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In vivo administration of LPS and β-glucan generates the expression of a serum lectin and its cellular receptor in *Cherax quadricarinatus*

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ABSTRACT

In crustaceans, it has been suggested that specific protection against pathogens could be triggered by vaccines and biological response modifiers; although the specific mechanisms of this protection have not been clarified yet. In the crayfish *Cherax quadricarinatus*, a humoral lectin (CqL) binds its own granular hemocytes through a specific receptor (CqLR) and increases the production of reactive oxygen species (ROS). In the present study, we challenged *in vivo* crayfishes with immunostimulants, β-glucan (200 μg/kg) or LPS (20 μg/kg), and identified the participation of cellular and humoral mechanisms. The stimulants generated a complex modification in the total hemocytes count (THC), as well as in the proportion of hemocyte subsets. At 2 h after the challenge, the largest value in THC was observed in either challenged crayfishes. Furthermore, at the same time, hyaline hemocytes were the most abundant subset in the hemolymph; after 6 h, granular hemocytes (GH) were the most abundant hemocyte subset. It has been observed that a specific subset of GH possesses a CqLR that has been related to ROS production. After 2 and 6 h of the β-glucan challenge, a significant increase in CqLR expression was observed in the three circulating hemocyte subsets; also, an increased expression of CqL was detected in a granular hemocytes sub-population. After 2 and 6 h of stimulation, the specific activity of the serum lectin challenged with β-glucan was 250% and 160% higher than in the LPS-treated-group, respectively ($P < 0.05$). Hemocytes from challenged crayfishes were stimulated *ex vivo* with CqL, ROS production was 180% higher in hemocytes treated with β-glucan + CqL than in hemocytes treated with LPS + CqL ($P < 0.05$). The results evidence the effectivity of immune stimulators to activate specific crayfish defense mechanisms, the participation of CqL and its receptor (CqLR) could play an important role in the regulation of immune cellular functions, like ROS production, in *Cherax quadricarinatus*.

1. Introduction

Crustaceans are a worldwide economically relevant group in aquaculture continuously affected by several diseases that impact their production [1]. These organisms possess an immune system capable of identifying and eliminating pathogens through pathogen recognition receptors (PRRs), which, in turn, are able to recognize pathogen-associated molecular patterns (PAMP), such as lipopolysaccharides, β-glucans, peptidoglycans, and genetic material [2,3].

In crustaceans, humoral immune mechanisms, such as the prophenoloxidase system (proPO), coagulation, antimicrobial peptides, and lectins, play a crucial role in the elimination of pathogens [4]. Hemocytes participate in cellular immunity, they have been classified in three main cellular populations based primarily on morphological criteria [5]. The granular hemocytes (GH) are the biggest cells in the hemolymph, they have a kidney-shaped nucleus and cytoplasm filled with large granules; semi-granular hemocytes (SGH) present a central nucleus surrounded by cytoplasmatic granules of different sizes and shapes; hyaline hemocytes (HH) are the smallest hemocytes, their cytoplasm lacks granules [5]. These cells can exert phagocytosis, which consists in internalizing and eliminating pathogens by oxygen-dependent and oxygen-independent mechanisms, and encapsulation, which generates multiple layers of hemocytes that participate in the elimination of pathogens through the secretion of cytotoxic [6,7]. It has been suggested that lectins regulate humoral and cellular mechanisms [8]. Some lectins show affinity for LPS and enhance phagocytosis [9,10], encapsulation [11], and the proPO system [12]. *Cherax quadricarinatus* lectin (CqL) interacts with its membrane receptor in a subset

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of granular hemocytes and triggers specific kinases leading to intracellular signaling and producing reactive oxygen species (ROS), a relevant mechanism that participates in the elimination of pathogens [13,14].

The Gram-negative binding proteins (GNBPs) and β-glucan receptor proteins (βGRP) are PRRs in invertebrates, including crustaceans [15]. Most of these proteins are secreted into the hemolymph, but at least one may be membrane bound via a GPI-linked anchor; besides, they present immunomodulating activity and are implicated in a variety of immune responses in invertebrates, including the activation of the proPO cascade and anti-microbial peptide production [16–18]. Expression of defense proteins has been suggested to be induced upon infection with yeast or bacteria [19]; however, the mechanism by which binding of β-glucan triggers humoral or cellular innate immune responses in crustaceans has not been identified clearly.

This work was aimed at identifying the potential role of biological response modifiers, LPS and β-glucan, to regulate humoral and cellular immune responses by activating lectin expression and its effect on hemocytes function, as well as determining the specific mechanisms that allow immunostimulants to increase the activity of the immune system resulting in protection against crustacean pathogens [20].

