Rapid regulation of hemocyte homeostasis in crayfish and its manipulation by viral infection

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A B S T R A C T

In crustaceans, the number of circulating hemocytes changes rapidly in response to pathogen infection and injury, but the underlying mechanism remains unclear. In this study, we investigated the regulation of hemocytes homeostasis in crayfish after hemolymph withdrawal. We showed that the circulating hemocytes increased by over 2 folds within 1 h post hemolymph withdrawal and returned to normal level within 8 h. New hemocytes produced by hemopoiesis accounted for ~6.5% of the total replenishment, implying a major role of sessile hemocytes in rapid hemocyte supply. Moreover, when hemocytes were transplanted, the extra cells were efficiently stored, mainly in the gill. These stored cells could be released into circulation immediately on demand. Notably, the rapid regulation of hemocyte homeostasis was abolished by white spot syndrome virus infection. These data indicate that the adjustment between the sessile and circulating pools of hemocytes may be the major route for the rapid regulation of circulating hemocytes in crayfish, and this process may be altered by pathogen infection.

1. Introduction

Hemocytes are the primary immune cells of crustaceans, which clear pathogens by phagocytosis, nodulation, encapsulation, and melanization. Based on their morphological features, crustacean hemocytes are classified into three groups: semi-granular cells (SGCs), granular cells (GCs), and hyaline cells [1]. In crayfish, SGCs and GCs are the dominant types of hemocytes [2]. Although the circulating cells are thought to be mature hemocytes for a long time, our recent findings demonstrate that SGCs and GCs are functional immune cells representing different developmental stages of the GC lineage [3].

The number of circulating hemocytes changes rapidly in response to different stimuli, including pathogen infection, pathogen-associated molecule pattern (PAMP) challenge, injection of saline buffer, blood drawing, and wounding. Although the number of hemocytes varies in different ways under different circumstances, there is a common rule. Immediately after an injury, crustaceans mobilize their hemocytes to the circulation, resulting in a rapid and significant increase of total hemocyte count (THC, the number of hemocytes per milliliter of hemolymph). This can occur in a few minutes [4,5]. If the injury is not pathogenic, such as an injection of buffer saline [6,7,8] or a small cut [9,10], THC will recover over time. Infection of pathogens or injection of PAMPs commonly leads to a temporary decrease of THC following the initial increase, which may be due to hemocyte consumption during immune responses [5,11,12]. If the challenge is too severe, the number of circulating hemocytes will continuously decline, and the animals may die [13–16]. Considering the critical role of hemocytes in immune responses, the regulation of hemocyte homeostasis is important to maintain a functional immune system.

Hemopoiesis is the major way for hemocyte replenishment [2]. Injection of PAMPs like lipopolysaccharide (LPS) [8] and β-glucan [4], which causes a dramatic reduction of circulating hemocytes, has been found to promote hemopoiesis in the hematopoietic tissue (HPT). Additionally, injection of PBS, which induces an increase in circulating hemocytes, also enhances cell proliferation in HPT to a lower extent [8]. Whether the circulating hemocytes in crustaceans have the ability to

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proliferate remains debatable. Although the hemocytes in crayfish scarcely proliferate [3,4], a small portion of hemocytes in peneaíd shrimp and crab may be proliferating cells, and their proliferation can be promoted by pathogen infection [17–19]. However, cell differentiation and proliferation are time-consuming processes. We found that in crayfish hemocyte precursors take at least 3 days to finish their development in HPT [3]. Whether new hemocyte production alone is sufficient to meet such a large and rapid demand remains a question. Some previous reports suggest that the number of circulating hemocytes is, in part, regulated by hemocytes stored in the tissues [8,20]. Yet, a more detailed study is required.

