7 × 10<sup>-8</sup>, 9.6 × 10<sup>-8</sup>, 4.8 × 10<sup>-8</sup>, 1.0 × 10<sup>-7</sup> and 1.0 × 10<sup>-7</sup> M, respectively. The *K<sub>d</sub>*<sub>2</sub> of rMnSVWC<sup>P57A</sup> toward these polysaccharides were 6.5 × 10<sup>-8</sup>, 4.0 × 10<sup>-7</sup>, 1.3 × 10<sup>-7</sup>, 6.6 × 10<sup>-8</sup>, 3.8 × 10<sup>-7</sup> and 2.4 × 10<sup>-7</sup> M, respectively. Correspondingly, the PAMP-binding activities of rMnSVWC<sup>P57A</sup> significantly decreased after the Pro-57 residue in the EPN motif was mutated into alanine.
However, no binding activity of recombinant GSH S-transferase to polysaccharides was detected even if at high concentration.

**Microbial agglutinating activity of rMnSVWC**

FITC-labeled microbes were incubated with the tested proteins to check the possible agglutination. Immunofluorescence microscopy further demonstrated strong agglutinating activities of rMnSVWC toward all four of the bacteria and a yeast in the presence of Ca\(^{2+}\) (Fig. 3). No agglutination was observed in the BSA control groups or the Ca\(^{2+}\)-free control groups, suggesting that the agglutinating activities of rMnSVWC toward microbes were Ca\(^{2+}\) dependent.

**MnSVWC enhance bacterial clearance in vivo**

To test whether the ability to bind and agglutinate bacteria was indeed associated to the protective role of MnSVWC, the bacterial colonization level in the hemolymph was studied. As shown in Fig. 4A, when rMnSVWC-coated E. coli was injected into the prawn, the number of colonized bacteria in the hemolymph was significantly lower than that in control at 1 h post injection, but not at 30 min. The results manifested that coating with the rMnSVWC significantly facilitated the clearance of bacteria in vivo. It was further verified by testing the bacterial colonization ability with RNAi model. The results of quantitative real-time PCR (qRT-PCR) showed that injection of MnSVWC double-strand RNA (dsRNA) effectively knocked down the expression of the target gene at 24 h (Fig. 4B). Then, E. coli expressing GFP was injected into the MnSVWC-RNAi prawns and control individuals. The hemolymph was drawn and plated onto resistant Luria-Bertani (LB) agar plates to determine the colony number. The results showed that dsRNA-mediated MnSVWC gene silencing reduced the ability of prawns to remove bacteria from the hemolymph compared with control (Fig. 4C).

**Expression of antimicrobial peptide and NF-κB-like transcription factor genes is not mediated by MnSVWC**

To investigate whether MnSVWC participates in bacterial clearance by regulating the expression of antimicrobial
peptides (AMPs) and NF-κB–like transcription factor genes, the expression of anti–lipopolysaccharide factor (ALF), crustin, dorsal, and relish was analyzed by qRT-PCR in MnSVWC-silenced prawn (Fig. 4D). The results showed that all these tested genes were up-regulated in MnSVWC-silenced shrimp following challenge by A. victoria. These data suggest that MnSVWC does not participate in the regulation of the expression of antimicrobial peptides.

MnSVWC promoted the hemocytes’ phagocytosis

To see whether MnSVWC can function as a potential opsonin, its effect on hemocytes’ phagocytosis was investigated. FITC-labeled A. victoria was incubated with rMnSVWC or BSA as control protein and injected into the body of prawns. Then the hemocytes was collected at 1 h after bacterial injection and analyzed using a confocal microscope. Phagocytosis signals were indicated with green fluorescence in hemocytes, resulting from FITC on the surface of bacteria (Fig. 5F). As shown in Fig. 5B, rMnSVWC coating led to a higher percentage of hemocytes’ phagocytosis (48.2%) compared with the controls (28.6%) in vivo. The result demonstrated that MnSVWC promoted hemocytes’ phagocytosis. In brief, these above results suggested that MnSVWC could be involved in the innate defense response in the prawn via agglutinating and opsonizing invading bacteria.