2. Materials and methods

2.1. Organisms

Adult male *C. quadricarinatus* crayfishes (49.3 g ± 4.9) in intermolt stage were obtained from the "Las Cruces" fish farm, Jojutla, Morelos, Mexico. Before the experimental study, the organisms were acclimated to laboratory conditions for 2 weeks in aerated ponds (O_2 > 5.0 mg/L) at 28 °C and fed once a day with a commercial diet (Api Camarón Intensivo 40, Malta Cleyton, Mexico) containing 40% protein.

2.2. Preparation of hemocytes and serum samples

Hemolymph was aseptically withdrawn from the pericardial area of the crayfish with a 3-mL syringe and a 21-gauge needle. For serum, the sample (1 mL) was obtained without anticoagulant solution. It was allowed to coagulate for 30 min and, then, the supernatant was gently removed from the sample, the sample was cleared by centrifugation at 16,000 *g* at 4 °C for 10 min to remove particles remaining in the sample. The supernatant was stored at −80 °C.

For hemocyte samples, 1 mL of the hemolymph was mixed with 1 mL of anticoagulant solution to prevent cell lysis and coagulation during the process (0.45 M sodium chloride, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 20 mM ethylenediaminetetraacetic acid, pH 4.5) [21,22]. Hemocytes were washed with modified Van Harreveld solution (VHS; 270 mM NaCl, 25.7 mM $CaCl_2$, 4.6 mM KCl_2, 2.6 mM $MgCl_2$, pH 8) and centrifuged three times at 300 *g* for 10 min at 4 °C. Cellular viability was tested by the trypan blue dye exclusion method (> 95% viability).

2.3. Production of polyclonal anti-CqL antibodies

The lectin (CqL) was purified from the hemolymph of *C. quadricarinatus* by affinity chromatography using stroma of Wistar rat erythrocytes [14]. For the antibodies against CqL, a female rabbit, New Zealand strain, was immunized with 1 mg/kg of weight with the purified CqL in Freund's complete adjuvant (FCA) (Gibco Laboratories Inc., Grand Island, NY, USA). Four immunizations at 15-day intervals were performed. Two weeks after the last immunization, the rabbit was bled from the marginal vein of the ear obtaining 50 mL of blood. Samples were allowed to coagulate at 4 °C.

The antibodies were purified by precipitation with a 35% ammonium sulfate saturated solution. The precipitation was repeated twice and finally suspended in 2 mL of saline solution, the antibodies were dialyzed extensively against saline solution, the dialyzed proteins were centrifuged at 13,200 *g* for 10 min at 4 °C to eliminate denatured proteins and, finally, the antibodies were stored at −20 °C.

2.4. Immune challenge of freshwater crayfish C. quadricarinatus

In the *in vivo* model, six adult male *C. quadricarinatus* crayfishes were used for each group: they received either 100 μL of lipopolysaccharides from *Escherichia coli* (LPS) (Sigma Aldrich, St. Louis, MO, USA, cat no. Z-4250) at optimal doses determined previously (20 μg/kg) or β-glucan from *Saccharomyces cerevisiae* (200 μg/kg) (Sigma Aldrich, cat no. L-3012); a control group received 100 μL of VHS. Hemolymph samples were collected 2–24 h after immune challenge.

2.5. Total and differential counts of hemocytes

Hemocytes were washed by centrifugation with VHS at 300 *g* at 4 °C for 10 min and suspended in 1 mL of VHS. Total hemocyte count (THC) was performed with 10 μL of cells suspension in a Neubauer Chamber in triplicate. For the differential hemocyte count (DHC), 1×10^5 cells were placed per well and incubated for 15 min at 4 °C, then, the hemocytes were fixed with 1% paraformaldehyde in phosphate buffer saline (PBS, 0.01 M Na_2PO_4, 0.15 M NaCl, pH 7.3) for 10 min. Hundred cells were counted in three random fields in an inverted microscope (DM2000 model; Leica, Cambridge, UK) equipped with a DFC/300FX camera (Leica, Cambridge, UK). The hemocytes classification was performed following the morphological criteria previously reported [5].

2.6. Hemagglutinating activity of serum

For hemagglutinating tests, erythrocytes were obtained from a rabbit procured from the animal facilities of the School of Medicine, UNAM, Mexico. Blood was collected in sterile Alsever's solution (100 mM glucose, 20 mM NaCl, and 30 mM sodium citrate, pH 7.2), and erythrocytes were washed four times with PBS at 300 *g* for 10 min. Hemagglutinating activity of the serum was assayed in microtiter U plates (NUNC, Denmark) by a two-fold serial dilution. The agglutinating activity was tested with 2% erythrocyte suspension in PBS and reported as the inverse of the last dilution showing visible agglutinating activity. Specific activity = HAU/mg of serum protein, where HAU is the inverse of the last dilution that showed agglutination activity.