Regulation of hemocyte homeostasis by sessile hemocytes or a hemocyte reservoir has been reported in some invertebrates including insects and mollusks. In the larval stage of Drosophila, the majority of hemocytes are sessile cells attaching to the cuticular epidermis. These sessile hemocytes are in dynamic exchange with the circulating hemocytes. The attached cells can be mobilized to circulation upon infection [21–25]. Research in the snail Pomacea canaliculata showed that the number of circulating hemocytes remained unaltered after repeating bleeding. The authors proposed that the “ampulla”, a saccular structure developed on the anterior aorta, might act as a hemocyte reservoir, and control the number of circulating hemocytes [26]. The existence of a hemocyte reservoir has also been assumed in cephalopods [27].

In this study, we investigated the rapid regulation of hemocyte homeostasis in crayfish by performing hemolymph withdrawal and hemocytes transplantation. We showed that adjustment between the sessile and circulating pools of hemocytes may be the major route for the rapid regulation of circulating hemocytes in crustaceans, which can be altered by pathogen infection.

2. Materials and Methods

2.1. Animals

Cherax quadricarinatus were obtained from Shantou Jixiong Aquatic Corporation. The animals were maintained in freshwater at 25 °C and fed daily with a commercial diet. Healthy juvenile males (5±2 g) were used in the live-imaging analysis of hemocyte dynamic in gill. Inter-molt adult males (50±5 g) were used in the rest experiments.

2.2. Preparation of crayfish plasma

Crayfish were pre-chilled on ice for 10 min. Hemolymph was withdrawn with a cold syringe and then centrifuged at 600 g for 5 min at 4 °C. The supernatant (plasma) was collected, sterilized by passing through a 0.22-μm filter, and stored at -80 °C [28]. The plasma was warmed to room temperature before use.

2.3. Hemolymph withdrawal assay

For both long-term and short-term analyses, 12 animals were divided into two groups, 6 individuals in each group. A hemolymph sample (150 μL) was collected from each crayfish before hemolymph withdrawal (0 h post hemolymph withdrawal, hpw) with a syringe preload with an equal volume of 8% paraformaldehyde (PFA). Then hemolymph (~5% v/w of body weight) was taken from each animal in the experimental group, and the same volume of normal saline was replenished immediately. Then at each time point, 150 μL hemolymph sample was taken for analysis from each animal in both groups. After fixation for 10 minutes at room temperature, the cell concentration in each sample was measured by an Orflo’s Maxi Z cell counter, and the THC at each time point was determined. Then hemocytes in each sample were pelleted by centrifugation at 300 g for 3 min and resuspended in PBS. The THC percentage was determined by a BD FACS Calibur flow cytometer, and 10000 cells were analyzed for each sample [29]. For all crayfish, hemolymph was taken from the abdominal cavity.

2.4. EdU incorporation and hemolymph withdrawal

Five crayfish were injected with 5-ethyl-2'-deoxyuridine (EdU) (ThermoFisher) at a dose of 15 μg/g body weight. Three days post-injection, 150 μL of hemolymph was taken from each animal and fixed (0 hpw). Five animals were bled as described in 2.3. One hour later, 150 μL of hemolymph was taken from each animal and fixed (1 hpw). Then, the HPT was removed, HPT cells were dissociated by collagenase treatment [4], and fixed with 4% PFA. After fixation for 10 min at room temperature, the cell concentration in each hemolymph sample was measured. THC was determined for each animal as described in 2.3. For EdU staining, fixed cells were collected by centrifugation at 300 g for 3 min, and permeabilized with 0.5% Triton X-100 for 20 min. Cells were then stained with Click-IT™ EdU Alexa Fluor® 488 Flow Cytometry Assay Kit (ThermoFisher) following the manufacturer’s instruction. The GC percentage and EdU-positive ratio of circulating hemocytes, and the EdU-positive ratio of HPT cells were analyzed by flow cytometry. For each sample, 10000 cells were analyzed. For all crayfish, EdU was injected into the abdominal cavity, and hemolymph was taken near the site of injection.