Hemocyte-binding activity of rMnSVWC

To examine the possible mechanism responsible for the above observation, the binding of MnSVWC to hemocytes was assessed in vivo by injecting the recombinant protein into the prawn. At 30 min post injection, the hemocytes was isolated from the hemolymph and the presence of surface-bound rMnSVWC was examined by immunocytochemistry. The images in Fig. 6 showed the green signal on the edge of the hemocytes collected from prawn injected with rMnSVWC. These results indicated that the rMnSVWC can bind to the hemocyte surface.

Discussion

SVWCs are widely found in arthropods and proposed to be responsive to a number of environmental stresses such as infection or starvation (10, 14, 15). Although many studies have declared the involvement of SVWCs in antiviral or antibacterial immunity in insects and crustaceans, the underlying mechanism still remains unclear, especially the crosstalk of SVWCs and another group of pathogen-responsive proteins, PRRs, was not clearly explained in the literature. PRRs are well studied in invertebrates who lack an adaptive immunity to prevent
pathogen invasion, and they are identified as a class of germ line–encoded receptors that recognize PAMPs and thus play a crucial role in the proper function of the innate immune system (16, 17). After the PAMPs’ recognition by PRRs, the expressions of AMPs are often regulated by the Toll and IMD pathways, such as Gram-negative–binding protein and peptidoglycan recognition proteins, short and long (18, 19).

There are some studies that tried to link SVWCs with PRRs by AMP regulation pathways. A SVWC gene in the bumblebee Bombus terrestris is reported to be involved in both host antiviral immunity and basal AMPs’ expression. Silencing of SVWC gene in the bumblebee results significant down-regulation of all four AMP genes, abaecin, apidaecin, defensing, and hymenoptaecin, in fat bodies. Meanwhile, down-regulation of pelle (Toll pathway) but no change of relish (IMD pathway) are observed after SVWC is silenced (14). In a previous study, the authors speculated that the Toll pathway was a potential candidate to be influenced by SVWC for regulating the antiviral activity (14). However, their recent research indicates that the expression alteration of hymenoptaecin in bumblebees after Israeli acute paralysis virus infection is not regulated by the SVWC-related immune activities (20). In the present research, the expression levels of two AMP genes, ALF and crustin, as well two NF-κB–like transcription factor genes, dorsal (Toll pathway) and relish (IMD pathway) are slightly up-regulated in the MnSVWC-silenced gill. Overall, it is still mysterious how the SVWCs interact with the PRRs in the innate immunity of invertebrates, and no SVWC is considered as PRR in the previous studies.

Orthologues of MnSVWC are identified in a series of representative species of arthropods by Blastp procedure against NCBI’s nonredundant database. A sequence alignment of the SVWC domain from these homologues shows a conserved eight-cysteine motif whose pattern complies with the typical pattern C-(17, 32)-C-(4, 5)-C-(10, 11)-C-(6, 10)-C-(12, 14)-(F/Y)-P-X-C-(2, 5)-C in D. melanogaster, which testified MnSVWC is a typical single von Willebrand factor type C protein. Notably, an EPN motif appears in the SVWC domain of MnSVWC, but not in other homologous sequences tested in the present research. The EPN motif, which is widely present in
carbohydrate recognition domain (CRD) of C-lectins and has a predicted ligand-binding specificity for mannose (21), leads us to examine the potential role of MnSVWC as a PRR for recognizing microbes.

C-type lectins (CTLs), a large group of proteins with Ca\(^{2+}\) (C-type)-dependent carbohydrate binding ability (22), can act as PRRs and display immunoregulatory properties such as microbial agglutination, nodule formation, encapsulation, cell adhesion, opsonization enhancement, antibacterial or antiviral response, and prophenoloxidase activation (23). CTLs contain single or multiple CRDs, which include a characteristic double-loop structure stabilized by two or three conserved disulfide bonds, and four Ca\(^{2+}\)-binding sites for carbohydrate binding (21, 24). The carbohydrate-binding site of many C-type CRDs contains two remarkable amino acid motifs: EPN or Gln-Pro-Asp, which has a predicted ligand-binding specificity for mannose or galactose, respectively (16).

