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Selective expression of a "correct cloud" of Dscam in crayfish survivors after second exposure to the same pathogen

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ABSTRACT

Arthropod hypervariable Dscam (Down syndrome cell adhesion molecule) may be involved in adaptive-like immune characteristics, namely immune priming, enabling the host to "learn" and "remember" pathogens previously encountered in arthropods. However, expression of Dscam in immune-primed arthropods after a second challenge has apparently not been confirmed. Herein, working with Dscam of Australian freshwater crayfish (*Cherax quadricarinatus*, i.e. *Cq*Dscam), we further investigated whether immune priming is mediated by "clouds" of appropriate (or "correct") *Cq*Dscam isoforms. In crayfish that survived a first WSSV challenge (immune priming), long-lasting *Cq*Dscam expression remained higher after a second WSSV challenge. Selective *Cq*Dscam isoforms were also induced after both challenges. Based on pathogen binding assays, these WSSV-induced *Cq*Dscam isoforms had a higher WSSV binding ability, perhaps mainly mediated by Ig3-spliced variants. We therefore hypothesized that in these crayfish survivors, an unknown selection process was generating a "correct cloud" of *Cq*Dscam against a previously encountered pathogen.

1. Introduction

An immune response with specificity and immunological memory, also known as adaptive or acquired immunity, was historically regarded as being restricted to vertebrates [1,2]. However, there is increasing evidence that invertebrates may also have an alternative adaptive-like immune response. Four decades ago, it was reported that lobster hemocytes had an enhanced phagocytic activity when they re-encountered foreign antigens following previous exposure [3]. In addition, it was remarkable that graft rejection in earthworms was accelerated after secondary grafting [4] and that chance of re-infection in copepods was reduced with re-exposure to a previously encountered strain of parasites, but not unrelated parasites [5]. Moreover, resistance against pathogen infections was enhanced in shrimp and crayfish when vaccine-like treatments were tested [6,7]. Vaccination confers immunological memory against pathogen antigens, enhancing protection when the host encounters the corresponding pathogen [8]. These phenomena related to immune specificity and immune-memory in invertebrates implied a "primed" immune response [9,10].

Immune priming (also known as innate immune memory) means, after exposure to a pathogen, host immunity "learns" something from the first exposure [11,12]. Therefore, when the same pathogen is re-encountered, host immunity with this "memory" can be elicited and apparently enhanced [13], thereby increasing host survival rate or pathogen clearance. In vertebrates, T and B cells and antibodies confer this type of immunological memory; however, none of these are present in invertebrates [10]. Therefore, molecular mechanisms of immune specificity and immune-memory in invertebrates are inherently distinct from those in vertebrates. It was recently reported that shrimp surviving white spot syndrome virus (WSSV) had uncharacterized humoral neutralizing factors in hemolymph 2–8 wk after exposure to the virus [14,15]. More than 10 y later, work in crayfish concluded this unknown humoral neutralizing factor may be an immune-related protein called Down syndrome cell adhesion molecule (Dscam) [16].

In *Drosophila*, mosquitoes, shrimp and crab (only in arthropods but not in other invertebrates), Dscam consisting of the extracellular region and the cytoplasmic tail expressed hypervariable variants through alternative splicing. Variable regions of the Dscam extracellular region are located in the N-terminal Ig2 domain, the N-terminal Ig3 domain and the entire Ig7 domain, whereas those of the cytoplasmic tail are at a transmembrane domain and some cytoplasmic tail elements [17–21]. Subsequently, hypervariability of Dscam extracellular regions was

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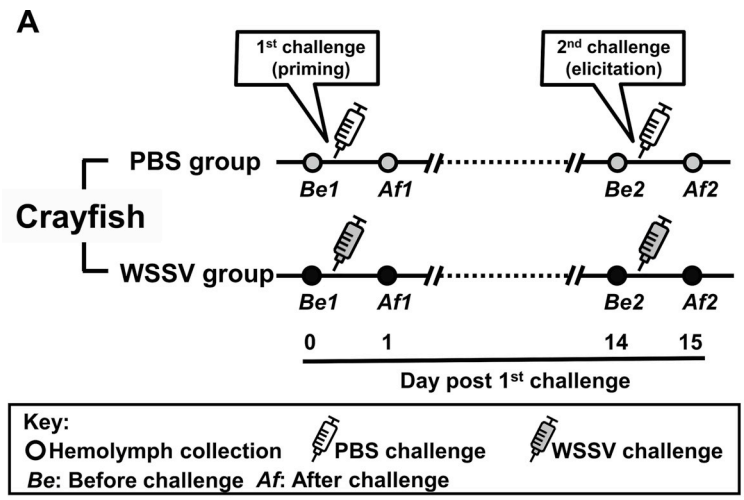

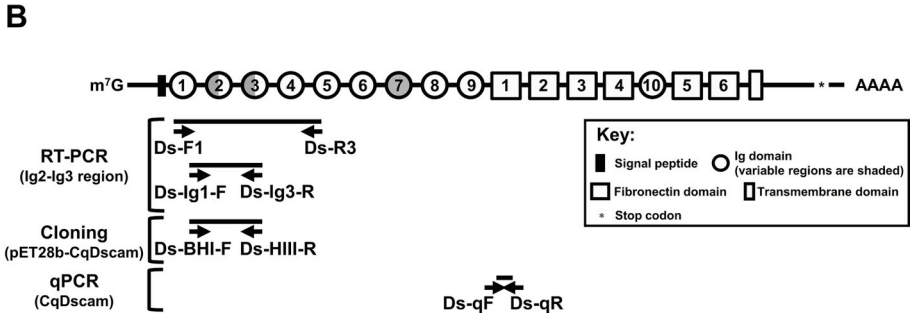

Fig. 1. (A) Schematic diagram of the experimental design for immune priming (first challenge) and immune elicitation (second challenge) in crayfish. At day 0, hemolymph *Be1* was collected from each individual crayfish before the first challenge. After hemolymph collection, one group of crayfish was challenged with WSSV, whereas the control group was given PBS, all by intramuscular injection. One day after the first challenge, hemolymph samples *(Af1)* were collected from each individual. At 14 d after the first challenge, hemolymph was again collected from survivors *(Be2)*, after which the WSSV-treated group was challenged with WSSV for a second time, whereas the PBS-treated group was injected with PBS. One day later, hemolymph samples *(Af2)* were again collected from each individual. Hemocytes from each hemolymph sample were subjected to Dscam expression analysis. (B) Schematic diagram showing locations of primers used for RT-PCR, cloning and real-time PCR (qPCR) relative to the gene structure of *Cq*Dscam.