Calculation

\[ m: \text{THC at 0 hpw} \quad n: \text{THC at 1 hpw} \]

\[ E_0: \text{the percentage of EdU incorporated hemocytes at 0 h} \]

\[ E_1: \text{the percentage of EdU incorporated hemocytes at 1 h} \]

\[ \Delta E = E_1 - E_0 \]

v: total hemolymph volume

p: the percentage of hemolymph being removed

q: the EdU incorporation rate in HPT

R_{hpw}: \text{The percentage of replenishment from HPT}

Hemocyte loss = mpv

Total replenishment = nv-mv+mpv

Replenishment from HPT = (nvE_1-mvE_0+mpvE_0)/q

\[
E_0 + \Delta E = \frac{(nvE_1 - mvE_0 + mpvE_0)/q}{nv - mv + mpv} = \frac{E_0 + \Delta E + \frac{(1-p_0)}{p_0} \Delta E}{q}
\]

2.5. Hemocyte transplantation

About 2 mL of hemolymph was collected from each donor crayfish with a syringe preload with an equal volume of anticoagulant solution (26 mM sodium citrate, 30 mM citric acid, 100 mM glucose, 140 mM NaCl, pH 5.8). The hemocytes were collected by centrifugation at 300 g for 3 min at room temperature and resuspended in 1 mL of anticoagulant solution. Then the cells were labeled with ThermoFisher’s Vybrant® CFDA SE Cell Tracer Kit (10 μM carboxy-fluorescein diacetate, succinimidy l ester (CFDA SE) in anticoagulant solution for 20 min). Cells were collected by centrifugation at 300 g for 3 min, resuspended in normal saline, and ready for transplantation [3].

Before transplantation (0 h post-transplantation, 0 h.p.t.), 150 μL hemolymph was collected from each recipient with a syringe preload with an equal volume of 8% PFA. The CFDA SE-labeled hemocytes were transplanted at a dose of 1 × 10⁷ cells/individual. The total volume of cell suspension injected into each animal was ~150 μL. In the control group, animals were injected with an equal volume of normal saline. Then at each time point, 150 μL hemolymph was taken from each animal. After fixation, the cell concentration in each sample was measured. THC at each time point was determined. The ratio of CFDA SE-labeled circulating hemocytes was analyzed by flow cytometry, and 10000 cells were analyzed for each sample. For all crayfish, hemocytes
suspension was injected into the abdominal cavity, and hemolymph was taken near the site of injection. Four crayfish were used in each group.

2.6. Tissue sectioning

Five crayfish were transplanted with $1 \times 10^7$ CFDA SE-labeled hemocytes (as in 2.5). Two hours later, the hemolymph, antenna gland, HPT, gill, muscle, nerve, intestine, and hepatopancreas samples were collected and fixed with 4% PFA at 4 °C overnight. The tissue samples were cut into 5 μm sections using a Leica CM1850 cryostat slicer. The sections were stained with 4′,6-diamidino-2-phenylindole (DAPI) and examined by fluorescent microscopy. For each tissue, over 10 sections were investigated. For each section, over 200 cells were counted. The ratio of CFDA SE-labeled cells in each tissue was calculated. The ratio of CFDA SE-labeled hemocytes in hemolymph was measured by flow cytometry and over 10000 cells were analyzed in each sample.

2.7. Analysis of the dynamic of hemocytes in gill

2.7.1. The dynamic of hemocytes after hemolymph withdrawal

Six animals were divided into two groups. Hoechst 33342 was injected into each animal at a dose of 5 μg/g body weight to label all the cells. Four hours later (0 h post-transplantation, h.p.t.), 150 μL of hemolymph was taken. Then, $1 \times 10^7$ CFDA SE-labeled hemocytes were