In our study, MnSVWC appears to have no homology with C-type lectins, but the properties of MnSVWC are very similar to those of C-type lectin PRRs. For example, MnSVWC exhibited strong binding activities to a variety of microbes, including G\(^+\) and G\(^-\) bacteria and yeast as well, and broad binding spectrum toward diverse PAMPs, which was essential for pathogen recognition during innate immune response. MnSVWC is able to extensively bind toward LPS, Dap-type PGN, Lys-type PGN, D-galactose, D-mannan, and β-1,3-glucan, indicating a more extensive microbial-binding profile than that of previously reported PRRs. rMnSVWC\(^{P25A}\) shows that the destruction of EPN motif with proline (Pro) is substituted by alanine (Ala), results in reduced affinity rather than altered specificity to all detected ligands. For the lectin family, EPN motif has been hypothesized to confer binding specificity toward mannose. However, some studies suggest that the mutation of EPN does not affect the PAMP-binding activity of lectins (25, 26). Our present work indicates that the MnSVWC with a putative EPN motif has a lower affinity to mannose compared with LPS, PGN, and even D-galactose. So, we speculate that MnSVWC might have more sites involved in PAMPs binding.

More pronounced MnSVWC displays strong Ca\(^{2+}\)-dependent bacterial agglutination activity, an original function of a CTL would prevent the spreading of bacteria in the hemolymph. In invertebrates, agglutination reaction is reported to be an important innate immune way of attacking the foreign pathogen, which is well-known to be mediated by lectins. In this study, the rMnSVWC caused agglutination of all the tested microbes, including G\(^+\) and G\(^-\) bacteria and yeast, suggesting a possible role in the prevention of microbial spread and colonization in prawn. Moreover, the agglutination might also inhibit pathogen transmission along with the hemolymph circulation and allow for quick reorganization and effective mechanical elimination by the host. Our result proposes a protective role of MnSVWC through its agglutinating ability, which is confirmed by further bacterial clearance experiments, coating by rMnSVWC promoted the bacterial elimination, whereas silencing of MnSVWC reduced the clearance rate in prawns.

In addition to agglutination, phagocytosis is another pivotal way to clear invading pathogens, which is usually triggered by the recognition of the PAMPs. The pattern recognition can be directly mediated via receptors on the cell surface, or indirectly mediated via soluble opsonins (27). The mammalian soluble lgs are classical opsonins, and some C-type lectins from invertebrates are also reported to be opsonins by promoting the phagocytosis of phagocytes to the invading microbe (26, 28). In the current research, we found that rMnSVWC could remarkably enhance the phagocytosis rate of hemocytes to bacteria in vivo, indicating the involvement of MnSVWC in innate immunity through its opsonic ability. Because MnSVWC can act as a class of extracellular soluble opsonization factor, it should be able to bind to the hemocyte surface. The results of immunohistochemical analysis support this hypothesis. As the binding ability of MnSVWC links the bacteria and hemocytes together, it is reasonable to explain that phagocytosis toward bacteria is enhanced.

We did not find any evidence that MnSVWC could regulate the expressions of the genes in IMD or Toll pathway, or the AMP genes. In prawns, there might be a novel pathway involving MnSVWC, which is completely independent on the known IMD and Toll pathways. The signal transducers that are downstream of MnSVWC need to be investigated in further studies.

Conclusion

In summary, MnSVWC, a member of SVWCs family cloned from the river prawn M. nipponense, is identified as a novel type of PRR with broad-spectrum and sensitive recognition ability to diverse PAMPs and microbes. MnSVWC facilitates the removal of invading pathogen by mediation of cellular immune response, including agglutination and phagocytosis, rather than by the regulation of Toll or IMD pathways, or antimicrobial peptide genes.

