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RESEARCH REPORT

Expression profiles of immune-related genes in coelomocytes during regeneration after evisceration in *Apostichopus japonicus*

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Abstract

A representative echinoderm, the sea cucumber *Apostichopus japonicus* has a special regeneration mechanism. The sea cucumber has no specific immune tissues or organs. Coelomocytes of sea cucumbers are involved in their cellular and humoral immunity. In this study, expression profiles of the main immune-related factors in sea cucumber coelomocytes were detected during coelomocyte regeneration after evisceration in *A. japonicus*. Immune-related factors Cu/Zn superoxide dismutase (Cu/Zn SOD), Catalase (CAT), C-type lysozyme (C-LYZ), Cathepsin D (CTSD), Melanotransferrin (MTF), Toll-like receptor (TLR), C-type lectin (C-LCT), Complement 3 (C3), Myeloid differentiation factor 88 (MYD88), Nuclear factor kappa-B (NF-κB), NF-κB inhibitor (IKB), TNF receptor-associated factor 6 (TRAF6), Peroxiredoxins (PRX), Nitric oxide synthase(NOS), Caspase-2 (CASP-2), Phenoloxidase (PO), and Glutathione Peroxidase(GPX), Heat shock protein 70 (HSP70) were detected by real-time fluorescent quantitative PCR at different time points during regeneration. The main immune-related genes in sea cucumber coelomocytes were significantly differentially expressed after evisceration, and an upregulation was observed for the majority of the considered genes. In summary, the discharge of viscera had a significant effect on expression of immune-related genes of sea cucumber coelomocytes. The expression level of each gene had a certain correlation with the sea cucumber regeneration process. The results provide reference data for the immune response of coelomocytes during regeneration.

Key Words: sea cucumber; regeneration; coelomocyte; immune-related factors; expression profile

Introduction

The sea cucumber *Apostichopus japonicus* is classified in the Phylum Echinodermata, Class Holothuroidea and is a representative echinoderm (Zhang *et al.*, 2017). Sea cucumbers have a defense mechanism against environmental changes or pathogenic infection. In hostile or detrimental environments such as high salt stress or anaerobic conditions, the internal organs of sea cucumbers are excreted (Sun *et al.*, 2013). Sea cucumbers in an appropriate environment regenerate new organs, including intestines, respiratory trees and gonads (Yuan *et al.*, 2019). The sea cucumbers regeneration mechanism is an important model for studying the cellular and molecular mechanisms of regeneration (Garcia-Arraras *et al.*, 1998). Sea cucumbers lack specific immune tissues and organs. Coelomocytes and their immune factors are important in hostile or detrimental environmental conditions (Xue *et al.*, 2015). And there was an interested question about what the expression profiles of the main immune-related factors in sea cucumber coelomocytes would be after removing the internal organs.

When sea cucumbers were infected by pathogens, coelomocytes defend against them by phagocytosis, identified coelomocyte immune factors attack foreign substances in coelomic fluid, remove invading microorganisms and repair damage (Jiang *et al.*, 2018). Cu/Zn superoxide dismutase (Cu/Zn-SOD) (Gao *et al.*, 2014), catalase (CAT) (Gao *et al.*, 2014), C-type lysozyme (C-LYZ) (Tian *et al.*, 2014), Cathepsin D (CTSD) (Jiang *et al.*, 2018), Melanotransferrin (MTF) (Qiu *et al.*, 2014), Toll-like receptor (TLR) (Jiang *et al.*, 2018), C-type lectin

