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Immunotoxicity of nanoparticle $nTiO_2$ to a commercial marine bivalve species, *Tegillarca granosa*

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ABSTRACT

The increasing production and extensive application of nanoparticles (NPs) inevitably leads to increased release of NPs into the marine environment and therefore poses a potential threat to marine organisms, especially the sessile benthic bivalves. However, the impacts of NPs on the immunity of commercial and ecological important bivalve species, *Tegillarca granosa*, still remain unknown to date. In addition, the molecular mechanism of the immunotoxicity of NPs still remains unclear in marine invertebrates. Therefore, the immunotoxicity of $nTiO_2$ exposure to *T. granosa* at environmental realistic concentrations was investigated in the present study. Results obtained showed that the total number, phagocytic activity, and red granulocytes ratio of the haemocytes were significantly reduced after 30 days $nTiO_2$ exposures at the concentrations of 10 and 100 μg/L. Furthermore, the expressions of genes encoding Pattern Recognition Receptors (PPRs) and downstream immune-related molecules were significantly down-regulated by $nTiO_2$ exposures, indicating a reduced sensitivity to pathogen challenges. In conclusion, evident immunotoxicity of $nTiO_2$ to *T. granosa* at environmental realistic concentrations was detected by the present study. In addition, the gene expression analysis suggests that the PRRs (both TLRs and RIG1 investigated) may be the molecules for NPs recognition in marine invertebrates.

1. Introduction

The extensive application of nanoparticles (NPs) boosts the NPs' production, which has been predicted to keep growing to more than half a million tons per year by 2020 (www.nanoproject.org). This would inevitably lead to an increased release of NPs into the environment [1]. Through the process of precipitation and surface runoff, atmospheric and ground NPs will converge into its ultimate sink, the ocean, and pose a potential threat to marine organisms and ecosystems [2]. As a result, great attention has been drawn to the toxicological and ecological impacts of NPs on marine organisms recently [3,4]. Previous studies demonstrated that biological processes and physiological functions such as fertilization [5–7], metabolism [8], larval development [9–11], immune responses [12–14], growth [8,15], and even survival [8,9,15] of various marine organisms could be affected by NP pollutants.

Due to the high ionic strength and low organic carbon content of seawater, NPs in the marine environment will aggregate and settle down to the seafloor [3]. The precipitation process of NPs along with bioturbation and resuspension leads to a high concentration of NPs in the sediment-water interface [16], where many benthic marine species such as sessile marine bivalves inhabit. Due to the abundance in estuaries and coastal zones and great ability of internalizing nano and micro-scale particles [13], benthic sessile marine bivalves are generally regarded as model organisms to characterize the potentially biological and ecological impacts of NPs [3,17]. Previous studies showed that NPs' exposure could hamper the immune responses [18], cause DNA damage [14], and alter protein expression [19] of marine bivalves such as *Mytilus galloprovincialis*, *M. edulis*, and *Crassostrea gigas*.

Among all these physiological processes, the immune responses of marine bivalves are shown to be sensitive to NP exposures [20,21]. For example, rapid increases in extracellular lysozyme release, reactive oxygen species (ROS) and nitric oxide (NO) production were observed in *M. galloprovincialis* haemocytes after short-term exposure (1–4 h) to NPs (carbon black, C60 fullerene, TiO_2 and SiO_2) at the concentration range of 1–10 μg/ml [13], indicating a significant immunotoxicity of NPs to the

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mediterranean mussel *M. galloprovincialis*. Similarly, immunotoxicity of NPs in terms of reduced phagocytic activity has also been detected for different types of NPs (i.e. CdS/CdTe quantum dots, Ag and TiO_2 NPs) in *M. galloprovincialis* [2,22,23], *M. edulis* [24], *C. virginica* [25], and *Scapharca subcrenata* [26]. Since possessing a sound immunity is essential for the survival of bivalve individuals, NP pollution may therefore render marine bivalve species more susceptible to pathogen challenges and subsequently pose a threat to the aquaculture of commercial marine bivalves.