Experimental Procedures

**Prawns, tissue sampling, and immune challenge**

Healthy wild freshwater prawns, M. nipponense, with average body weight of ~3.5 g were obtained from Baiyang Lake in Hebei province, China. The prawns were cultured in 50-l aquariums with aerated freshwater at 22 ± 1°C and fed with artificial grains twice a day for 7 to 10 days prior to experimentation in the laboratory. For tissue distribution analysis, hemolymph was drawn from 10 healthy prawns in an equal volume of anticoagulant (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7.0) and hemocytes were immediately isolated by centrifugation at 800 × g for 10 min. Meanwhile, intestine, muscle, Gill, and hepatopancreas were dissected out from 6 prawns. Hemocytes were directly collected for the test of phagocytosis or preserved in liquid nitrogen with other tissues together for further detection of gene expression.

For immunostimulation tests, 50 healthy prawns were challenged according to a previously reported procedure (29). Briefly, acclimated prawns were injected with a suspension of A. victoria (2 × 10\(^7\) colony forming units/milliliter, 6.5 μl/g) in physiologic saline solution into the last abdominal segment of each prawn. Six surviving individuals were randomly sampled at 0, 3, 6, 12, 24, and 48 h post injections and gills were dissected for qRT-PCR analysis.
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RNA isolation, cDNA cloning, and bioinformatics analyses

Total RNA was extracted from various tissues with TRIzol reagent (Invitrogen) following the manufacturer’s protocol and treated with RQ1 RNase-Free DNase (Promega) to remove contaminated DNA. cDNA was synthesized from 3 μg total RNA by M-MLV reverse transcriptase (Promega) following the manufacturer’s protocol with a universal primer AOLF (Table S1).

A transcript of gene-encoding SVWC protein (MnSVWC), which was significantly enhanced by bacterial challenge, was screened out from the M. nipponense transcriptome database SRX142691 in NCBI. The 3’-end of MnSVWC cDNA was obtained by 3’ RACE with gene-specific primer SVWC-F1 and 3’ adapter primer AP. PCR products were gel-purified, cloned into the pMD19-T simple vector (TaKaRa, China), and sequenced by Shanghai Sangon Company (China). According to the expression sequence tag sequence and the obtained sequence by 3’ RACE, a pair of gene-specific primers SVWC-F2/R1 were designed for verifying the full-length cDNA sequence by PCR and sequencing.

The deduced amino acid sequence basing the completed cDNA sequence was obtained by using an ORF finder program (RRID:SCR_016643). The homology search of the Nr and SwissPort database was performed using Blastp algorithm at NCBI (RRID:SCR_004870). The presence and location of signal peptide was predicted using the SignalP 4.1 program (RRID:SCR_015644). Physicochemical characteristics of the deduced protein were analyzed with the Expert Protein Analysis System (RRID:SCR_012880). The protein domains were searched via web CD search tools, including SMART for Ensembl database (RRID:SCR_005026) and Batch for NCBI database (RRID:SCR_018756). The GenBank protein sequences that can be significantly aligned with MnSVWC were collected to do multiple sequences alignment by ClustalW program. An unrooted phylogenetic tree based on the alignments was constructed by the maximum likelihood algorithm using the MEGA 5.0 program.

qRT-PCR analysis

Expression levels of MnSVWC mRNA were measured by qRT-PCR on a LightCycler system (Roche) using the SYBR Green kit (TaKaRa, China). The primer sequences used for amplifying target gene (RtSVWC-F/R) and β-actin gene (Ractin-F/R) as internal control were listed in Table S1. Expression levels of the target genes were calculated by comparing the cycle threshold value (Ct) with the reference gene β-actin. All samples were normalized to the ΔCt value of β-actin to obtain a ΔΔCt value (ΔCt target – ΔCt reference). The final relative expression was calculated using the following formula: $F = 2^{-\Delta\Delta Ct}$ (30). All the results were generated with three technical replicates.