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Table 1 Primers for qRT-PCR

Immune-related factors	Accession No.	Primer sequences (from 5′ to 3′)	References
Pattern recognition receptors (PRRs)			
Toll-like receptor	JQ743247	F: ACGAAAGCGATTTAGCC R: GAGCCCGTGGTGAGATG	Jiang *et al.*, 2018
Myeloid differentiation factor 88	KF032818.1	F: GGAAACGAGAGGAGGAGAGACG R: TCCAGACAGTAGCAGACGAAAGC	Lu *et al.*, 2013
TNF receptor-associated factor 6	KF032819.1	F: AGGAGCGGGAAAGGAAGCAG R: TAGCCGTAGAGCGCCGTGTAG	Lu *et al.*, 2013
NF-κB	JF828765.1	F: TGAAGGTGGTATGCGTCTGG R: TTGGGCTGCTCGGTTATG	Li *et al.*, 2018
IKB	KF032816	F: ACAGGAGTCGTTTGATGATTGG R: GTTTCTTCTTGTGTTTGGCGTTC	Lu *et al.*,2013
Complement 3	HQ214156	F: GCGTTGTTTCGTTCAACAAGGGGA R: GCCATTCACTGGAGGTGTGGCA	Jiang *et al.*, 2018
Cytochrome B	7802877	F: TGAGCCGCAACAGTAATC R: AAGGGAAAAGGAAGTGAAAG	Wang *et al.*,2016
Enzymes			
Phenoloxidase	KF040052	F: CAGCAGTTACAAGTGGGATG R: CCAGTCACGAAGACCAGAAT	Jiang *et al.*,2014
Cu/Zn superoxide dismutase	JX097096	F: TCGGGCACTATTACCTTC R: ACCATTATCATCGGCTTC	Jiang *et al.*, 2018
Catalase	JQ776634	F: CTCCCAACTACTTCCCAAAC R: GTCCGACAAGACCTCACG	Jiang *et al.*, 2018
C-type lysozyme	KF773759	F: GTACCACGGAGCAGGAGT R: CACAGACAAGCGGAGACC	Jiang *et al.*, 2018
Cathepsin D	JF430592	F: CTCCCAACTACTTCCCAAAC R: GTCCGACAAGACCTCACG	Jiang *et al.*, 2018
Glutathione peroxidase	JF769857	F: GGATGTGTGTGTCTAGTGGTGAA R: GAATTACTCCCAGGTTCCTGACT	Wang *et al.*, 2011
Nitric oxide synthase	KT366016.1	F: TTGGGCTGCTCGGTTATG R: TGATGCAAGAGACTGCTGGA	Li *et al.*, 2018
Peroxiredoxins	JF769853	F: TATTCACTCCTGGCTGCTCTAAG R: TGTGAAGTCACAGCAGGTATCAG	Wang *et al.*, 2011
Other immune-related factors			
Caspase 2	KC972624	F: CAAGTTCCACACGACACAGG R: GCAGTCTTTGTTCGTTCCGT	Li *et al.*, 2018
Heat shock protein 70	EU930813.1	F: GCCGCATCCCTTGTAAGAAG R: AGTTCAAATTGACCGAGGCG	Li *et al.*, 2018
Melanotransferrin	HQ260578	F: GGTGGGTGATGCCTGTTG R: AGCTGAGGTGGGTTCGTT	Jiang *et al.*, 2018
C-type lectin	HQ728281	F: TCGGATCTAACTTGGACG R: TTACCCTGCGAATGACTT	Jiang *et al.*, 2018

(C-LCT) (Wei, *et al.*, 2015), Complement 3 (C3) (Zhou *et al.*, 2011), Myeloid differentiation factor 88 (MYD88) (Lu *et al.*, 2013b), NF-κB (Wang *et al.*, 2013), NF-κB inhibitor (IKB) (Lu*, et al.*, 2013), TNF receptor-associated factor 6 (TRAF6) (Lu *et al.*, 2013a), Peroxiredoxins (PRX) (Wang *et al.*, 2011), Nitric oxide synthase (NOS) (Shao *et al.*, 2016), Caspase-2 (CASP-2) (Ye *et al.*, 2016), Phenoloxidase (PO) (Jiang *et al.*, 2014), Glutathione peroxidase (GPX) (Wang *et al.*, 2011b), Heat shock protein 70 (Li *et al.*, 2018) and other immune-related factors are reported to be involved in response and removal of invading pathogens. Based on our previous study on the regeneration of coelomocytes after evisceration by *A. japonicas* (Li *et al.*, 2018), we selected 18 immune-related factors reported to be involved in pattern recognition receptor pathways, immune enzymes, and other immune-related factors. Expression profiles of immune-related genes in regeneration after evisceration were used to research immunological characteristics and immune defense mechanisms of the immune factors in coelomocytes after evisceration. This study provides reference data for studying the immune regulation mechanism of echinoderm regeneration.

Materials and methods

Experimental animals and sampling

Healthy *Apostichopus japonicus* (55 ± 10.2 g, mean ± SD) were collected from a local aquatic farm (Dalian, Liaoning Province, China) and acclimated in an indoor aquarium with well-aerated sea water at 17 - 19 °C for 1 week before experiments. To study expression of coelomocyte immune-related factors during regeneration, evisceration was induced by intracoelomic injection of 1.2 mL 0.35 M KCl. Total coelomocytes from *A. japonicus* at pre-evisceration, 2 hours post evisceration (hpe), 6 hpe, 12 hpe, 36 hpe, 3 days post evisceration (dpe), 5 dpe, 7 dpe, 14 dpe, 21 dpe, and 35 dpe were sampled. The health sea cucumber before evisceration was as the control group (0 hpe), and each group had three biological replications. The coelomocyte sample was put into RNase-free centrifuge tubes. After centrifugation at 5000 rpm for 10 minutes at 4 °C, the supernatants were removed. And the coelomocyte precipitations were stored at -80 °C for further analysis.

Total RNA extraction and cDNA synthesis

Samples of coelomocytes were taken from -80 °C. Total RNA was extracted by Triozl (Life Technologies, USA). RNA integrity was detected by 1 % agarose gel electrophoresis. Concentration and purity of RNA were determined by a micro nucleic acid analyzer (Nano Drop 8000, Thermo, USA). Coelomocyte RNA was reverse transcribed using the PrimeScript™ RT reagent kit gDNA Eraser (Takara, Japan). Reverse transcription occurred as follows: First-strand cDNA was synthesized using PrimeScript™ 1st strand cDNA Synthesis Kits (Takara, Japan) as a 10.0 μL mixture (1.0 μL 50 μmol/L Oligo dT primer, 1.0 μL 10 mmol/L dNTP mixture, 0.8 μg total RNA and the remaining RNase-free ddH_2O complemented the total volume of 10 μL) incubated at 42 °C for 2 min. To the 10.0 μL mixture, 4.0 μL 5 × PrimeScript™ buffer, 0.5 μL 40 U/μL RNase inhibitor, 1.0 μL 200 U/μL primeScript™ RNase and 4.5 μL RNase-free dH_2O were mixed for incubation at 42 °C for 15 min, 85 °C for 5 s and cooling on ice. Products were used as cDNA and stored at -20 °C.