Fig. 1. Rapid hemocyte replenishment after hemolymph withdrawal

Long-term (A, B, C) and short-term (D, E, F) analyses were done to see the replenishment of circulating hemocytes after hemolymph withdrawal. Hemolymph samples were collected from the animals before hemolymph withdrawal (0 h). Hemolymph (5% (v/w) of body weight) was taken from each animal in the experimental group, and the same volume of normal saline was replenished immediately. The animals in the control group remained untreated. Hemolymph samples were then taken at different time points. The change in the THC (A, B, D, E) and GC percentage (C, F) were determined for each animal. The values are expressed as means±S.D. (n=6). In each plot, different symbols indicate the data from different animals (A, B, D, E).
transplanted as described in 2.5. Two hours later (0 min post hemolymph withdrawal, 0 mpw), 150 μL of hemolymph sample was taken again and the top of a gill was cut. Another portion of hemolymph (5% of body weight) was taken from each animal in the experimental group, and an equal volume of crayfish plasma was replenished. Then, 150 μL of hemolymph sample was taken (1 mpw). Hemolymph was collected from the control animals as well. Sixty minutes later (60 mpw), 150 μL hemolymph, and another sample of gill were taken from animals in both groups. For the hemolymph samples, THC and CFDA SE-labeled ratios were analyzed. The gill samples were examined by fluorescent microscopy. For all crayfish, hemocytes suspension was injected into the abdominal cavity, and hemolymph was taken near the site of injection.

2.7.2. Live-imaging analysis

Juvenile crayfish were transplanted with 1 × 10⁶ CFDA SE-labeled hemocytes as described in 2.5. One hour later, the animals were anesthetized by immersed in 1.3 mmol/L eugenol for about 30 minutes. Then the animals were placed on a petri dish containing eugenol solution. For each animal, one side of the carapace was carefully removed, and the gill was examined under a fluorescent microscope. The video of hemocyte movement in the gill was taken using a cell phone.

2.8. Regulation of hemocyte homeostasis during viral infection

White spot syndrome virus (WSSV-CN03) was propagated and purified as described previously [30]. Eight crayfish were injected with WSSV-CN03 at a dose of 1000 copies/g body weight. Five crayfish were injected with an equal volume of normal saline. Two days later, hemolymph (150 μL) was taken from each animal to stimulate the change in circulating hemocytes. THC was measured at 0 h, 15 min, and 1 h. The viral copies in the hemolymph samples were determined by real-time quantitative PCR using the White Spot Syndrome Virus (WSSV) Fluorescent Quantitative PCR Detection Kit purchased from Xiamen Zhihulianfeng Biotechnology Co., Ltd. (Xiamen, China).

2.9. Statistical analysis

All data are presented as means ± standard deviation (SD). When three or more treatments were compared, they were examined by one-way ANOVA (Brown-Forsythe test). Multiple comparison (Tukey) test was conducted to compare the significant differences. When two groups were compared, their difference was examined using Student’s t-Test. Differences were considered statistically significant when p<0.05.

3. Results

3.1. Rapid hemocyte replenishment after hemolymph withdrawal

To investigate the regulation of hemocyte homeostasis in crayfish, we removed a relatively large amount of hemolymph (~5% (v/w) of body weight) from the animals, replenished it with an equal volume of normal saline, and analyzed the change in the number of circulating hemocytes (see 2.3 for details). Instead of a decrease of hemocytes, we saw a ~2-fold increase in THC at 2 hpw (Figure 1 A). A similar increase was also observed in the control group (only a 150 μL sample was taken at 0 hpw) (Figure 1 B). The THC recovered to the normal level within 24 h for both groups. To determine the minimum time required for hemocyte replenishment, we further analyzed the change of THC within 2 h post hemolymph removal. Surprisingly, we did not see a decline in hemocyte number, even at 5 mpw (Figure 1 D). In contrast, THC raised dramatically, peaked at 15 mpw, and remained high till 60 mpw. Similarly, the THC in the control group raised gradually and peaked at 60 mpw (Figure 1 E). The increase was over 2 folds in our experiments. Moreover, the GC ratio slightly increased in both the bleeding and control groups (Figure 1 C and F) and recovered within 24 h. Although THC and the variation range were different among animals, the pattern