2.7. Immunohistochemistry assay in hemocytes

To detect the CqLR, hemocytes from the hemolymph were washed three times in VHS and centrifuged at 300 *g* for 10 min at 4 °C. Hemocytes (1×10^5 cells) were placed on glass slides at 4 °C for 10 min and fixed in 1% paraformaldehyde in PBS at 4 °C for 10 min. Then, fixed hemocytes were washed with PBS-T (PBS and 0.1% Tween 20) and incubated in 5% bovine albumin IgG-free PBS for 30 min, followed by washing with PBS-T. The slides were incubated with 5 μg/mL of biotin-labeled CqL in VHS for 2 h at 28 °C. Next, slides were washed three times with PBS-T and incubated with 2 μg/mL of Alexa Fluor 594-streptavidin labeled for 2 h at 37 °C.

To detect CqL, permeabilized hemocytes (1×10^5 cells) with Triton X-100 (Sigma), were incubated with *anti*-CqL polyclonal antibodies for 2 h at 37 °C. The slides were washed three times with PBS-T and then incubated with 1 μg/mL of Alexa Fluor 594-coupled anti-rabbit IgG antibodies for 1 h at 37 °C. Finally, the slides were washed four times with PBS-T, mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA), and observed in an inverted microscope (DM2000 model) equipped with a DFC/300FX camera (Leica).

The morphological features of hemocyte populations were identified by light microscopy following the morphological criteria previously reported [5]. The fluorescence intensity of *anti*-CqL antibodies was estimated by randomly counting the mean fluorescence of 30 granular cells per condition using the program ImageJ (Maryland, USA). Immunohistochemistry images were projected on a two-dimensional plane and merged using a pseudo-color display using LAS AF software (Version 3.1, Leica): blue for DAPI and red for Alexa Fluor 594. Control assays for the specificity of the recognition were performed using CqL incubated previously with 2.5 μM bovine submaxillary mucin (BSM) 30 min before the assays.

2.8. Determination of ROS production in hemocytes

Hemocytes (1×10^5 cells) were placed in each well of a 96-well plate (Sigma Aldrich) and incubated for 15 min at 4 °C. Hemocytes were then washed with VHS, and 0.2% nitroblue tetrazolium (NBT) in PBS was added and incubated for 30 min at 37 °C. The cells were washed with 200 μL of PBS and fixed with 70% methanol for 10 min. To dissolve the formazan (reduced NBT), 120 μL of 2 M potassium hydroxide (KOH) and 140 μL of dimethyl sulfoxide were added. Finally, each well was read at 630 nm using an automatic enzyme-linked immunosorbent assay (ELISA) reader (Dynatech MR5000; Labsystems, Helsinki, Finland). ROS production was estimated from the amount of formazan per monolayer in each condition, it was expressed as nanomoles of reduced NBT (nMNBTr), considering an extinction factor of 0.1 OD at 630 nm representing 1.9 nm of O^{2-} [23].

2.9. Statistical analysis

Quantitative data were expressed as mean ± standard error of mean (SEM). Statistical significance was determined using a one-way analysis of variance (ANOVA) or two-way ANOVA test using the Statistical Package for the Social Sciences (SPSS), version 20 (SPSS Inc., IL, USA). Values were considered statistically significant at $P < 0.05$. All experiments were performed with pooled hemocytes and were independently repeated 3 times. In all experiments, the VHS group and control group (only sample taking) did not present significant differences.

3. Results

3.1. Characterization of crayfish hemocytes challenged with LPS or β-glucan

To identify the participation of CqL and its cellular receptor, adult male *C. quadricarinatus* were challenged with LPS or β glucan in an *in vivo* model. In crayfish treated with β-glucan (200 μg/mL as optimal dose) or LPS (20 μg/mL as optimal dose) after 2 h and 6 h, the THC increased as compared to the VHS group; at 12 h post-challenge, the LPS group had lower THC than the VHS group. But at 24 h after the stimulation, no significant differences between the β-glucan or LPS groups and the VHS group were observed (Fig. 1). The results suggest a response of the crayfish to the stimuli, however, to gain insight of the specific response by hemocyte populations to LPS or β-glucan, we performed DHC.

Granular hemocytes (GH), after 6 h, presented a 41% increase in the presence of β-glucan, and of 45% with LPS. After 12 h, a slight increase with β-glucan was observed as compared to the VHS group (Fig. 2A). In hyaline cells, after 2 h, a 20% increase was observed with β-glucan administration and of 17% with LPS; after 12 h, the values were similar to those of the VHS group (Fig. 2B). Semigranular hemocytes (SGH) increased (68%) with the β-glucan stimulation at 6 h, whereas LPS-treated crayfish showed no increases in SGH throughout the experiment (Fig. 2C).