Quantitative real-time PCR

Relative mRNA levels of immune-related factors were determined by quantitative real-time PCR (qRT-PCR). Primer pairs for immune-related factors were reported (Table 1) and verified for amplicon sizes and specificity by melting curve analysis and agarose gel electrophoresis. All cDNA sample for qRT-PCR, we used SYBR Premix Ex Taq™ II Kits (TaKaRa, Japan) in 20 μL reactions of 10 μL 2 × SYBR Premix Ex Taq™ II, 0.8 μL 10 μmol/L forward and reverse primer, 0.4 μL ROX Reference Dye II, 1 μL cDNA template and 7 μL sterile distilled water. The program was 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s, 55 °C for 25 s and 72 °C for 25 s. Each samples amplification melting curve was a single peak, and the Ct value was collected, followed by data processing and analysis.

Statistical analysis

The Ct values of amplification curves were analyzed by the $2^{-\Delta\Delta ct}$ method. All experimental data were expressed as mean ± SD and all experimental data were analyzed with SPSS 19.0 version (SPSS Inc., IL, USA), including *t*-tests for comparison of two sets of data, one-way ANOVA for multiple sets. The level of significance was defined as $P < 0.05$.

Results

Expression of major immune genes related to pattern recognition receptors in sea cucumbers

Expression of major immune factors in the pattern recognition receptor in sea cucumber coelomocytes after evisceration was shown in Figure 1. After evisceration, expression of TLR in sea cucumbers was significantly up-regulated and was 3.26 times than the control group at 3 dpe ($P < 0.05$). Expression of TLR in sea cucumbers was significantly down-regulated and was 0.0813 times the control group at 5 dpe, 0.1090 times at 7 dpe and 0.0274 times at 35 dpe. Expression of IKB after evisceration was significantly higher than the control group at 3.65 times at 6 hpe, 2.42 times at 12 hpe, 2.91 times at 3 dpe, and 4.44 times at 21 dpe ($P < 0.05$). Expression of NF-κB after evisceration was significantly higher than the control group at 12 hpe, 36 hpe and 3 dpe ($P < 0.05$). The highest value was at 3 dpe, at 11.15 times the control group. Expression of C3 after evisceration was significantly higher than the control group at 6, 12, and 36 hpe and 3, 5, 7, 14, and 21 dpe ($P < 0.05$). The highest value was at 3 dpe, at 22.30 times the control group. Expression of MYD88 after evisceration was significantly higher than the control group at 6 and 36 hpe and 3 dpe ($P < 0.05$). The highest value was at 3 dpe, at 26.27 times the control group. Expression of the TRAF6 gene after evisceration was significantly higher than the control at 12 and 36 hpe, and 3 dpe ($P < 0.05$). The highest value was at 3 dpe, at 43.04 times the control group. Within 35 days of regeneration in sea cucumbers, the overall trends for IKB, NF-κB, C3, MYD88, and TRAF6 were up-regulation. TLR was significantly up-regulated first, then significantly down-regulated. These six genes were all up-regulated at 3 dpe. C3 was continuously up-regulated throughout the regeneration process except for the initial 2 hpe and 35 dpe. Expression of C3 was significantly up-regulated within 35 days of regeneration.

Expression of immune enzyme genes of sea cucumbers after evisceration

Expression of immune enzymes in coelomocytes after evisceration was shown in Figure 2. Expression of CAT first rose, then decreased at 2 to 12 hpe, 36 hpe to 5 dpe, and 5 dpe to 35 dpe, with peaks at 6 hpe and at 3 dpe and 14 dpe. Expression of CAT was highest at 3 dpe, at 8.38 times the control group ($P < 0.05$). Expression of CTSD rose first and then decreased at 2 to 12 hpe, 36 hpe to 5 dpe, and 5 dpe to 35 dpe, with peaks at 6 hpe, and 3 and 14 dpe. Expression of CTSD was the highest at 6 hpe, at 4.85 times the control group ($P < 0.05$). Expression of C-LYZ rose first and then decreased at 2 to 12 hpe, 36 hpe to 5 dpe and 5 dpe to 35dpe, with peaks at 6 hpe, and 3 dpe and 14 dpe ($P < 0.05$). Expression of C-LYZ was highest at 3 dpe,