| Crayfish THC (cells/mL) | THC Fold change | Percentage of GCs EdU-incorporation rate in hemocytes | Increase in EdU-incorporation rate in hemocytes | EdU-incorporation rate in HPT Replenishment from HPT |
|------------------------|-----------------|-----------------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| 0 h 3.92 × 10⁶         | 1.41 × 10⁷      | 3.61                                                | 18.4%                                         | 10.2%                                         |
| 1 h 7.96 × 10⁶         | 3.43            | 2.33%                                               | 27.4%                                         | 2.49%                                         |
| 0 h 2.32 × 10⁶         | 4.28 × 10⁶      | 3.43                                                | 27.4%                                         | 2.49%                                         |
| 1 h 4.34 × 10⁶         | 3.43            | 2.33%                                               | 27.4%                                         | 2.49%                                         |
| 0 h 4.76 × 10⁶         | 9.14 × 10⁶      | 1.92                                                | 22.1%                                         | 0.49%                                         |
| 1 h 8.76 × 10⁶         | 3.56            | 0.99%                                               | 8.76%                                         | 1.92%                                         |
| 0 h 2.54 × 10⁶         | 8.76 × 10⁶      | 3.45                                                | 26.7%                                         | 2.91%                                         |
| 1 h 3.54 × 10⁶         | 3.48            | 0.89%                                               | 3.54 × 10⁶                                    | 0.89%                                         |

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was similar. The experiments were repeated, and similar results were obtained. These data indicate that the hemocyte homeostasis is regulated very rapidly in response to stimulations, and a large number of cells can be replenished to the circulation within an hour. Thus, in the following experiments, samples were taken at 1 h.p.w to evaluate the hemocyte replenishment in this rapid process.

3.2. Hematopoiesis is not the main source of rapid hemocyte supply

To investigate whether the hemocytes rapidly replenished to the circulation are produced by hematopoiesis, we labeled the cells in HPT by EdU incorporation and performed the hemolymph withdrawal assay three days later (see 2.4 for details). Because the circulating hemocytes in crayfish do not proliferate, they cannot incorporate EdU [3]. Therefore, we can track the new hemocytes released from HPT and calculate their number. In this experiment, THC increased by over 2 folds (on average) at 1 h.p.w, while the GC ratio slightly increased. The EdU-incorporation rate in HPT cells was 47~93%. The EdU-incorporation rate of circulating hemocytes before hemolymph withdrawal was 0.5~2.9% and it slightly raised to 0.5~5.0% at 1 h.p.w (Table 1). As described in 2.4, the replenishment from HPT can be calculated using the following formula.

\[ R_{\text{hpt}} = \frac{E_0 + \Delta E + \frac{1-p}{1+t} \Delta E}{q} \]

In this formula, the values of \( E_0 \), \( \Delta E \), \( q \), and \( t \) were known. However, we could not measure the amount of hemolymph in the animals accurately. Thus, we did not know the exact percentage (p) of hemolymph being removed. Therefore, instead of calculating the exact percentage of replenishment from HPT, we calculated its upper limit. Because \( 0<p<1 \) and \( t\geq 1.9 \) (Table 1), the upper limit of replenishment from HPT (\( R_{\text{hpt}}^{\text{up}} \)) was \( (E_0 + \Delta E + 1/(t-1) \Delta E)/q \), which was 0.89~6.20% in our experiment. These data suggest that HPT is not the main source for the rapid hemocyte supply.