confirmed to contribute in this immune specificity of Dscam, whereas that of Dscam cytoplasmic regions may be responsible for the switch between membrane-bound versus soluble forms, as well as between signal transductions of immune response or neuronal guidance [16,20,22–24].

Regarding Dscam-mediated immune specificity, several reports suggested that after pathogen challenge, Dscam with specific combinations of Ig2 and Ig3 spliced variants were induced and functioned as a pattern recognition receptor with pathogen-specific recognition [18,20,22,24,25]. Potential release and adhesion mechanisms to explain how arthropod Dscam triggered phagocytosis were proposed and tested, suggesting that after secreted soluble Dscam binds with a pathogen, the complex will further interact with membrane-bound Dscam via a hemophilic interaction, resulting in pathogen-specific phagocytosis to remove specific invaders [20,26].

Although improved survival rates were reported due to enhanced immunity when the host was subjected to a second challenge with the same pathogen, factor(s) that mediated the enhanced immunity was not determined [15,27]. To evaluate potential involvement of Dscam in immune "memory", Ng et al. (2014) [16] reported that after the first challenge with white spot syndrome virus (WSSV), Australian freshwater crayfish (*Cherax quadricarinatus*) Dscam (*Cq*Dscam) provided prolonged (up to 2 mo) immune protection, i.e. anti-WSSV neutralizing ability. Compared to short-lifespan arthropods (*Drosophila* and mosquitoes), crustaceans with a relatively longer lifespan are more likely to re-encounter a previous pathogen and therefore may be more suitable subjects to study immune priming [16,28,29]. Regardless, to our knowledge, involvement of Dscam in immune priming combined with a second challenge has not been reported.

Therefore, in the present study, we performed a pioneering study, using *C. quadricarinatu* that had survived a first WSSV infection and we determined whether there were *Cq*Dscam responses in an immune priming-challenge test after a second WSSV infection. We investigated effects of WSSV priming to mediate a selective expression of appropriate "clouds" of *Cq*Dscam against a previously encountered pathogen and also evaluated WSSV binding ability of these selected WSSV-induced *Cq*Dscam isoforms.

2. Materials and methods

2.1. Experimental animals

Cherax quadricarinatus (Australian freshwater crayfish, ~3 cm long) were obtained from local crayfish culture farms in Pingtung, Taiwan. Before the experiment, crayfish were maintained in an aerated water tank system at 26–28 °C for at least 7 days. Unfortunately, no SPF crayfish were available. On Day 0, expression level of VP28 was very low, indicating that there was only a low-grade, persistent infection in these crayfish. We randomly allocated crayfish into tanks and injected them with either PBS or WSSV solution. Consequently, any effects of persistent infection should be equivalent between these groups.

2.2. WSSV virus inoculum

Standard virus inoculum stock of white spot syndrome virus (WSSV) Taiwan isolate (GeneBank accession number AF440570) used in this study was provided by the International Center for the Scientific Development of Shrimp Aquaculture, National Cheng Kung University, Taiwan. Experimental inoculum was prepared from virus inoculum stock (3.3×10^4 WSSV copies/μl) by 10^9 dilutions with cold phosphate-buffered saline (PBS; 137 mM NCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4).

2.3. Immune priming and second challenge with WSSV

A schematic diagram of the *C. quadricarinatus* immune priming challenge (first injection) followed by a second challenge (second injection) is shown in Fig. 1A. Briefly, crayfish were randomly allocated into two groups, the PBS-injected control group and WSSV-injected experimental group. Individuals cultured in the same tank were identified by fluorescent visible implant elastomer (VIE) tags injected into a semi-transparent abdominal area. For immune priming, after *Be* samples (designated *Be*1 were collected from each individual on day 0, crayfish were given an intramuscular injection (50 μl) of either PBS saline or diluted WSSV inoculum. At 1 d post challenge, *Af1* hemolymph samples were collected. Fourteen days after the first challenge, survivors were injected with a second dose of PBS saline or WSSV inoculum with *Be2* and *Af2* samples collected after first exposure.

2.4. RNA extraction and cDNA synthesis

Immediately after each hemolymph sample was collected, it was centrifuged (10,000 × *g* for 3 min) and the pellet subjected to RNA extraction and cDNA synthesis with Superscript II (Invitrogen) and Anchored oligo dTv primer (Table 1). The cDNA samples were stored at −20 °C until used to determine *Cq*Dscam Ig2/Ig3 combinations by RT-PCR as well as mRNA expression of Dscam, proPO and EF1α and WSSV VP28 by real-time PCR. Primer sets are listed in Table 1.

2.5. Diversity and combinations of Ig2 and Ig3 spliced variants of CqDscam

Diversity and combinations of Ig2 and Ig3 spliced variant analysis were determined and analyzed as described [16]. Briefly, fragments containing Ig2 and Ig3 spliced variants were amplified from each hemocyte cDNA, using the primer set Ds-F1/Ds-R3 for the first PCR and the primer set Ds-Ig1-F/Ds-Ig3-R for the nested RT-PCR (Table 1; Fig. 1B). Amplicons were cloned into RBC T&A cloning vector (RBC Bioscience) and 20 individual colonies from each sample were sequenced. Genedoc software was used to align resulting sequences. Obtained sequences of Ig2 and Ig3 spliced variants are shown (Tables S1 and S2). Variable isoform definition was followed by Ng et al. (2014) [16].