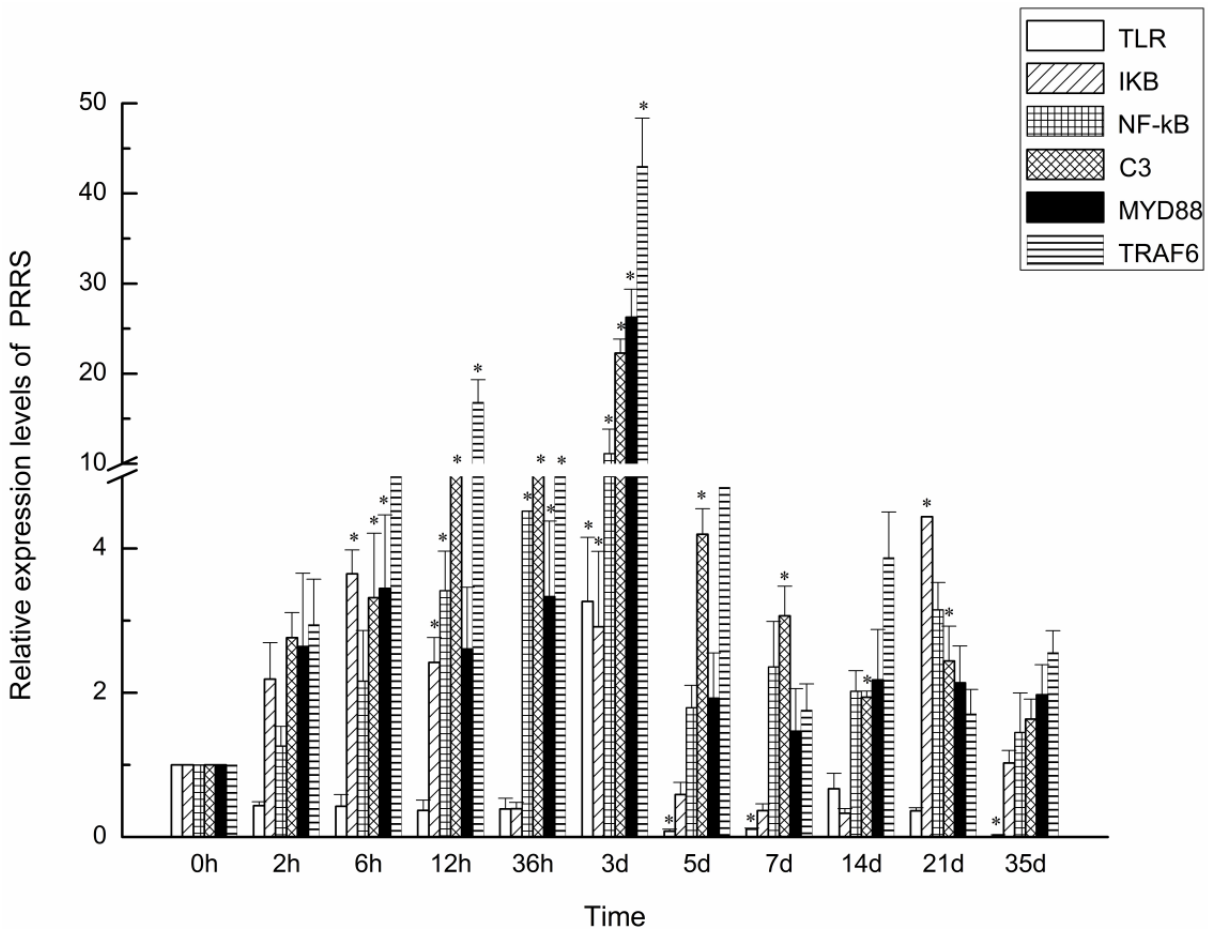

Fig. 1 Expression of major immune genes related to pattern recognition receptors in sea cucumber coelomocytes after evisceration. Abbreviations: Toll-like receptor (TLR), Nuclear factor kappa-B (NF-κB), NF-κB inhibitor (IKB), Complement 3 (C3), Myeloid differentiation factor 88 (MYD88), TNF receptor-associated factor 6 (TRAF6)

figat 2.46 times the control group (P < 0.05). Expression of GPX rose and then decreased at 2 to 12 hpe, 36 hpe to 7 dpe, and 7 dpe to 35 dpe, with peaks at 6 hpe, and 3 dpe and 14 dpe. Expression of GPX was highest at 3 dpe, at 24.17 times the control group (P < 0.05).Expression of PO rose and then decreased at 2 to 12 hpe, 36 hpe to 7 dpe, and 7 to 21 dpe, with peaks at 6 hpe and 3 and 14 dpe. Expression of PO was highest at 3 dpe, at 9.69 times the control group (P < 0.05). In addition, expression of PRX was significantly lower than the control group at 3 dpe (P < 0.05), rising first and then decreasing at 2 to 12 hpe, 12 hpe to 7 dpe, and 7 to 35 dpe, with peaks at 6 hpe, and 3 and 14 dpe (P < 0.05). Expression of PRX was highest at 3 dpe, at 2.21 times the control group (P < 0.05). Expression of PRX was lowest at 35 dpe, at 0.12 times the control group (P < 0.05). Expression of Cu/Zn SOD rose and then decreased at 2 to 12 hpe, 36 hpe to 5 dpe, and 7 to 35 dpe, with peaks at 6 hpe, and 3 and 14 dpe. The expression of Cu/Zn SOD was the highest value at 14 dpe, at 5.12 times of the control group (P < 0.05). Expression of NOS rose and then decreased at 2 to 12 hpe, 36 h to 5 d, and 14 to 35 dpe, with peaks at 6 hpe, and 3 and 21 dpe. Expression of Cu/Zn SOD was highest at 21 dpe, at 57.18 times the control group (P < 0.05). Within 35 days of regeneration, the overall trend for CAT, CTSD, C-LYZ, GPX, PO, Cu/Zn SOD, NOS was up-regulation. These seven genes, except for Cu/Zn SOD, were significantly up-regulated at 3 dpe. Cu/Zn SOD was significantly up-regulated at 14 dpe. PRX was significantly up-regulated at 3 dpe, while was significantly down-regulated at other time points.