Expression and purification of recombinant MnSVWC and MnSVWC<sup>P57A</sup>

A 351-bp DNA fragment coding for the mature peptide of MnSVWC was amplified by primers ExSVWC-F and ExSVWC-R (Table S1). The purified PCR product was digested with Hind III and EcoR I, and then subcloned into a pET-30a expression vector digested with the same enzymes. Meanwhile, a mutant MnSVWC<sup>P57A</sup> expressing plasmid was constructed. Primers ExSVWC-F and Mu-R were used to amplify fragment I; Mu-F and ExSVWC-R were employed to amplify fragment II. The full length of mutant MnSVWC<sup>P57A</sup> DNA fragment was obtained by overlapping PCR using the two purified DNA fragments as the templates, and then the product was digested and ligated to pET-30a. After that, the constructed plasmids (pET30a-MnSVWC and pET30a-MnSVWC<sup>P57A</sup>) were transformed into competent cells of E. coli BL21 (DE3) for fusion expression. Recombinant proteins were induced with 1 mM IPTG at 37°C for 4 h. Bacteria were harvested and lysed by sonication in PBS (pH7.4). The lysate was centrifuged at 10,000 × g for 10 min, analyzed by 15% SDS-PAGE, and visualized with Coomassie Brilliant Blue R250. rMnSVWC and rMnSVWC<sup>P57A</sup> were purified and desalted using Ni-NTA Agarose (GenScript, China) and Sephadex G-25 Resin (GE Healthcare), respectively. Protein concentration was determined by the Bradford method with BSA as a standard.

Microbial binding assay

The bacteria-binding assay was performed according to the method described by Wang et al. (31), with slight modifications. Briefly, G− bacteria (E. coli and A. victoria), G+ bacteria (S. aureus and B. subtilis), and yeast P. pastoris were used to test the binding activity of rMnSVWC. The bacteria were cultured in LB medium overnight and then were collected by centrifugation at 6000 × g for 5 min. After rinsing by PBS four times, the bacteria were resuspended in PBS and adjusted to A<sub>600</sub> = 2.0. The purified rMnSVWC (400 μl, 1 mg/ml) was mixed with 400 μl bacteria by shaking at 28°C for 1 h. Then the bacteria were collected by centrifugation at 6000 × g for 5 min and washed four times by PBS. The last eluate and the bacterial precipitate were subjected to 15% SDS-PAGE. The proteins in the gel were transferred to a nitrocellulose membrane for Western blot analysis using an anti-histidine antibody and an alkaline phosphatase-conjugated horse anti-mouse IgG antibody (ZSGB Bio, China) as the primary and secondary antibodies. rMnSVWC protein was purified and also subjected to Western blot analysis as a positive control.

Polysaccharide-binding assay

To test the binding ability of rMnSVWC to different microbial components, ELISA were performed as described previously (32). Eight PAMPs including LPS, Lys-type, and Dap-type PGN from S. aureus and E. coli, d-galactose, d-mannan and β-1,3-glucan, while soluble starch and D-trehalose served as the negative control. All the test ligands were provided by Merck and used in the assays. Each of these ligands was coated on the wells of flat-bottomed 96-well microtiter plates at 37°C overnight, and then evaporated at 60°C for 30 min. Every well was coated with 2 μg ligand in a volume of 50 μl. Each well was blocked with 200 μl BSA (1 mg/ml) in TBS for 2 h at 37°C and then washed four times with 200 μl TBS. The purified rMnSVWC (diluted with TBS containing 0.1 mg/ml BSA, 50 μl/well) was added and incubated at room temperature for 3 h. Plates were washed four times with binding buffer (200 μl/
well), and then 100 μl of horseradish peroxidase–labeled anti-His antiserum (1:2000 diluted) was added and incubated at 37°C for 1 h. After the plates were washed again four times with binding buffer, the color reaction was developed by 3,3’-5,5’-tetramethylbenzidine liquid substrate in citric acid–α-naphthol buffer (0.01%) and then stopped by adding 2 m H2SO4. The absorbance was read at 450 nm. Kd, the ligand concentration when half of the receptor was bound by the ligand, was used to assess the affinity of SVWC to polysaccharide. Kd was calculated using GraphPad Prism 5 with nonlinear regression curve fit and a one-site binding model analysis. A = Amax [L]/(Kd + [L]), where A is the absorbance at 450 nm and [L] is the concentration of the rMnSVWC. To investigate the effect of EPN motif in MnSVWC on the polysaccharide-binding activity, the mutant rMnSVWC-P57A was subjected to the same detection. The negative control experiment was performed using a recombinant His-tagged GSH S-transferase instead of the rMnSVWC or rMnSVWC-P57A protein. Each binding assay was performed three times.