2.6. Quantification of host genes and WSSV VP28 by real-time PCR

The mRNA expression levels of *Cq*Dscam and *Cq*proPO, important innate non-specific immune factors, were determined by real-time PCR using specific primer sets Ds-qF/Ds-qR and PO-qF/PO-qR, respectively (Table 1 and Fig. 1B). In addition, WSSV VP28, used as an indicator of virus replication, was amplified using the specific primer set VP28-qF/VP28-qR, whereas the housekeeping gene EF1α was amplified using the specific primer set EF1α-qF/EF1α-qR (Table 1). Real-time PCR reactions were performed using a Bio-Rad detection system. Data for *Cq*Dscam, *Cq*proPO and WSSV VP28 were normalized against the housekeeping gene EF1α and expressed as $2^{-\Delta Ct}$.

2.7. Construction and expression of recombinant Ig2-and Ig3 -spliced variants of CqDscam

An *Escherichia coli* expression system was used to produce recombinant Ig2 and Ig3 spliced variants of *Cq*Dscam, as described [22]. First, the primer set Ds-BHI-F/Ds-HIII-R (Table 1) was used to amplify the *Cq*Dscam Ig1-Ig3 partial sequence fragment of each selected combination from its corresponding plasmid. Restriction enzymes BamHI and HindIII were used to digest Ig1-Ig3 partial sequence fragments and treated fragments were ligated to the pET-28b (+) vector. Recombinant plasmids were transformed into *Escherichia coli* BL21 (DE3) CodonPlus-RIL cells (Stratagene), with expression of recombinant proteins induced by IPTG, according to manufacturer's instructions. After IPTG induction, bacteria pellets were collected and re-suspended in 1X PBS with 10% glycerol and subjected to the first sonication. After centrifugation at 10,000 × *g* for 10 min at 4 °C, bacteria pellets were re-suspended in 1X PBS with 1.5% N-Lauroylsarcosin for the second sonication. After centrifugation, supernatants were collected and subjected to buffer replacement and protein concentration using 10-kDa ultrafiltration membranes (MILLIPORE). SDS-PAGE and Western blotting were conducted for quality checking. Concentrations of each sample (0.25 μg of each recombinant protein) were measured by SDS-PAGE (using BSA standards) and stained with Coomassie Brilliant Blue R-250 (Fig. S1A). For Western blotting, the SDS-PAGE was transferred to PVDF membranes and reacted with primary antibody (rabbit anti-His antibody) and alkaline phosphatase-conjugated secondary antibody (Fig. S1A). Recombinant EGFP protein was expressed and treated by the same method and used as a control protein. Quantified recombinant *Cq*Dscam isoforms and rEGFP were then used in pathogen-binding assays (described below).

2.8. In vitro pathogen binding assay

The pathogen binding assay used herein against WSSV and bacteria was modified from Hung et al. (2013) [22] and Ng et al. (2014) [16]. For the WSSV binding assay, WSSV virions were purified as described [16]. Each recombinant protein (0.5 μg) was mixed with a total of 0.4 μg purified WSSV virions (~37,000 genome copies) and PBS was added to a final volume of 100 μl. For bacteria binding assays, when

Table 1
Primers used in this study.

Gene	Primer name	Primer sequence (5′-3′)[a]	Usage
*Cq*Dscam	Ds-F1	5′- GACAACCGTGTGGACTTCAGC-3′	RT-PCR [Ig2-Ig3 region]
	Ds-R3	5′- GAACTGCAGAGATGTTCAAG-3′	RT-PCR [Ig2-Ig3 region]
	Ds-Ig1-F	5′- CTGATGTTCCCTCCCTTC-3′	RT-PCR [Ig2-Ig3 region]
	Ds-Ig3-R	5′- CACGTATTATTAGGGTAC-3′	RT-PCR [Ig2-Ig3 region]
	Ds-BHI-F	5′- CCGGATCCGATGTTCCCTCCCTTC -3′	Cloning of pET28b-*Cq*Dscam
	Ds-HIII-R	5′- CCAAGCTTCACGTATTATTAGGGTAC -3′	Cloning of pET28b-*Cq*Dscam
	Ds-qF	5′- GGACGCCTCCCTATAATGGAA-3′	qPCR for *Cq*Dscam
	Ds-qR	5′- CGGCTCAACTTGTATTCAACAATG-3′	qPCR for *Cq*Dscam
*Cq*proPO	PO-qF	5′- TGCCAATGCCGTGTCCTT-3′	qPCR for *Cq*proPO
	PO-qR	5′-CATGGGTCTCTTGTCAGGGTACT -3′	qPCR for *Cq*proPO
*Cq*EF1α	EF1α-qF	5′- GGAGAATTTGAAGCTGGGATTTC-3′	qPCR for *Cq*EF1α
	EF1α-qR	5′- CAACTGCTTCACACCCAAGGT-3′	qPCR for *Cq*EF1α
WSSV VP28	VP28-qF	5′- AGTTGGCACCTTTGTGTGTGGTA-3′	qPCR for WSSV VP28
	VP28-qR	5′- TTTCCACCGGCGGTAGCT-3′	qPCR for WSSV VP28
Others	Anchored oligo dTv	5′-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTv-3′	cDNA synthesis

[a] The restriction site is underlined.