3.2. Expression of CqL and its receptor (CqLR) in hemocytes

The expression of the receptor for CqL in hemocytes was determined by immunohistochemistry (Fig. 3A). After 2 h post-challenge with β-

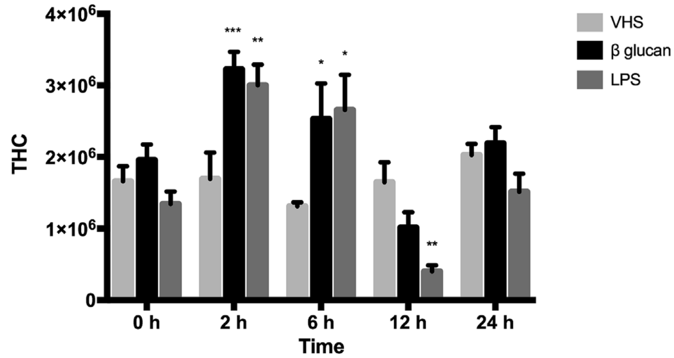


Fig. 1. Variation of total hemocytes count (THC) in crayfishes challenged with immunostimulants. Data are mean ± SEM of three experiments. The asterisk indicates values that are different compared with the VHS group (*$P < 0.05$, **$P < 0.025$, ***$P < 0.001$).

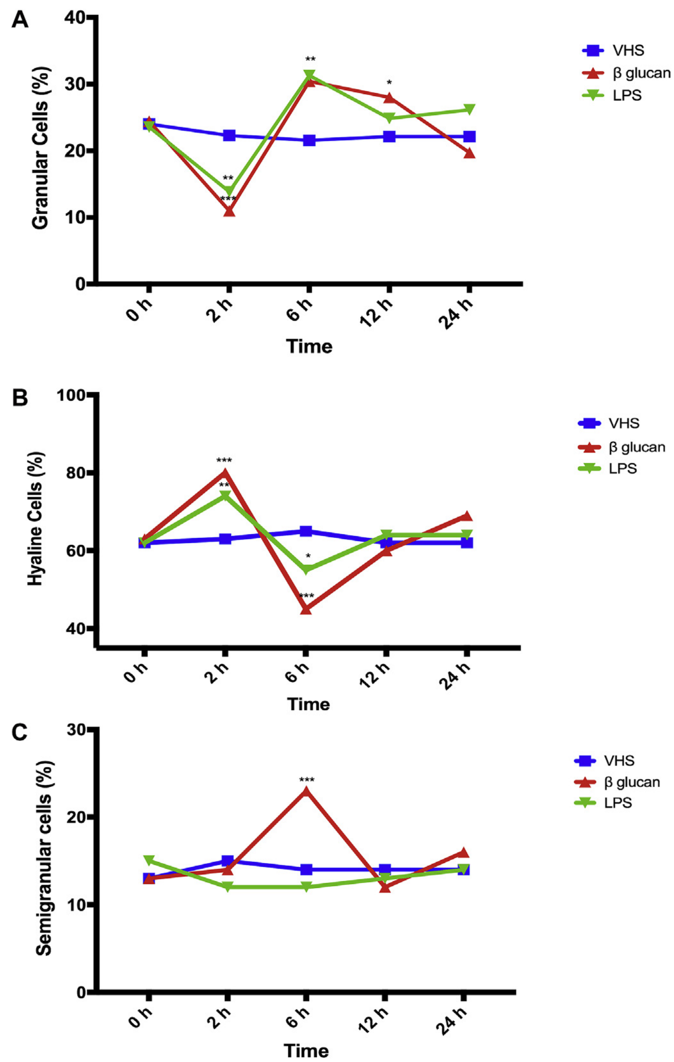
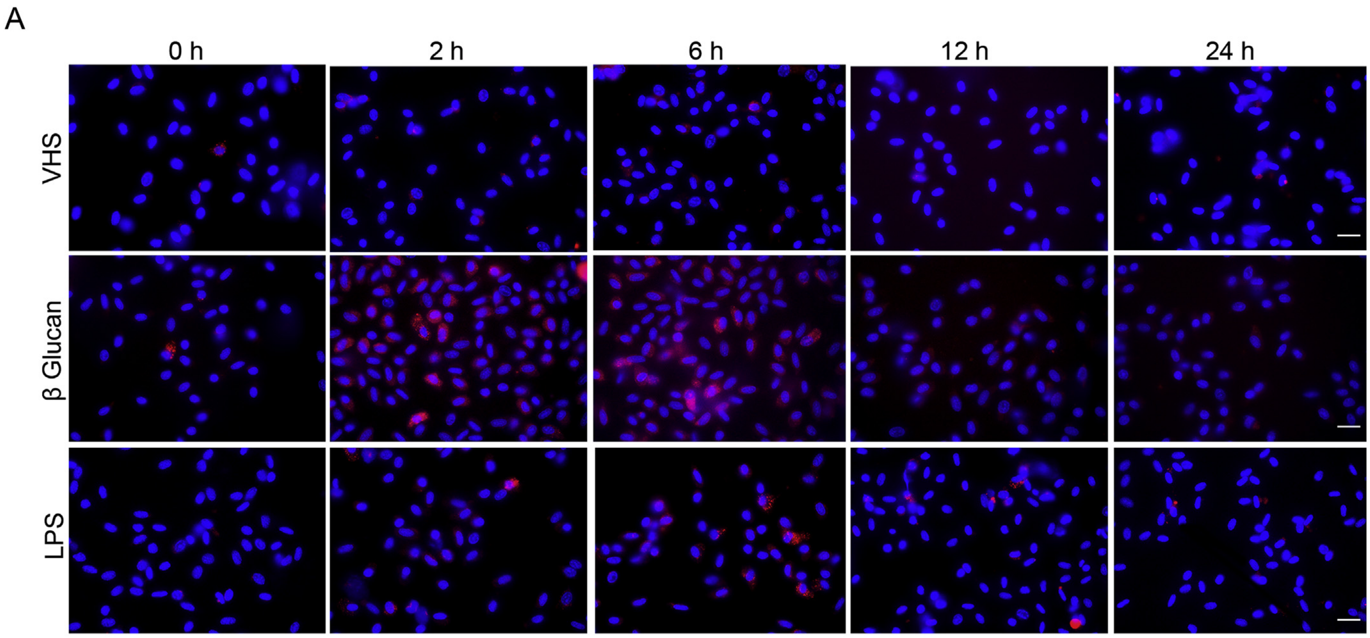