The blood clam, *Tegillarca granosa*, is a traditional aquaculture marine bivalve species and is widely distributed throughout the Indo-Pacific region [27–30]. Since lives in the intertidal mudflat, where pollutants such as NPs are often concentrated [31,32], the sessile blood clam may be under the threat of exposure to a high concentration of NPs. However, to the best of our knowledge, it still remains unknown whether the immunity of *T. granosa* will be affected by exposure to NPs or not.

Among various NPs, titanium dioxide NPs ($nTiO_2$) are widely used in a variety of products such as paints, coatings, plastics, papers, inks, foods, pharmaceuticals, cosmetics, and toothpastes [33,34]. Therefore, a huge amount of $nTiO_2$ is released into the aquatic environment and the concentration may reach to as high as several μg/L [35]. Though the immunotoxicity of $nTiO_2$ has been reported in a variety of bivalve species recently [2,23,36], either an unrealistic high dose (usually above 1 mg/L) or short duration (usually 4–7 days) [3,4] of exposure were generally adopted in these investigations.

Furthermore, the molecular mechanism manifesting the immunotoxicity of NPs still remains poorly understood in marine invertebrates. Though some studies have demonstrated that NPs may be immunotoxic to marine bivalves through suppressing immune-related responding molecules such as superoxide dismutases (SOD), catalase (CAT), glutathione peroxidase (GPx), Mytilin, and Myticin [2], it is still unclear how NPs are recognized by the immune system of marine invertebrates. More recently, *in vitro* hybridization and X-ray structure analysis suggest that NPs may be recognized by the Pattern Recognition Receptors (PRRs) such as Toll-like receptors (TLRs) in mammals [37]. However, empirical studies are necessary to verify whether it is the case in marine invertebrates as well.

Therefore, in order to gain a better understanding of the toxicological and ecological impacts of NPs on marine bivalve species. The present study was conducted to explore whether longer term (30 days) $nTiO_2$ exposure would affect the immune ability of blood clams, and if so, whether the observed impacts could be partially explained by alterations in the expressions of immune-related genes encoding PRRs and downstream responding molecules.

2. Methods

2.1. Characterization analysis of nanoparticle $nTiO_2$

The $nTiO_2$ used in the present study was purchased from Shanghai Klamar Reagent Co. Ltd, China. The $nTiO_2$ was dissolved in ultrapure water followed by sonication for 15 min to prepare a stock solution (1 g/L) and diluted to test solutions with 0.1 μm membrane-filtered seawater (pH 8.10, salinity 21.5‰) immediately prior to use. Particle morphology and size were determined by transmission electron microscopy (TEM, JEM-1230, JEOL, Tokyo, Japan). The crystal structure of the particles was identified by X-ray powder diffractometry (XRD, Rigaku D/MAX 2550/PC, Tokyo, Japan) and the surface area was obtained by Brunauer-Emmett-Teller (BET) adsorption measurements (Micromeritic TriStarII 3020, Micrometritics Instrument Corp., Norcross, GA) Following the methods of Canesi [13]. The physicochemical properties of $nTiO_2$ used in the present study are shown in Table 1 and Fig. 1.

2.2. Collection and acclimation of blood clams

Adult *T. granosa* (9.8 ± 1.5 g) were obtained from Yueqing Bay (28°28′ N and 121°11′ E), Wenzhou, China, in June 2016. After cleaning off the epizoa, the animals were acclimated for one week in a 1000 L plastic tank with flowing sand filtered seawater prior to the respective treatments (25.0 ± 0.5 °C, pH 8.10 ± 0.05, salinity at 21.5 ± 0.4‰). The animals were fed with microalgae (*Tetraselmis chuii*) at the satiation feed rate twice a day during the acclimation period.

2.3. Exposure of blood clams to $nTiO_2$

According to previous studies [2,38,39], 10 and 100 μg/L were chosen to simulate the environmental realistic concentrations of $nTiO_2$ in the present study. The working concentrations of $nTiO_2$ in seawater (listed in Table 1) were measured using inductively coupled plasma atomic emission spectrometry (ICP-MS, PE NexION 300X, USA), following the methods described by Wang [40].