OD600 reached 1.6, *E. coli*, *Staphylococcus aureus* and *Vibrio parahaemolyticus*, were collected and pelleted by centrifugation (4500 × *g* for 10 min at 4 °C). After washing three times with PBS, pellets were re-suspended in PBS with each recombinant protein (0.5 μg) and PBS added to a final volume of 100 μl. Mixtures were incubated overnight at 4 °C under gentle agitation and then pelleted by centrifugation (15,000 × *g* for 30 min at 4 °C for WSSV and 4500 × *g* for 10 min at 4 °C for bacteria). After removal of supernatant, pellets were re-suspended in 100 μl of carbonate/bicarbonate buffer, pH 9.6. Suspensions were then coated onto 96-well polystyrene microplates and incubated for 2 h at 37 °C. To detect recombinant proteins that bound tested pathogens, wells were blocked with blocking buffer (2% BSA in PBST) and then incubated with anti-His antibody for 1 h at room temperature. After washing and incubating with a goat anti-rabbit HRP-conjugated secondary antibody, signal was developed by adding TMB (3,3′,5,5′-Tetramethylbenzidine) substrate (Sigma) for 3 min in the dark at room temperature and subsequently stopped using an equal volume of 1 N HCl. Signal values were determined by optical absorbance at 450 nm. All samples were analyzed in duplicate.

2.9. Statistical analyses

Differences in pathogen binding affinities of various recombinant proteins were analyzed by ANOVA, with Tukey's multiple-comparison test used to locate differences (SPSS computer software). For all analyses, $P < 0.05$ was considered significant.

3. Results and discussion

A vaccination-like response was reported in shrimp and crayfish, although only observational data, e.g. survival or phagocytic rates, were used to evaluate responses to immune priming [30]. Without investigating molecular mechanisms, it is difficult to elucidate immune priming in invertebrates [31]. Regarding immune factors involved in vertebrate immune memory, their protection level returned to baseline after pathogen first exposure, but rapidly invoked a more robust second response if the host re-encountered the same pathogen [13,32]. Since Dscam have been proposed to have a role in arthropod immunity with specificity [26,33], in this study, we aimed to investigate whether *Cq*Dscam was involved in immune priming with WSSV, which is able to elicit enhanced protection in surviving crayfish when re-encountered with a previous pathogen.

Here, we conducted an immune priming test. Individual crayfish were primed by given a first dose of live WSSV, followed 14 d later by a second challenge with another dose of live WSSV (Fig. 1A). A prophenoloxidase (proPO) response occurs within 6 h post infection, whereas *Cq*Dscam started to respond between 24 and 48 h after WSSV infection [16,34]; therefore, mRNA expression level of WSSV VP28, *Cq*Dscam and proPO were assessed before (*Be*) or 1 day after (*Af*) both first and second challenges in the same individual crayfish (Fig. 1A). From *Be1* through *Af2*, there was a mostly consistent trend of a steady increase in levels of WSSV VP28 in all WSSV-challenged crayfish (n = 9; W1 ~ W9) (Fig. 2). In parallel, within the same interval, the *Cq*Dscam mRNA level was also induced in most individual crayfish in the WSSV group. However, there was no such trend in levels of prophenoloxidase (proPO) mRNA expression (Fig. 2), although innate immune factors, e.g. PO-related factors and antimicrobial peptides (AMPs), are considered underpinning molecules for immune priming in insects [31]. In control crayfish treated with PBS (n = 4; P1 ~ P4; Fig. 3), there was no consistent upward trend in mRNA expression levels of either *Cq*Dscam or proPO.

Based on the consistent increase in *Cq*Dscam between *Af1* and *Af2* (Fig. 2), we inferred that long-term priming of *Cq*Dscam was induced by the first WSSV challenge and that it persisted for at least 14 d. This was consistent with our previous study in which *Cq*Dscam conferred prolonged immune protection (up to 2 mo) against WSSV in crayfish [16]. Notwithstanding, increased Dscam expression did not make crayfish resistant to WSSV (i.e. it did not prevent virus replication).

Dscam achieves mRNA diversity by producing thousands of isoforms through mutually exclusive alternative splicing in arthropods [21,35]. There are still knowledge gaps regarding Dscam as the immune factor mediating immune priming in arthropods; for example, evidence that Dscam isoforms can be expressed more intensely after pathogen re-exposure [29,36]. To investigate whether immune priming with WSSV induced expression of selective *Cq*Dscam isoforms in crayfish, spliced variants of the *Cq*Dscam Ig2-Ig3 region before and after the 1st and 2nd challenges were amplified, cloned and sequenced. Spliced variants induced after the first WSSV infection and then induced again after the second WSSV infection are highlighted (Fig. 4A). Furthermore, spliced variants induced after both first and second injections of PBS in the control group are also shown (Fig. 4B). A complete list of all PBS- and WSSV-induced spliced variants is provided (Table 2). Some spliced variants were induced only by WSSV or only by PBS, whereas others were induced by both. Despite substantial differences among individual crayfish, we inferred that priming might mediate a selective expression of "correct cloud" of *Cq*Dscam against previous stimulation. Currently, several signaling pathways, e.g. Imd and Toll pathways, may be involved in alternative splicing of arthropod Dscam after the first challenge [25]. It will be interesting to determine if these host pathways (or novel ones) are involved in expression of "selective" Dscam isoforms after the second pathogen challenge.

To determine whether WSSV-selective spliced variants of the *Cq*Dscam Ig2-Ig3 region bound more specifically to WSSV than to other pathogens, several combinations of *Cq*Dscam Ig2-Ig3 spliced variants were expressed as recombinant proteins and subjected to pathogen binding assays with WSSV, *E. coli*, *S. aureus* and *V. parahaemolyticus*. WSSV-selective rDscam isoforms, WW1 (r*Cq*Dscam$_{2.11,\ 3.1}$), WW2 (r*Cq*Dscam$_{2.11,\ 3.35}$) and WW3 (r*Cq*Dscam$_{2.11,\ 3.16}$), consisted of a single WSSV re-induced Ig2 spliced variant (Ig2-11) combined with one of three WSSV re-induced Ig3 spliced variants (Ig3-1, Ig3-35, Ig3-16; Table 3). Ideally, PBS-selective control rDscam isoforms would have likewise consisted of PBS re-induced Ig2 and Ig3 variants. However, as this kind of combination was not available, control rDscam isoforms, PW1 (r*Cq*Dscam$_{2.15,\ 3.3}$) and PW2 (r*Cq*Dscam$_{2.15,\ 3.16}$), instead consisted of a single PBS re-induced Ig2 spliced variant (Ig2-15) combined with WSSV re-induced Ig3 spliced variants (Ig3-3, Ig3-16; Table 3). Amino acid sequences and expression of the r*Cq*Dscams are shown in Table S3 and Fig. S1, respectively. Recombinant EGFP protein (rEGFP) was expressed as the non-specific binding control.