Fig. 2. Variation of hemocyte subsets by differential hemocytes count (DHC) in crayfishes challenged with immunostimulants. A) Granular hemocytes. B) Hyaline hemocytes. C) Semigranular hemocytes. Data are mean ± SEM of three experiments. The asterisk indicates values that are different compared with the VHS group (*$P < 0.05$, **$P < 0.025$, ***$P < 0.001$).

glucan, by performing a DHC of positive CqL labeling hemocytes, almost all granular and semigranular hemocytes were recognized by CqL; moreover, 24% of hyaline hemocytes were CqLR-positive (Fig. 3B).



B

		0 h	2 h	6 h	12 h	24 h
VHS	Hyaline	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Semigranular	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Granular	8.3 ± 0.5	11.3 ± 2.1	11 ± 1	9.3 ± 1.5	8.6 ± 1.1
β-glucan	Hyaline	0 ± 0	24.6 ± 4.6	28 ± 3	6 ± 2.6	2 ± 1
	Semigranular	0 ± 0	97 ± 3. 1	94.6 ± 3.1	13. ± 7.1	3.6 ± 3.1
	Granular	8.6 ± 1.1	99.3 ± 1.1	99 ± 1.7	46.6 ± 8.6	12 ± 4
LPS	Hyaline	0 ± 0	0 ± 0	1.3 ± 1.5	0 ± 0	0 ± 0
	Semigranular	0 ± 0	11.3 ± 3.3	12.3 ± 2.5	7 ± 3.4	4.3 ± 5.1
	Granular	7.6 ± 0.5	11.3 ± 1.1	14.6 ± 0.5	11 ± 2.6	9.3 ± 1.1

Fig. 3. Expression of CqL receptor (CqLR) in crayfish hemocyte subsets challenged with immunostimulants. A) Immunofluorescence labeled with CqL and visualized with Alexa Fluor 594 (red), nuclei were labeled with DAPI (blue). Bar = 20 μm. B) Differential hemocytes count of CqLR positive hemocytes. Data are mean ± SEM of three experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

After 6 h post-stimulation, the same proportion remained. LPS challenge did not induce a significant increase at any time.

Identification of CqL in hemocytes was performed using *anti*-CqL antibodies (Fig. 4A). In the VHS group, all hemocytes were labeled CqL-positive at all times post-challenge; however, at 2 h after β-glucan stimulation increased labeling was observed in a granular hemocytes subset; semigranular and hyaline hemocytes showed no labeling increase. Furthermore, this labeling of the subset of granular hemocytes remained at 6 h and 12 h post-challenge (Fig. 4B). In the LPS-treated group, no significant increase in CqL was identified at any time after the stimulation.

3.3. Specific activity of the serum lectin of *C. quadricarinatus*

Specific activity was determined by a hemagglutination test with rabbit erythrocytes. Two hours after crayfishes were challenged with β-glucan, specific activity of the serum increased 183% as compared to the VHS group; after 6 h, the values were higher (150%) than those of the VHS group; 12 and 24 h after β-glucan challenge, no differences were observed in the specific activity. LPS-challenged serum did not show significant increases at any time compared with the control (Fig. 5).