Expression of other immune factors in sea cucumber coelomocytes after evisceration

Expression of other immune factors in coelomocytes after evisceration was shown in Figure 3. In addition to CASP-2, all other immune factors studied showed a trend of increasing first and then decreasing at 2 to 12 hpe and 36 hpe to 5 dpe, peaking at 6 hpe and 3 dpe (P < 0.05), with significant up-regulation at 3 dpe (P < 0.05). The CASP-2 gene showed a downward trend within 35 days after evisceration, reaching the lowest value at 3 dpe, at 0.01 times the control group (P < 0.01). Expression of MTF rose first and then decreased at 2 to 12 hpe, and 36 hpe to 5 dpe, with peaks at 6 hpe and 3 dpe (P < 0.05). Expression of MTF was

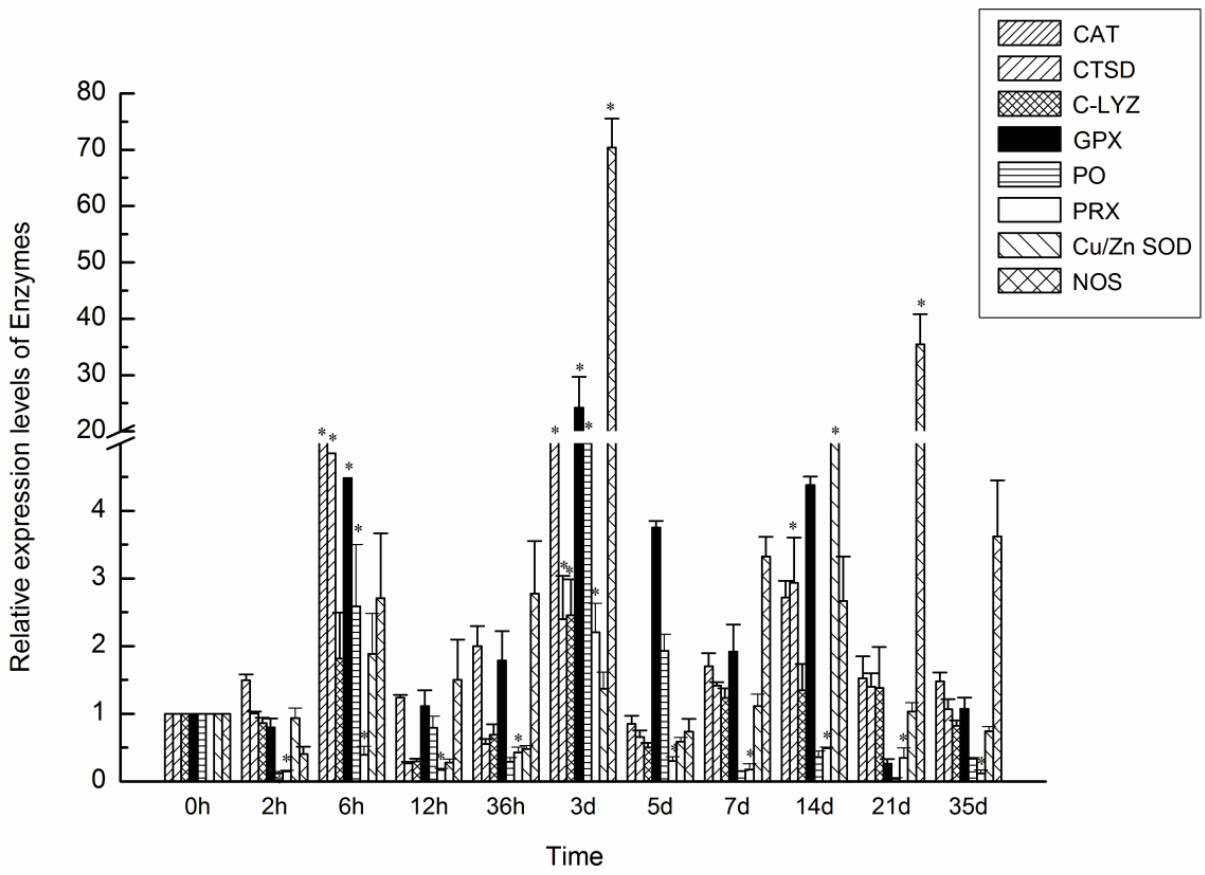

Fig. 2 Expression of immune enzymes in sea cucumber coelomocytes after evisceration. Abbreviations: Catalase (CAT), Cathepsin D (CTSD), C-type lysozyme (C-LYZ), Glutathione Peroxidase (GPX), Phenoloxidase (PO), Peroxiredoxins(PRX), Cu/Zn superoxide dismutase (Cu/Zn SOD), Nitric oxide synthase(NOS). *$P < 0.05$