**Microbial agglutination assay**

*E. coli*, *A. victoria*, *S. aureus*, *B. subtilis*, and *P. pastoris* were used again for agglutination tests. Microbes in mid-logarithmic phase were collected, heat killed (75°C for 30 min), washed with 0.1 M NaHCO3 (pH 9.0), and stained with FITC (Sigma) (1 mg/ml in 0.1 M NaHCO3) at 28°C for 1.5 h, with gentle shaking (33). The FITC-labeled microbes were washed with PBS three times to eliminate all free FITC and resuspended in PBS to A600 = 1.0. 50 μl microbe suspension was mixed with 50 μl rMnSVWC (1 mg/ml) in the presence or absence of 10 mM CaCl2. The mixtures were incubated at room temperature for about 1 h. BSA instead of rMnSVWC was used as a negative control. After incubation, agglutinating reactions were observed under a fluorescent microscope (Olympus BX51, Japan).

**Bacterial clearance assay**

The bacterial clearance assay was performed according to a previously described method with slight modifications (23). Briefly, the *E. coli* expressing GFP was cultured in LB medium with ampicillin. Bacteria in logarithmic growth phase were collected by centrifugation at 6000 × g for 5 min. After rinsing by PBS for three times, the bacteria were resuspended in PBS to A600 = 1.0, blended with a same volume of purified rMnSVWC (1 mg/ml), and then incubated by shaking at 28°C for 30 min. BSA instead of rMnSVWC was used as control. The mixture was injected into the body of *M. nipponense* with a microinjector. A group of 40 prawns were injected, and the injection volume was 5 μl/g body weight. Hemolymph was taken from each individual with an equal amount of filter-sterilized anticoagulant at 30 min and 1 h post injection. The hemolymph was diluted with PBS and then coated on the resistant plates. Colony counts were counted after being cultured overnight. To further investigate the roles of MnSVWC in innate immunity, we knocked down the expression of MnSVWC gene in *M. nipponense* by RNAi method. Primers dsSVWC-F/R were used to amplify the DNA fragment of MnSVWC for dsRNA (dsMnSVWC) synthesis. A T7 promoter sequence (5′-TACGACTCTAGATAGGG-3′) was introduced into both the primers. dsRNA was generated using a T7 transcription kit (Transgen Biotech, China) according to the manufacturer’s instructions. After purification, the dsRNA was dissolved in PBS at a final concentration of 2 μg/μl and injected into the abdominal segment of *M. nipponense* (4 μg/g). The dsRNA for GFP (dsGFP) was also injected in another group as the negative control. qRT-PCR was used to detect the RNAI efficiency at 24 h post dsRNA injection. At 24 h after RNAi, the prawns were injected with *E. coli* expressing GFP for bacterial clearance assay. Bacteria population in hemolymph was detected at 1 h post injection.

**Expression profiling of AMPs and NF-κB–like transcription factor genes**

After knockdown of MnSVWC, the prawns were challenged by *A. victoria*. The gill was collected to extract RNA at 12 h after bacterial injection. The expression level of AMP genes (ALF and crustin), and NF-κB–like transcription factor genes (relish and dorsal) were tested by qRT-PCR. Primer sequences for amplification of these immune genes and β-actin gene as internal control were listed in Table S1.