Although all of rDscam isoforms had a low binding affinity to *E. coli*, *S. aureus* and *V. parahaemolyticus,* the three WW combinations had higher binding affinity for WSSV (Fig. 5). Therefore, pathogen-induced Dscam isoforms bound to their respective eliciting pathogens, as reported in flies, mosquitos, shrimp and crabs, using binding assays with recombinant Dscam isoforms [18,20,22,24]. Furthermore, PW2 (r*Cq*Dscam$_{2.15,\ 3.16}$; Fig. 5A) which shared a WSSV-induced Ig3 spliced variant, i.e. [Ig 3.16] with WW3 (r*Cq*Dscam$_{3.11,\ 3.16}$), also had a relatively strong WSSV binding ability, compared to that of PW1 (r*Cq*Dscam$_{2.11,\ 3.1}$) which shared a PBS-induced Ig2 spliced variant, i.e. [Ig 2.15]. In mosquitos, inclusion of pathogen-induced Ig2 spliced variants, defined as high induced variants after pathogen challenge, were more effective against parasites than Ig3-or Ig7-spliced variants [25,37]. The first four Ig domains (Ig1 ~ Ig4) of the N-terminal of Dscam protein have a horseshoe configuration, as determined with X-ray structure analysis [38]. Two variable Ig domains, Ig2 and Ig3, both contribute to composite surface epitopes, namely epitope I and epitope II. Epitope I is contributed by N-terminus of variable spliced variants of these two Ig domains, whereas epitope II is contributed by a C-terminus [38]. These authors proposed that epitope I was important to homophilic binding specificity, whereas epitope II may be involved in heterophilic, non-Dscam binding, based on its structure and relatively rapid-evolving sequence variability. These sequence logos of two

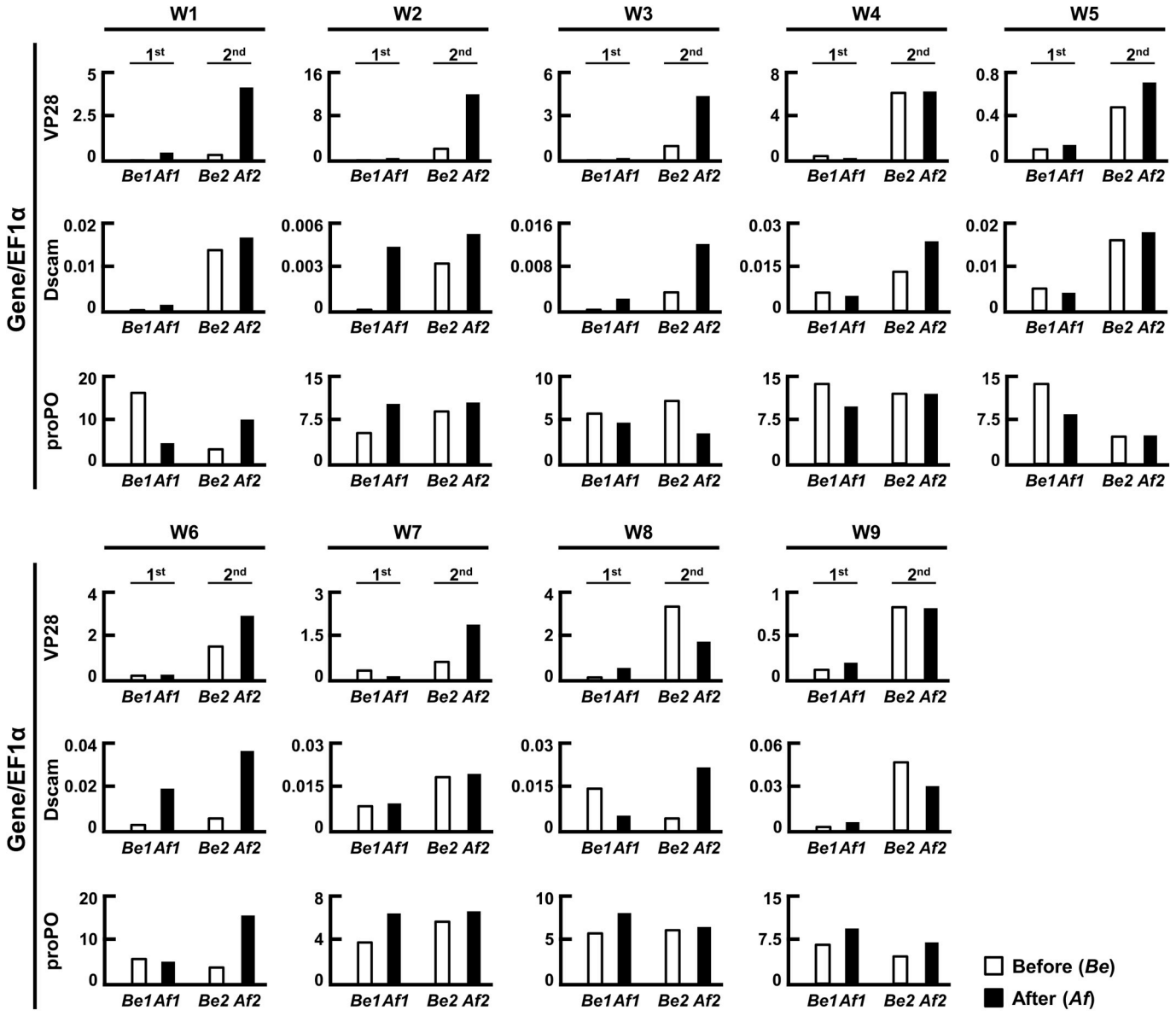

Fig. 2. Effects of first and second WSSV challenges on mRNA expression levels of VP28, *Cq*Dscam and *Cq*proPO in crayfish W1–W9. Samples of hemocytes were collected from the same individual crayfish at indicated time points. Bar graphs of relative mRNA expression levels (real-time PCR).