highest at 3 dpe, at 15.36 times the control group ($P < 0.05$). Expression of HSP70 rose and then decreased at 2 to 12 hpe, and 36 hpe to 5 dpe, with peaks at 6 hpe and 3 dpe. Expression of HSP70 was highest value at 3 dpe, at 49.78 times the control group ($P < 0.05$). Expression of C-type lectin rose and then decreased at 2 to 12 hpe, and 36 hpe to 5 dpe, with peaks at 6 hpe and 3 dpe. Expression level of HSP70 was highest at 3 dpe, at 114.49 times the control group ($P < 0.01$). Within 35 days of regeneration, the overall trend for MTF, HSP70, and C-type lectin were up-regulation, with significant up-regulation at 3 dpe. CASP-2 was significantly down-regulated at 3 dpe.

Discussion

Pattern recognition receptor-associated immune factor

After evisceration, sea cucumber bodies were sensitive and fragile. The pattern recognition receptor (PRRs) pathway of sea cucumbers was important for evisceration and regeneration because sea cucumbers were echinoderms that lack acquired immunity, and their defense mechanism relies on PRRs to identify and eliminate pathogens. In this study, TLR was significantly up-regulated at 3 dpe after evisceration and then significantly decreased, which might be a protective mechanism that was conducive to sea cucumber regeneration. Significant up-regulation of TLR might be because when microorganisms destroy physical barriers of the sea cucumber, TLR activated a signaling pathway of immune cells by significantly up-regulating and specifically recognizing the conserved molecular structure of the pathogen PAMP (Liu *et al.*, 2017), which allowed sea cucumbers to eliminate more pathogens, thereby promoting regeneration. In our previous study, the total number of luminal cells in sea cucumbers returned to previous levels 6 hours after evisceration and the ability to divide cells 3 days after evisceration was relatively stronger than at other time points (Li *et al.*, 2018). TLR4 was down-regulated when it differentiates into human neural stem cells *in vivo* (Grasselli*, et al.*, 2018). In addition, expression of TLR4 was down-regulated during differentiation of cervical tumor cells into cervical tumors (Ma *et al.*, 2018). Therefore, we hypothesized that on the third day after evisceration, sea cucumber coelomocytes began to differentiate and form coelomocytes with different immune functions. In this study, both TRAF6 and MYD88 were significantly up-regulated in the PRRs pathway compared with the control group, and the difference