In total, three experimental treatment groups containing nominally 0 (control), 10 and 100 μg/L $nTiO_2$ were conducted, respectively. For each treatment, three replicates were performed. In brief, after one week of acclimation, 270 clams were randomly divided into 9 plastic tanks (3 treatments × 3 replicates) with a total seawater volume of 40 L at desired $nTiO_2$ concentrations. During the experiment, the seawater was changed daily with pre-prepared seawater containing corresponding concentrations of $nTiO_2$ and the animals were fed with *T. chuii* before the replacement of water. An exposure duration of 30 days was adopted and no individual mortality was observed throughout the experimental period.

2.4. Haemocyte counts analysis

A haemocyte count analysis was performed following the method of Liu [41]. Briefly, five individuals were randomly taken out from each experimental trial after the 30 days' exposure to different doses of $nTiO_2$. After being rinsed with 0.1 M phosphate buffer saline (PBS, pH at 7.4) solution, 50 μL haemolymph was extracted from the cavity of the individual using a 1 ml sterile syringe and then immediately transferred into a 1.5 ml centrifuge tube pre-filled with 50 μL 2.5% glutaraldehyde on ice. After adding 900 μL PBS, a wet mount of the fixed haemolymph was made with a Neubauer haemocytometer (XB-K-25, Anxin Optical Instrument) and subsequently observed under a Nikon eclipse E600 microscopy at a magnification of 200 × for the estimation of total haemocytes counts.

Similarly, after mixing 700 μL freshly extracted haemocytes with 300 μL 2.5% glutaraldehyde, the mixtures were centrifuged at

Table 1
Physicochemical properties of TiO_2 nanoparticles used in the present study.

Property	TiO_2 NPs
Average diameter	35 ± 5 nm
BET surface area	60.65 $m^2\ g^{-1}$
Crystal structure	Anatase
Purity	99.8%
Working Ti concentration	
at control	2.8 ± 0.1 μg/L
at 10 μg/L	9.2 ± 0.2 μg/L
at 100 μg/L	102 ± 0.4 μg/L

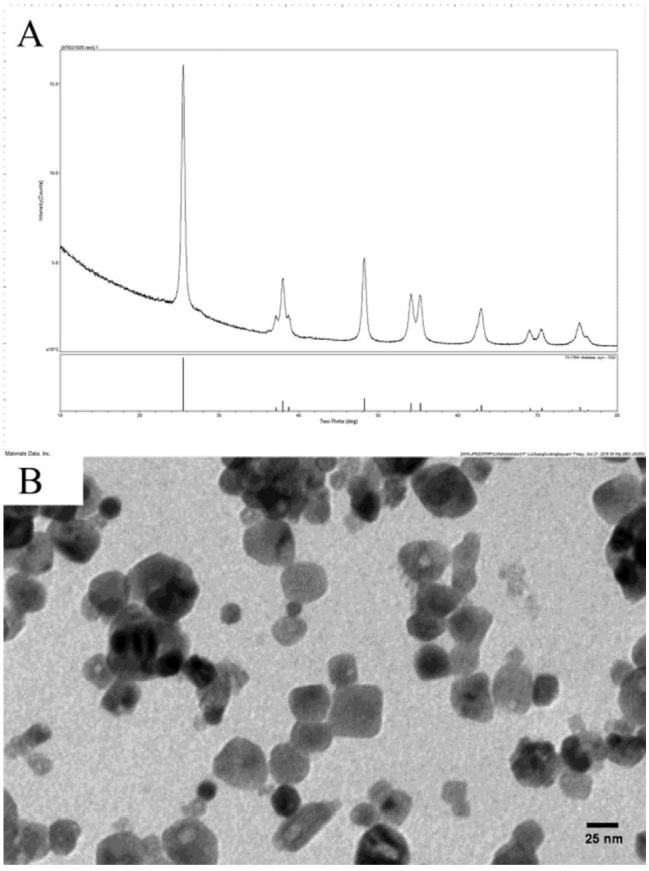

Fig. 1. X-ray diffractogram (A) and TEM micrograph (B) of the $nTiO_2$ samples.