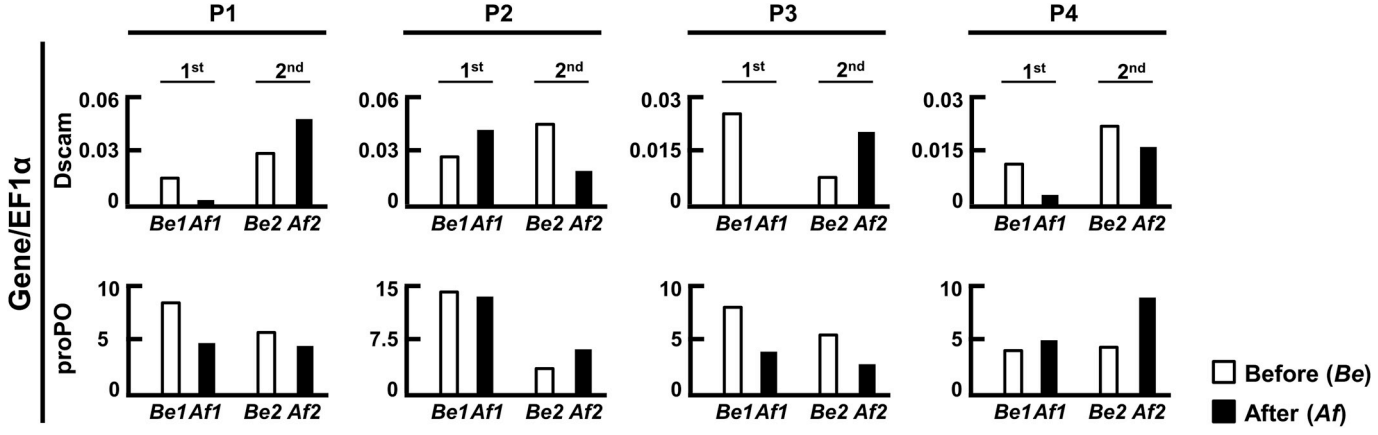

Fig. 3. Effects of first and second PBS challenges on mRNA expression levels of *Cq*Dscam and CqproPO in crayfish P1–P4. Samples of hemocytes were collected from the same individual crayfish at indicated time points. Relative mRNA expression levels were determined by real-time PCR.