**Phagocytosis assay in vivo**

FITC-labeled *A. victoria* was coated with rMnSVWC or BSA as control, following the method above and used to determine the phagocytic capacity of hemocytes. The rMnSVWC-binding *A. victoria* was injected into the body of *M. nipponense* and hemolymph was drawn at 1 h after injection. Hemocytes were collected by centrifugation at 800 × g for 15 min, and resuspended in PBS. The cell suspension (50 μl) was added to the poly-lysine–treated slide and placed in a wet box for 30 min at room temperature. Droplets on the slide were absorbed and 200 μl polyformaldehyde (4%) was added. Hemocytes were fixed in a wet box for 20 min. Polyformaldehyde was absorbed and dyed with 4,6-diamino-2-phenyl indole (DAPI) after PBS cleaning. The hemocytes were monitored and the fluorescent images were taken using a confocal microscope (Olympus FV3000, Japan). The phagocytosis rate of 200 hemocytes was in statistical perspective. Phagocytosis rate was defined as (the hemocytes ingesting bacteria/all hemocytes observed or tested) × 100%.

**Hemocytes-binding assay**

The binding capacity of rMnSVWC to hemocytes was investigated in vivo by injecting rMnSVWC into prawns (10 μg/g) as described by Shi et al. (33). Hemolymph was collected at 30 min post injection, and the hemocytes were isolated according the method described above. The hemocytes were fixed with 1% paraformaldehyde in PBS for at least 10 min. The fixed hemocytes (50 μl) were dripped onto a poly-lysine–treated slide and allowed to settle for 30 min at room temperature. After blocking with 3% BSA in PBS for 30 min, an anti-His mouse antibody (1:1000 in PBS containing 3% BSA) was added and incubated for 8 h at 4°C, and the slides were washed six times with PBS. After blocking with 3% BSA in PBS for 5 min, FITC-conjugated horse anti-mouse IgG (Sigma, 1:1000 in PBS)

**MnSVWC acts as an extracellular PRR**
containing 3% BSA) was added to the glass slide and incubated for 1 h at 37°C in the dark, washed six times with PBS for 10 min each. Then, hemocytes were incubated successively with DAPI (1:1000 dilution in PBS) for 10 min and 1,1'-diocadecyl-3,3',3', tetramethylindocarbocyanine perchlorate (Dil) (1:500 dilution in PBS) for 20 min to stain nuclei and membrane, washed again six times with PBS for 10 min each, and examined under fluorescence microscope (Olympus BX51, Japan).

**Statistical analysis**

The SPSS software (version 18.0) was used to analyze all data as mean ± S.E. Data were analyzed by a two-tailed Student’s t test or analysis of one-way analysis of variance (ANOVA).

**Data Availability**

All data are contained within the article and accompanying supporting information.

**Author contributions**—N. Q. and M. L. data curation; N. Q., H. S., and M. L. investigation; N. Q. and T. T. methodology; H. S. formal analysis; J. W. and F. L. writing-review and editing; T. T. writing—original draft; T. T. and F. L. project administration; F. L. supervision; F. L. funding acquisition.

**Funding and additional information** —This work was supported by National Natural Science Foundation of China Grant No. 31572327 (to F. L.) and Natural Science Foundation of Hebei Province Grant No. C2019201194 (to F. L.).

**Conflict of interest** —The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations** —The abbreviations used are: PRR, pattern recognition receptor; SVWC, single von Willebrand factor C-domain protein; PAMP, pathogen-associated molecular patterns; IMD, immune deficiency; EPN, Glu-Pro-Asn motif; RACE, rapid amplification of cDNA ends; LPS, lipopolysaccharide; VWC, C-domain of von Willebrand factor; CRD, carbohydrate recognition domain; CTL, C-type lectin; AMP, antimicrobial peptides; ALF, anti-lipopolysaccharide factor; qRT, quantitative real-time; LB, Luria-Bertani; IPTG, isopropyl-β-D-thiogalactopyranoside.

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