3.4. Production of ROS by hemocytes

The ROS production was determined in crayfish groups challenged with β-glucan or LPS in *ex vivo* assays. As indicated in Fig. 6A, after 2, 6, and 12 h of β-glucan challenge, ROS production increased 57%, 110%, and 93%, respectively, as compared with the VHS group. Similarly, after LPS-challenge, increases of 45%, 98%, 92% were observed at 2 h, 12 h, and 24 h, respectively.

To observe whether the presence of CqLR in hemocytes (Fig. 3A) had an effect on ROS production, hemocytes from the groups challenged with β-glucan or LPS were stimulated *ex vivo* with CqL for 60 min. As indicated in Fig. 6B, hemocytes from the β-glucan-treated group showed nMNBTr increases of 160%, 273%, 421%, and 242% at 2, 6, 12, and 24 h, respectively, as compared with the VHS group (Fig. 6B). In the hemocytes from LPS-activated crayfishes, the optimal ROS production induced by CqL was 250% and 205% higher at 12 and 24 h, respectively, than in hemocytes from VHS crayfishes.

4. Discussion

β-glucan and LPS from a variety of sources have been evaluated for their capacity to stimulate the immune system; in crustaceans, they have been evaluated as immunomodulators. In general, these molecules are classified as biological response modifiers [24]. Immune priming in shrimp after vaccination with bacteria has shown that inactivated bacterial pathogens can provide shrimp with enhanced protection against the corresponding pathogen. Although, a complete mechanistic explanation is not clear [20].

Hemocytes play an important role in the regulation of the immune response in crustaceans; the general classification mentions the presence of three hemocyte populations in crustaceans: hyaline, semi-