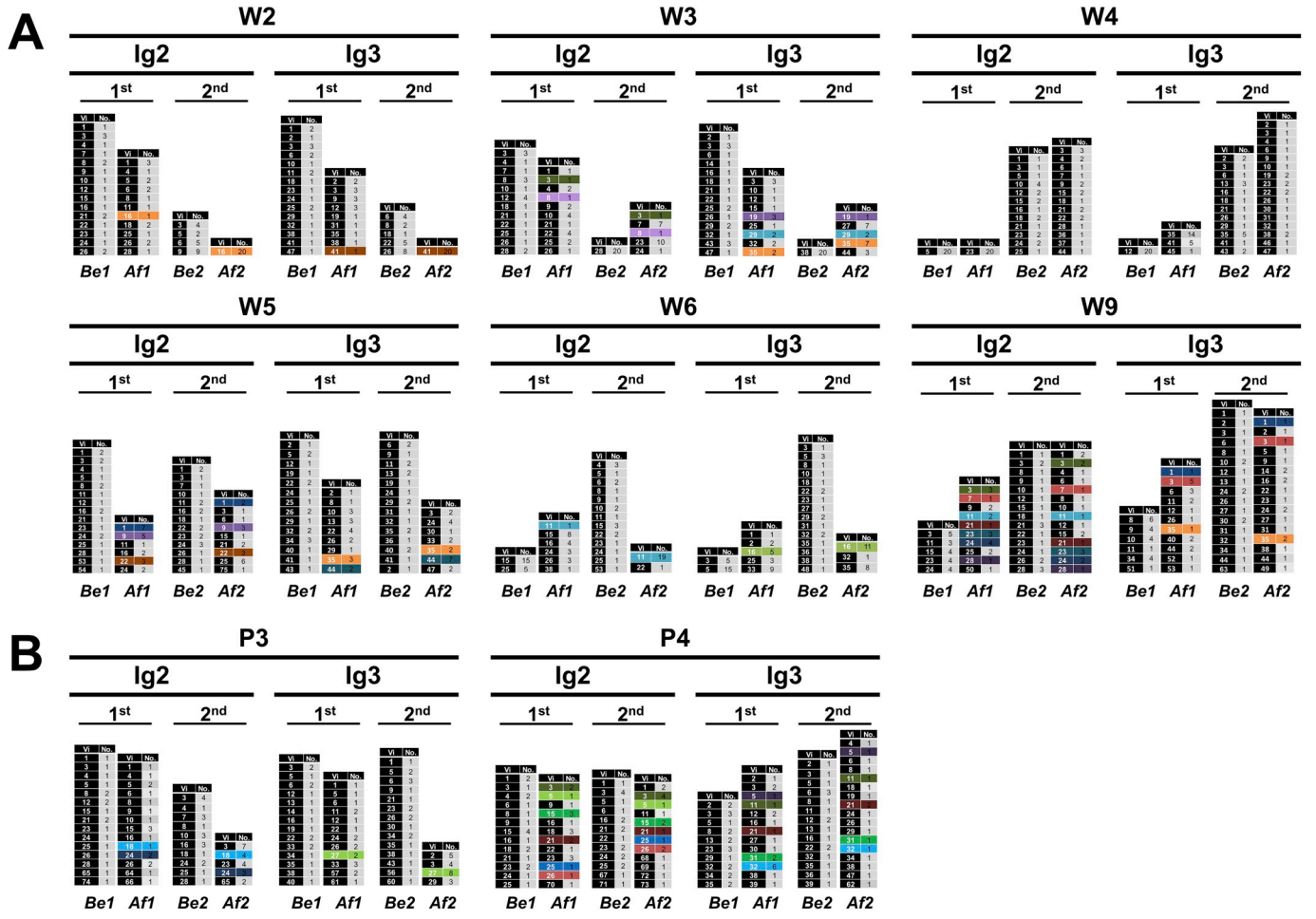

Fig. 4. *Cq*Dscam Ig2 and Ig3 spliced variants expressed in hemocytes of individual crayfish after first and second injections of (A) WSSV or (B) PBS. After Ig2 and Ig3 regions were amplified by PCR, 20 colonies from each sample were sent for sequencing. Ig2-or Ig3-spliced variants induced in both the first and second challenges are highlighted with a common color. Vi = splice variant number (see Ng et al., 2014 for variable isoform definitions); No. = number of colonies. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Spliced variants in Ig2 and Ig3 isoforms induced by PBS and WSSV 24 h after both the first and second challenges.

Ig2			Ig3		
Vi	PBS	WSSV	Vi	PBS	WSSV
1		+	1		+
7		+	3		+
8		+	16		+
9		+	19		+
11		+	29		+
16		+	35		+
22		+	41		+
23		+	44		+
28		+	5	+	
3	+	+	11	+	
21	+	+	21	+	
24	+	+	27	+	
5	+		31	+	
15	+		32	+	
18	+				
25	+				
26	+				

*Vi: spliced variant number.

Table 3
Ig2 + Ig3 combinations in r*Cq*Dscam isoforms used in a pathogen-binding assay.

*Cq*Dscam isoforms		Crayfish [c]
Name	Vi numbers of Ig2/Ig3 combination	
PW1[a]	**[Ig2-15] + [Ig3-3]**	**W4**
PW2[a]	**[Ig2-15] + [Ig3-16]**	**W4**
WW1[b]	**[Ig2-11] + [Ig3-1]**	**W6**
WW2[b]	**[Ig2-11] + [Ig3-35]**	**W6**
WW3[b]	**[Ig2-11] + [Ig3-16]**	**W6**

[a] PW1 and PW2 isoforms were comprised of an Ig2 variant re-induced (i.e. induced after both first and second challenge) by PBS and an Ig3 variant re-induced by WSSV.

[b] WW1, WW2 and WW3 isoforms were comprised of entirely Ig2 and Ig3 variants that were re-induced by WSSV.

[c] Crayfish from which isoforms were isolated.

epitopes located in Ig2-and Ig3-spliced variants were also in *Cq*Dscam (Fig. 6), suggesting it may function as proposed [38]. Our data also supported the hypothesis proposed by Meijers and his team, namely that Ig2 and Ig3 are important to Dscam-mediated immunity with specificity to dynamics in host–pathogen interactions. Compared to Ig2-spliced variants, Ig3-spliced variants of *Cq*Dscam may have an important role for sufficient WSSV binding (Fig. 5), suggesting that whereas it was clear that inclusion of the Ig2-spliced variant may increase binding ability to the particular pathogen, Ig3 variants may be involved in pathogen-binding specificity.

In conclusion, we confirmed that long-lasting *Cq*Dscam expression was induced upon priming survivors after a 2nd challenge with WSSV