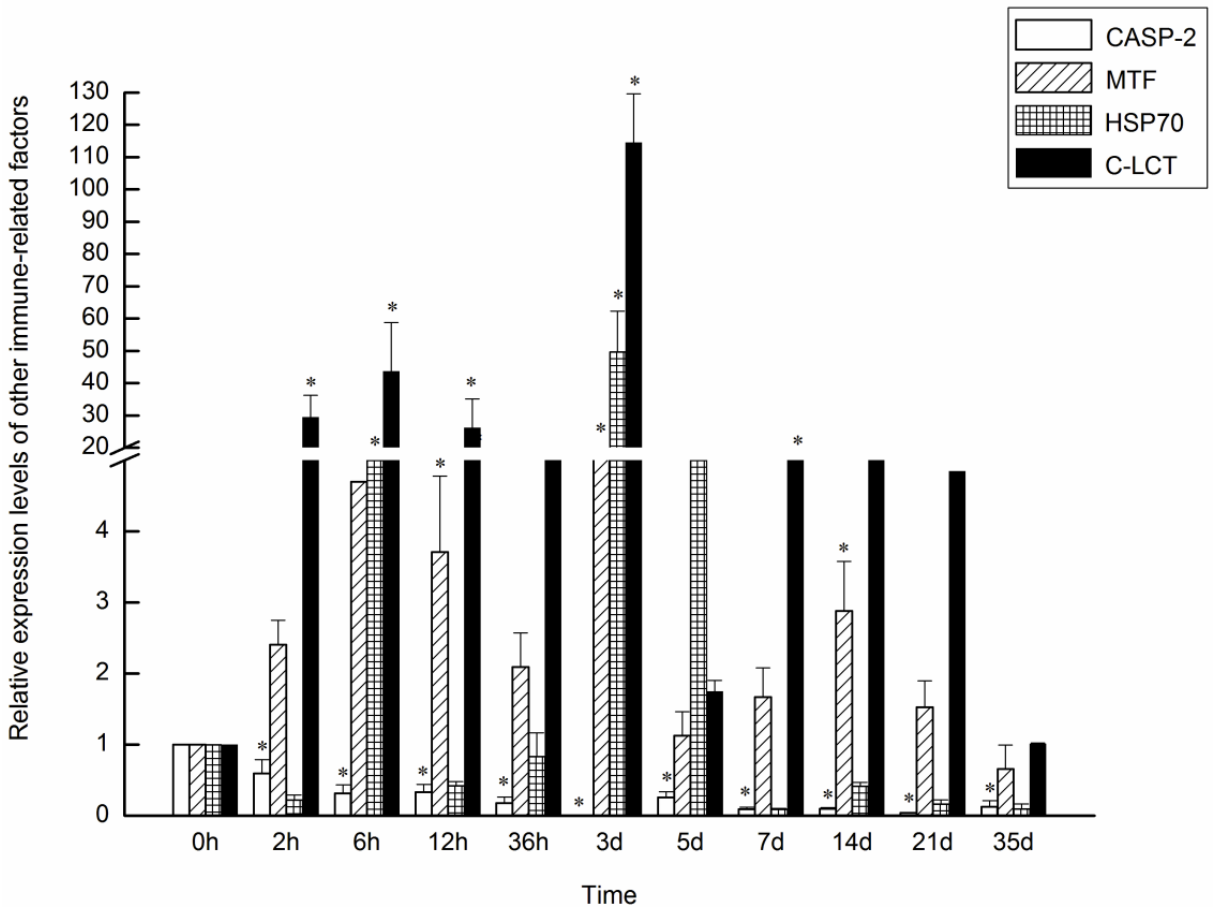

Fig. 3 Expression of other immune factors in sea cucumber coelomocytes after evisceration. Abbreviations: Caspase-2 (CASP-2), Melanotransferrin (MTF), C-type lectin (C-LCT), Heat shock protein 70 (HSP70)

fold was significantly higher than other genes; TRAF6 and MYD88 belonged to adaptor proteins, from which we can speculate The significant up-regulation of both TRAF6 and MYD88 in the PRRs pathway compared to the control group was to increase the sensitivity of the recognition pathway. TRAF6 was an ubiquitin-ligase. When activated, it produced short protein chains on itself and other proteins. TRAF6 acted as a switch that determines which signals are turned on in cells (Sun *et al.*, 2014). Studies showed that TRAF6 was involved in receptor-mediated activation of multiple signaling pathways in adult muscle fiber regeneration (Hindi *et al.*, 2012). When TRAF6 was knockout in adult mouse satellite cells, it caused severe muscle regeneration defects (Hindi*et al.*, 2016). When the expression level of TRAF6 was significantly decreased, the proliferation of zebrafish cardiomyocytes was significantly inhibited (Seeger *et al.*, 2013). These findings indicate that TRAF6 was important in the process of cell regeneration. MYD88 was a key linker molecule in the TLR signaling pathway and important in the transmission of upstream information and disease development. MYD88 was an adaptor protein that mediates Toll-like receptor and interleukin-1 receptor signaling. Up-regulation of MYD88 promoted fusion of exogenous myoblasts with damaged muscle fibers during muscle regeneration (Hindi *et al.*, 2017). We hypothesized that the up-regulation of TRAF6 and MYD88 in the regeneration process of sea cucumbers increased the sensitivity of the recognition pathway and enhanced the proliferation of coelomocytes, resulting in more immune responses that facilitated recovery of sea cucumbers. C3 was continuously up-regulated during the entire regeneration process except the first 2 hours after evisceration and the 35th day after regeneration. We hypothesized that the complement pathway was important in the process of regeneration of coelomocytes. *Pleurodeles waltl* complement component C3 was also expressed early in development and its expression increased with the development of the immune system (Gueguinou *et al.*, 2014). The hepatocyte proliferation ability of mice was impaired when C3 is defective, and proliferation of hepatocytes is restored when C3 was normal. Complement was activated in skeletal muscle injury in mice and was a key to skeletal muscle regeneration (Markiewski *et al.*, 2004). Genetic ablation of complement C3 resulted in impaired muscle regeneration following cardiac

cardiomyopathy-induced injury in mice (Zhang *et al.*, 2012). These studies indicated that the complement pathway was important in the process of regeneration, indicating the good agreement with our current results.