3.3. The transplanted hemocytes are rapidly stored

Since HPT is not the main source for the rapid replenishment of hemocytes, we assume that crayfish may have a “hemocyte reservoir”, which regulates the number of circulating hemocytes. This hypothesis was further tested by hemocyte transplantation experiment. Eight animals were used in the experiment. Four were transplanted with CFDA SE-labeled hemocytes at a dose of \( 1 \times 10^7 \) cells/individual. Since the average number of circulation hemocytes was \( 1.2 \times 10^6/\text{mL} \) at 0 h.p.t. (Fig. 2 A), the number of transplanted hemocytes was comparable to the number of cells in 8 mL of hemolymph. If these foreign cells were not stored, THC in the recipients would greatly raise. In our experiment, THC rose rapidly after hemocyte transplantation, peaked at 1~2 h.p.t., and returned to the initial level at 8 h.p.t.. However, a similar change was observed in the control animals, which received an injection of an equal volume of normal saline. The only difference is that the increase of THC was a bit larger in the transplanted animals at 0.5 h.p.t. (Fig. 2 A and B). In addition, the CFDA SE-labeled hemocytes only accounted for about 2~10% of the population (Fig. 2 C), even though such a large number of cells were transplanted. This experiment was repeated and similar results were obtained. These results indicate that there is a place in the body, where extra hemocytes are stored as the sessile population.

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**Fig. 2. Transplanted hemocytes are rapidly stored**

Crayfish in the experimental group (A, C), were transplanted with CFDA SE-labeled hemocytes at a dose of \( 1 \times 10^7 \) cells/individual. Animals in the control group (B) received an injection of an equal volume of normal saline. Hemolymph was taken at 0, 0.5, 1, 2, 4, and 8 h.p.t.. The THC (A and B) and CFDA SE-labeled ratio (C) of circulating hemocytes were analyzed at each time point. The values are expressed as means±S.D. (n=4). In each plot, different symbols indicate the data from different animals.
3.4. Gill may function as the main tissue where sessile hemocytes are stored

3.4.1. Transplanted hemocytes are enriched in gill

To locate the main tissue/organ for hemocyte storage, five crayfishes were transplanted with CFDA SE-labeled hemocytes at a dose of $1 \times 10^7$ cells/individual. Different tissues, including the antenna gland, HPT, gill, muscle, nerve, intestine, and hepatopancreas were collected 2 h.p.t., sectioned, stained, and investigated by fluorescent microscopy. As shown in Fig. 3, the percentages of CFDA-labeled hemocytes in HPT, muscle, nerve, intestine, and hepatopancreas were very low (<1.3%). The average percentage of CFDA SE-labeled hemocytes in the antennal gland was ~2.0%, similar to that in circulating hemocytes (~3.0%). This may be due to the large amount of hemolymph flowing through the antenna gland. Notably, CFDA SE-labeled hemocytes represented ~6.0% of the cells in the gill, which was significantly higher than that in other tissues. The average number of cells in crayfish gill tissue is about $2.0 \times 10^5$ cells/mg [31]. The average weight of the gill tissue in a 50 g-crayfish is ~500 mg, thus the total number of cells in the gill is about $1.0 \times 10^8$. The CFDA SE-labeled hemocytes in the gill were ~$6.0 \times 10^6$ in our experiment, representing ~60% of the transplanted cells. The enrichment of transplanted cells suggests that the gill may function as the main tissue where sessile hemocytes are stored.

3.4.2. Hemocytes are released from the gill on demand

A “hemocyte reservoir” that regulates homeostasis must be able to release hemocytes on demand. Therefore, we further investigated the ability of the gill to release hemocytes. The experimental process is shown in Fig. 4 A. Six crayfish were injected with Hoechst to label all the cells in the body and then transplanted with $1 \times 10^7$ of CFDA SE-labeled hemocytes. There was an increase of THC in all the animals immediately after hemocyte transplantation. Two hours post-transplantation, hemolymph (~5% (v/w) of body weight) was removed from three animals, while the other three remained untreated. Hemolymph withdrawal led to a significant decrease in THC at 1 mpw, and it recovered 1 h later (Fig. 4 B). During this period, the ratio of fluorescent hemocytes was relatively steady in circulation (< 10%) (Fig. 4 C). In addition, we examined the gill sample before and after hemolymph withdrawal. Compared to the control group (Fig. 4 D), there was an obvious decline of CFDA SE-labeled hemocytes in the gill after bleeding (Fig. 4 E), indicating the stored hemocytes are released on demand. This experiment was repeated and similar results were obtained.