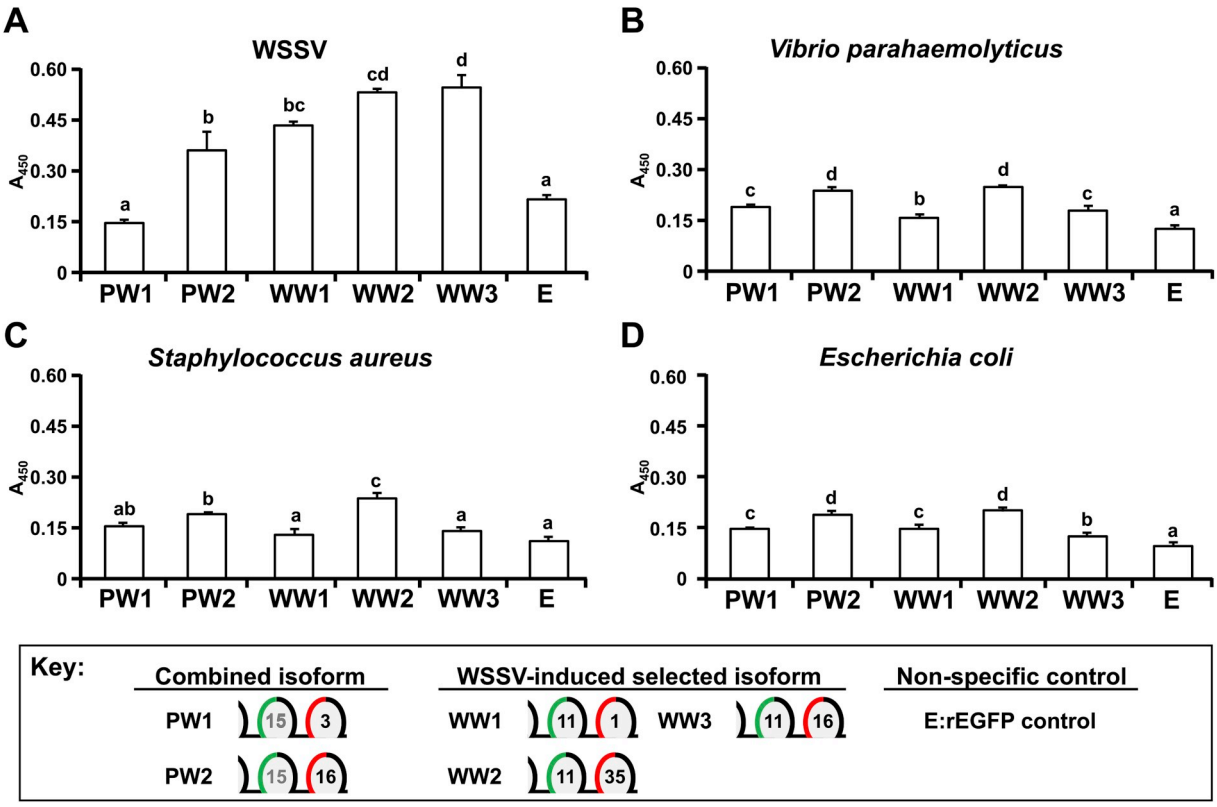

Fig. 5. Binding specificity of selected r*Cq*Dscam Ig2 + Ig3 isoforms against (A) WSSV, (B) *Vibrio parahaemolyticus*, (C) *Staphylococcus aureus* and (D) *Escherichia coli*. Pathogens were mixed with recombinant proteins overnight, then washed and coated onto ELISA plates. Optical density values (A450) represent the mean ± SD of duplicate ELISA assays. [a-d]Recombinant proteins without a common letter had different binding abilities ($P < 0.05$).

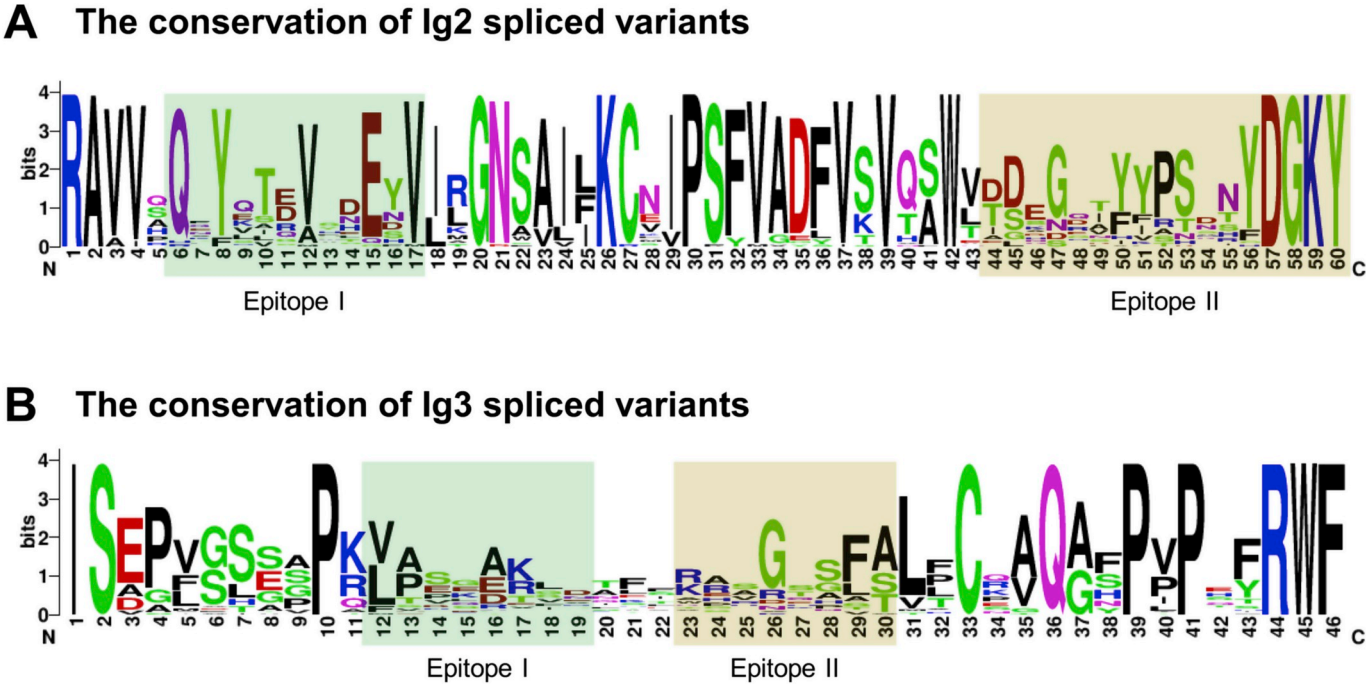

Fig. 6. *Cq*Dscam conservation of epitopes I and II of (A) Ig2 spliced variant and (B) Ig3 spliced variants. A total of 40 from 41 Ig2 variants with 60 amino acids and 37 from 52 Ig3 variants with 46 amino acids were used to compare sequence logo representation. The y-axis indicates units of evolutionary conservation.

in crayfish; that this phenomenon follows another hypothetical scenario for immune priming in invertebrates, i.e. enhanced survival from the second challenge with the same invader, may be due to induction of long-lasting defences from immune-priming, rather than the "true immune-memory" [31]. Despite differences of Dscam population and its expression pattern differing significantly among individual crayfish, WSSV priming contributed to long-lasting *Cq*Dscam mRNA expression that was maintained at a high level upon secondary infection. In

addition, results of selective *Cq*Dscam isoforms upon secondary infection yielded further insights into how Dscam may function as a highly variable immune molecule involved in immune priming in arthropods. For pathogen binding and recognition, not only Ig2-and Ig3-spliced variants of *Cq*Dscam may have important roles. Our observation raised the question regarding how the election process of spliced variants on mRNA was able to “create” the “correct” or appropriate *Cq*Dscam isoforms and whether epigenetic modifications were involved in selection. Nevertheless, we cannot exclude another possibility, which is a change in the hemocyte cell populations with “correct cloud” of *Cq*Dscam isoform after WSSV infection. Presence of a “correct cloud” of *Cq*Dscam isoform selection and/or maintenance of “appropriate” hemocytes to combat a second infection remains an open question.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.06.023.

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