Enzyme-related immune-related factors

After evisceration, the sea cucumber immune response was important to ensure normal regeneration. The immune response of the sea cucumber was significantly enhanced in regenerative tissue (Zhang *et al.*, 2017). Because of a lack of acquired immunity in invertebrates, sea cucumber coelomocytes immune enzyme factors were important in regeneration. In our study, all immunoenzyme genes were significantly expressed on the third day after evisceration, except for SOD, which was significantly expressed on the 14th day. In addition, the difference in expression of the NOS gene was significantly greater than other immunoenzyme-based genes and it was the most significantly up-regulated immune enzyme. NOS is expressed during peripheral nerve regeneration and functions in repairing nerve-induced regeneration (Gonzálezhernández *et al.*, 2015). In addition, NOS was significantly up-regulated in liver endothelial cells and regulates the proliferation of hepatic sinusoidal endothelial cells to regenerate the liver (Yokoi *et al.*, 2010). Therefore, we hypothesized that NOS participated in regeneration of sea cucumbers and was important in regeneration. PRX was down-regulated throughout the regeneration process and might be a protective mechanism to promote regeneration. PRX were a ubiquitous family of cysteine-dependent peroxidases with antioxidant and anti-apoptotic functions. Down-regulation of oxidoreductase mediated an increase in oxidative stress and induces apoptosis in cancer cells (Yoshida *et al.*, 2011). When dprx5 was inhibited, *Drosophila* were more sensitive to oxidative stress and had a higher rate of apoptosis (Radyuk *et al.*, 2009). In a study on the effect of peroxiredoxin I gene silencing on TGF-β1-induced fibroblast proliferation and collagen synthesis, when the Prx-1 gene was silenced, JNK activation was promoted by increased ROS levels, leading to cell proliferation (Chang *et al.*, 2006). These results indicated that inhibiting peroxiredoxins was beneficial to increasing body cells. In the antioxidant system of our study, CAT, CTSD, C-LYZ, GPX, PO, SOD, and NOS were up-regulated. We hypothesized that during regeneration, PRX induced apoptosis of damaged cells by down-regulation and enhanced the antioxidant system's defense response to adverse factors in regeneration, promoting regeneration. When genes for CAT, CTSD, C-LYZ, GPX, PO, SOD in the antioxidant system were up-regulated during 3 dpe of regeneration, PRX was also significantly up-regulated. The antioxidant activity of PRDX5 promotes the development of bovine SCNT embryos in vitro (Wang *et al.*, 2017). Therefore, it was speculated that the antioxidant system was over-expressed, resulting in damage to the sea cucumber body, thereby affecting the regeneration of sea cucumber. At this time, the up-regulation of PRX made the expression of sea cucumber antioxidant system relatively stable. It maintained the sea cucumber body's regenerative function. SOD in the antioxidant system was the first act in scavenging reactive oxygen species (Zhang *et al.*, 2007). In the antioxidant system in our study, CAT, CTSD, C-LYZ, GPX, PO, SOD and NOS were all up-regulated on the third day after the evisceration. SOD was significantly up-regulated on day 14 after evisceration. Of course, the function of this particular mechanism in regeneration remained to be further studied.

Other immune-related factors

After evisceration, sea cucumber bodies were rapidly enhanced for immunity against external pathogenic microorganisms, for a good environment for regeneration. This result indicated that C-LCT, HSP70, MTF, and CASP-2 were involved in regeneration of sea cucumbers. CASP-2 was consistently down-regulated during 35 days of regeneration and significantly down-regulated on 3 dpe. As the core enzyme of apoptosis mechanisms, the caspase protease system directly caused cell disintegration (Takle *et al.*, 2007). As a member of the caspases, caspase-2 induced apoptosis in response to both extrinsic and intrinsic signals. It was important in cell proliferation and differentiation. In studies involving caspase-2 in cell cycle promotion and AR activation, the activity of caspase-2 was important for the proliferation of androgen dependent prostate cancer cell lines LNCaP (Taghiyev *et al.*, 2011). Caspase-2 was necessary for skeletal muscle differentiation and muscle formation. The activity of caspase-2 was significantly increased during skeletal muscle cell differentiation (Boonstra *et al.*, 2018). These findings indicated that increased caspase-2 was conducive to cell proliferation. Whereas caspase-2 was down-regulated within 35 days of evisceration in this study, this might be due to the fact that the visceral regeneration of sea cucumber was similar to embryonic development, involving a large number of natural cell deaths, and a large number of new cells are formed, thereby forming new internal organs. Caspase-2 deficient mice accelerated cell death in motor neurons during development (Bergeron *et al.*, 1998). Caspase-2 was down-regulated during the 35 days' period of regeneration, causing apoptosis of naturally dead cells during regeneration to create new internal organs. And on the third day, caspase-2 was significantly down-regulated. This may indicate that the third day of the regeneration was the beginning of coelomocyte differentiation in sea cucumbers. All this results indicated down-regulation of caspase-2 was important in this process.

In this study, the selected immune-related genes in the coelomocytes of sea cucumber were significantly expressed at 3 dpe, except for Cu/Zn SOD, which was significantly up-regulated at 14 dpe. After evisceration, sea cucumber pattern recognition receptor-associated immune factors IKB, NF-κB, C3, MYD88, TRAF6 showed an overall upward trend within 35 days. TLR was first significantly up-regulated then significantly down-regulated. The overall trend for sea cucumber enzyme-related immune factors CAT, CTSD, C-LYZ, GPX, PO, Cu/Zn SOD, NOS was up-regulation. These seven genes were significantly upregulated at 3 dpe except

for Cu/Zn SOD, which was significantly up-regulated at 14 dpe. The other immune-related factors of sea cucumbers, MTF, HSP70, and C-LCT were up-regulated. The overall trend was up-regulation with significant up-regulation at 3 dpe. CASP2 was significantly down-regulated throughout this process.

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