Fig. 3. The transplanted hemocytes are enriched in the gill
Crayfish were transplanted with CFDA SE-labeled (green) hemocytes. Tissues were collected at 2 h.p.t., sectioned, stained with DAPI, and investigated by fluorescent microscopy. For each sample, over 10 sections were examined, and over 200 cells were counted for each section. The ratio of CFDA SE-labeled hemocytes in each tissue was calculated (A). The ratio of CFDA SE-labeled hemocytes in each tissue is shown as means±S.D. (10 sections). The columns of different colors indicate the data from different animals. The columns with different letters are significantly different (p<0.05, one-way ANOVA). Images of gill (B), antenna gland (C), and muscle (D) are shown. Bar, 150 μm.
3.4.3. There are sessile hemocytes in the gill

To confirm the existence of sessile hemocytes in the gill, we transplanted CFDA-labeled hemocytes into juvenile crayfish, and then investigated the movement of these allogenic cells in the gill. Although some of the cells moved rapidly with the hemolymph flow, the others did reside in the tissue during the observation period (Video 1). Similar results were observed in three animals.

3.5. WSSV infection abolishes the rapid regulation of hemocyte homeostasis

We further analyzed whether pathogen infection could affect the regulation of hemocyte homeostasis. Crayfish were infected with WSSV. Two days later, the average viral load in the infected animals was \(1 \times 10^4\) copies/mL. Hemolymph (150 μL) was taken from animals in both the WSSV-infected and control groups at 0 h to stimulate hemocyte replenishment, and the THC was measured at different time points. As shown in Fig. 5, the THC increased by over 2 folds at 1 h post-infection in the control group, while the THC in the infected group only showed a very slight increase. The experiment was repeated and similar results were obtained. These data indicate that the rapid regulation of hemocyte homeostasis is abolished by WSSV infection.

4. Discussion

In crustaceans, the number of circulating hemocytes changes rapidly in response to different stimuli. In some cases, the replenishment of a
In this study, we used crayfish as a model system to investigate the rapid regulation of hemocyte homeostasis in crustaceans. We first analyzed the change of THC in response to stimuli. Generally, crayfish responded to an acute loss of hemolymph by rapid recruitment of a large number of hemocytes, which is accomplished within an hour [4,5]. So far, the mechanism for the rapid regulation of circulating hemocytes remains unknown. The circulating hemocytes scarcely proliferate [3,4], and the ability for hematopoiesis to meet such a large demand in a short time is doubtful.

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response was weaker.

The number of hemocytes changes dramatically during pathogen infection, and the control of homeostasis is important for the health of the animals. WSSV infection leads to a sharp decline of circulating hemocytes at the late stage of infection [30,33,34]. Here we show that WSSV abolishes the rapid regulation of circulating hemocytes in the hosts at a relatively early stage when THC is still normal and the viral load is moderate. There are two possible explanations: (1) A lot of sessile hemocytes have been recruited into circulation upon infection and the storage is used up. (2) Gill cells are infected and destroyed by WSSV. The main tissue where sessile hemocytes reside is not functional.

In conclusion, there is a sessile population of hemocytes in crayfish, which is responsible for the rapid regulation of hemocytes homeostasis. Gill may be the main tissue where sessile hemocytes reside. Hemocyte homeostasis in crayfish may be controlled by a two-gear model: rapid regulation by sessile hemocytes, and routine/long-term replenishment by hematopoiesis. So far, we don’t know how many hemocytes are stored in the gill; whether sessile hemocytes are different from circulating hemocytes, and how they are recruited into circulation. Consideration of the central role of hemocytes in immune response, it will be important to study the sessile pool of hemocytes and the regulation of hemocyte homeostasis.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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