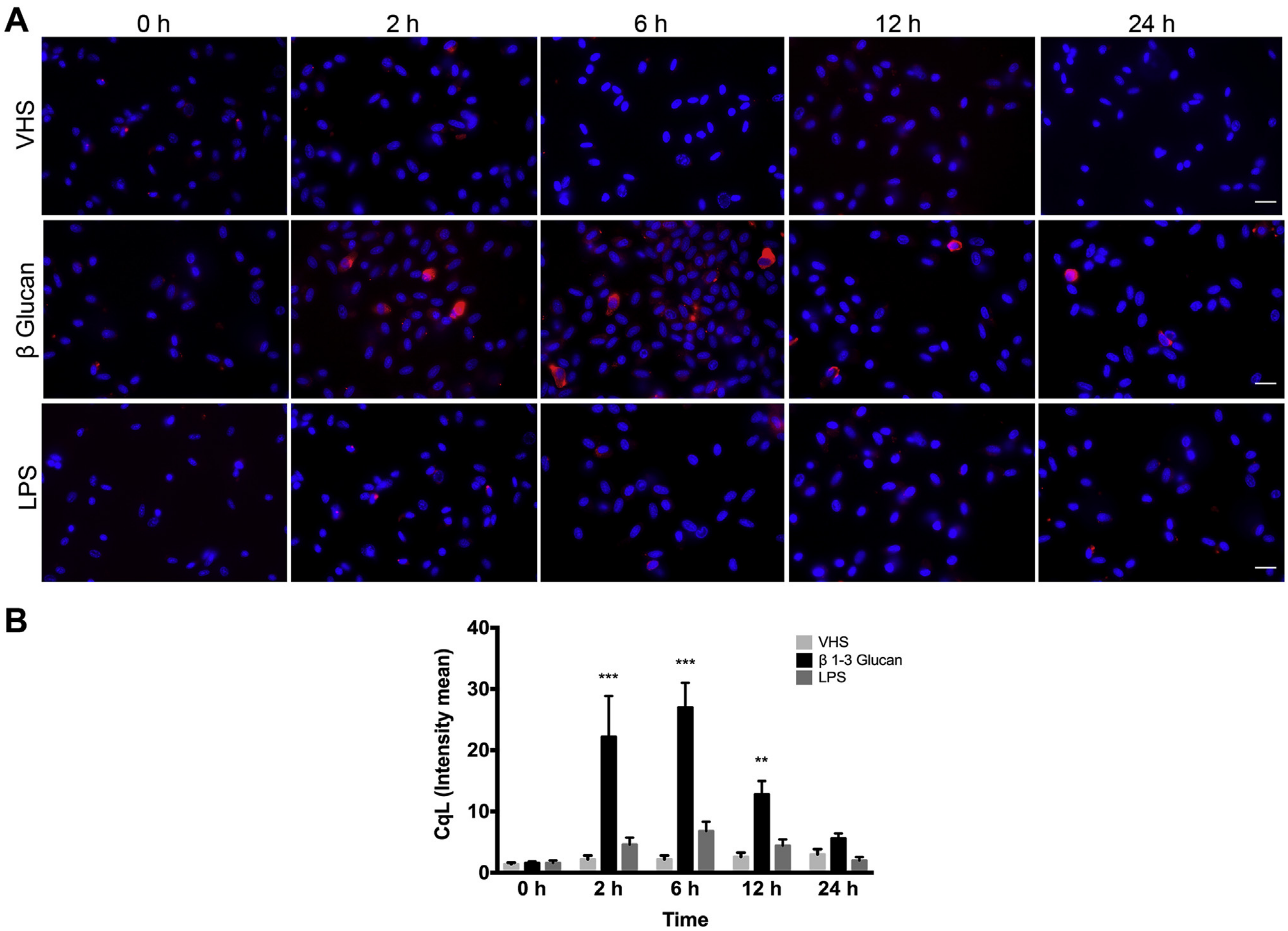


Fig. 4. Increase of the CqL lectin expression in granular hemocytes from crayfishes challenged with immunostimulants. A) Immunofluorescence labeled with *anti*-CqL polyclonal antibodies and visualized with Alexa Fluor 594 (red) and nuclei were labeled with DAPI (blue). Bar = 20 μm. B) Intensity of *anti*-CqL antibodies labeling was estimated from the fluorescence. Data are mean ± SEM of three experiments. The asterisk indicates values that are different compared with the VHS group (**$P < 0.025$, ***$P < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

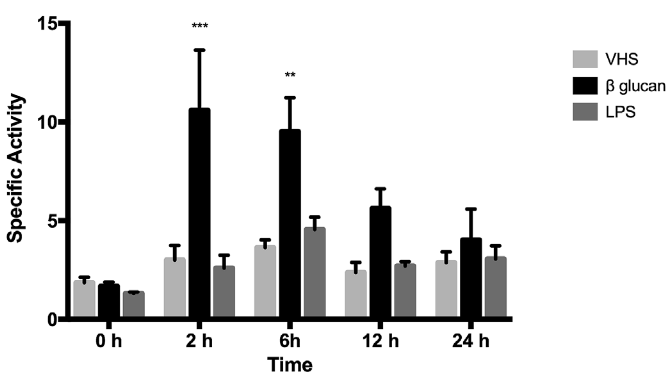


Fig. 5. Agglutination activity in hemolymph of crayfishes challenged with immunostimulants: Specific activity = HAU/mg protein, where HAU is the inverse of the last dilution that showed agglutination activity. Data are mean ± SEM of three experiments. The asterisk indicates values that are different compared with the VHS group (**$P < 0.025$, ***$P < 0.001$).

granular, and granular cells [25]. It has been suggested that lectins participate as key molecules that regulate the response against pathogens [7]. In *C. quadricarinatus* hemolymph, a serum lectin (CqL) has been purified that participates in the regulation of ROS production in a granular hemocytes subset [13,14]. In the present study, we identified the participation of CqL and its receptor in the immune mechanisms of crayfish *C. quadricarinatus* challenged with LPS or β-glucan.

It has been suggested that the total hemocytes count is affected by immunological challenges [26]. In the present study, β-glucan or LPS challenge increased THC after 2 and 6 h; however, at 12 h post-challenge, THC decreased markedly. Interestingly, after 24 h, the THC did not show significant differences as compared to the control group. In contrast, in *P. monodon* and *L. vannamei* shrimps stimulated with LPS, the THC values decreased after 24 h [27,28]. Under poly I:C challenge, the THC in the first 12 h was higher than in the control, and after 24–48 h, it was lower [29]. The hematopoietic process releases hemocytes during microbial infections or in presence of immunostimulants [5]. This process could be related to our results, suggesting that the increase of THC in presence of LPS and β-glucan plays a relevant role in the generation of hemocytes that participate in the immune response.

In the present report, a differential hemocyte count (DHC) was performed. Treatment with β-glucan increased the hyaline hemocytes proportion after 2 h and decreased at 6 h post-challenge; granular cells decreased after 2 h; however, this cellular subset increased after 6 h post-challenge. In the presence of LPS, at 6 h post-challenge there are mainly semigranular hemocytes. Several linage models have been proposed; however, the most supported model is that HH are a precursor of SGH and GH [5]. In other crustaceans, modifications in the presence of different hemocyte subsets induced by immunostimulants have been observed [29,30]. The variation of DHC values in *C. quadricarinatus* could be related to the hematopoietic process and the generation of specific hemocytes to act with each immunostimulant.

Lectin receptors have been identified in hemocytes from several crustacean organisms, however, their participation in the presence of

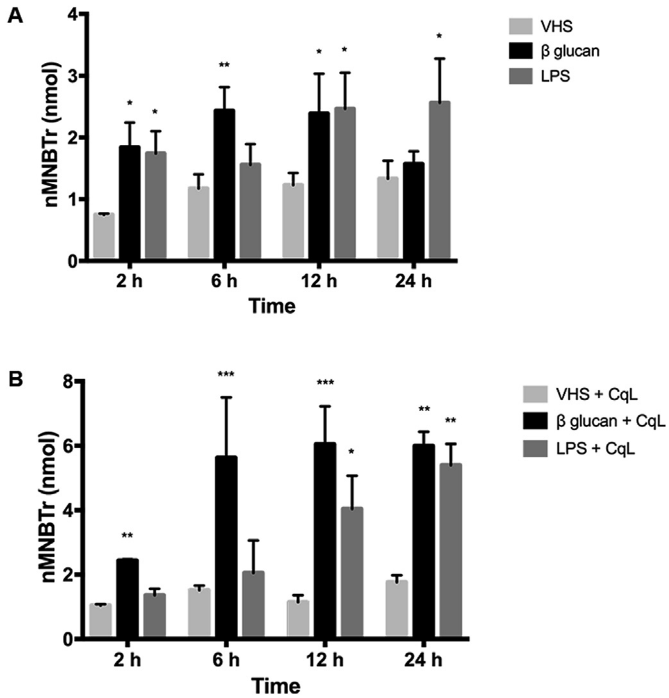


Fig. 6. Reactive oxygen species (ROS) production by hemocytes. A). Hemocytes obtained from crayfishes challenged with immunostimulants. Data are mean ± SEM of three experiments. The asterisk indicates values that are different compared with the VHS group (*$P < 0.05$, **$P < 0.025$). B) Hemocytes from the β-glucan and LPS groups were stimulated *ex vivo* with CqL for 60 min. Data are mean ± SEM of three experiments. The asterisk indicates values that are different compared with the VHS group (*$P < 0.05$, **$P < 0.025$, ***$P < 0.001$).

an immunological challenge remains unclear [8]. In the present report, a CqLR increase was observed in all hemocyte subsets after 2 h of β-glucan challenge, which remained until 12 h post-stimulation. A significant increase in CqL expression was observed in granular hemocytes. The presence of lectins and their receptor has been reported in hemocytes of other crustaceans [14,31–33]. Lectins have been identified also in hemocytes and seem to be synthetized after pathogen stimulation [32]. These results suggest that CqLR could be related to the immune response to β-glucan, leading to an increase of CqL expression in the granular subset of hemocytes and its secretion into the hemolymph.

Specific activity and quantification of CqL in the hemolymph increased after 2 h post- β-glucan challenge. An increase of lectin-related genes has been reported in crustaceans after different immune challenges [34]. In the freshwater prawn *M. rosenbergii*, an increase of the hemagglutination titer was observed after 24 h of *Aeromonas hydrophila* stimulation, and a decrease in presence of WSSV [35,36]. In *P. clarkii*, following challenge with *Vibrio anguillarum*, the presence of PcLec3 was increased in hemocytes and hemolymph [10]. The increase of CqL, as well as its hemagglutination activity, could result from its expression in hemocytes and, subsequently, from the secretion or degranulation of granular hemocytes; however, further research is needed to clarify whether CqL is expressed in other tissues or the specific activity increase is related to the expression of lectin isoforms [37].

In the present study, it was observed that β-glucan challenge induced ROS production in hemocytes earlier than LPS. Similar effects were identified in *P. monodon* at 3 h post-challenge with LPS [27]. Granular and semigranular hemocytes are the main subsets able to participate in the production of these reactive species [27,29,30]. In *M. rosenbergii*, LPS and β-glucan induce the production of ROS in granular and semigranular hemocytes [38]. In L. *vannamei*, WSSV and *Vibrio* increased ROS production [39].

It has been postulated that the interaction between CqL and its cellular receptor (CqLR) induces ROS production in a sub-population of granular hemocytes through specific signaling molecules [13,14]. In the present report, the hemocytes from the β-glucan group stimulated with CqL showed an early increase in ROS production. Interestingly, CqLR presents similarity with transglutaminase molecules [13]. It has been suggested that ROS could affect transglutaminase activity and regulate crustacean hematopoiesis [40]. Further research in needed to clarify whether lectins participate in this process and play a relevant role in the cellular immune response of *C. quadricarinatus*.

5. Conclusions

Invertebrates only have an innate immune system; the complexity of these systems is far from being fully understood. Research interest has focused on the immune response in the presence of immunostimulants. Our results suggest that CqL is a key molecule in the modulation of the response in the presence of β-glucan, instead of LPS, that does not show the same response. An increase of CqL activity was observed in the serum, and of its receptor in hemocytes, which showed a better capability of ROS production, suggesting that β-glucan, as immunostimulant, seems to trigger invertebrate immune factors by increasing the lectin activity and the capacity to increase its own receptor in hemocytes to induces ROS production. Furthermore, these results suggest that *C. quadricarinatus* could present specific immune response depending in the type of immunostimulant, CqL could participate in this response and, hence, participate in the protection of this crustacean against disease threats in aquaculture.

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