4000 rpm for 4 min. Blood smears were subsequently made with 50 μL blood precipitates. Once air dried, the blood smears were stained with Wright-Giemsa stain (G1020, Solarbio) for cell type composition analysis. The counts of various cell types were determined under a Nikon eclipse E600 light microscope at a magnification of 1000 × . According to the method described by Zhu [42], three major types of haemocytes, including red granulocyte, basophil granulocyte, and hyalinocyte, were identified in the present study. A total of more than 100 haemocyte cells were scored for each sample.

2.5. Phagocytosis assays

Following the methods described by Su [32] and Liu [41], Yeast (Instant dry yeast, AngelYeast) suspensions were prepared by dissolving 7 mg yeast powder in 1000 ml 0.1 M PBS. As described above, 100 μL haemolymph was extracted from the individual and then mixed with 100 μL Alsever's solution (ALS, Noble Ryder) in a 1.5 ml centrifuge tube. After adding 20 μL yeast suspension, the yeast-haemocyte mixtures were kept at room temperature (25 °C) for 30 min followed by incubation in a cool water bath at 4 °C for an hour. Subsequently, 100 μL 2.5% glutaraldehyde was added to arrest the process of phagocytosis. The blood smears were then prepared and stained with Wright's stain as described above. The phagocytic rate was estimated microscopically at a magnification of 1000 × with a Nikon eclipse E600 light microscope. Five individuals from each trial and more than 100 haemocyte cells for each individual were scored.

2.6. Gene expression analysis

Total RNA of *T. granosa* was extracted from haemocytes samples after 30 days of treatment according to the method of Su [32]. RNA integrity was verified by gel electrophoresis and quantified spectrophotometrically with NanoDrop 1000 UV/visible spectrophotometer (Thermo Scientific). First strand cDNA was synthesized from high-quality total RNA using the M-MLV First Strand Kit (Invitrogen, C28025-032) following protocols provided by the manufacturer. Real-time quantitative PCR was performed on the ABI StepOne Plus (Applied Biosystems, Darmstadt, Germany) in triplicates, with a volume of 20 μL reaction system consisting of 10 μL AceQ qPCR SYBR Green Master Mix (Vazyme Biotech Co., Piscataway, NJ, USA), 0.4 μL of each primer (10 μM), 0.4 μL of ROX Reference Dye 1, 2 μL of cDNA template, and 6.8 μL of double-distilled water. The following amplification protocol was used for the amplification: 95 °C for 5 min followed by 40 cycles at the indicated settings (95 °C for 10 s and 60 °C for 30 s). A melting curve analysis was used to confirm the specificity and reliability of the PCR products, and 18S rRNA was employed as a reference for the calculation of the relative expression levels [30,41]. Except IRAK4 and TRAF3, the corresponding primers sequences for all the genes investigated and the internal reference were obtained from published literatures [32,41]. The software Primer Premier 5.0 was used to design primers for IRAK4 and TRAF3 based on the sequences information from NCBI GenBank. The former and reverse primer sequences used for IRAK4 are CTGTGTCAGGTCATCAGCATT and GGTGGCAATGTGATAGGTTGT, respectively. Primers GACAGTGGTCAGCGACATCT (Former) and AAGAGGACAACCAGAGGCAATA (Reverse) were used for TRAF3. All primers were synthesized by Sangon Biotech (Shanghai, China).

2.7. Statistics

One-way ANOVAs followed by Tukey's post hoc tests were conducted to compare haemocyte counts, phagocytosis, and the percentage of three major types of haemocytes subjected to 30 days $nTiO_2$ exposures at different concentrations. For all the analyses, Levene's test and Shapiro-Wilk's test was used to verify the homogeneity and normality of variance, respectively. For cases where these assumptions were not satisfied by the raw data, the data were arcsine square root transformed prior to the analysis. Gene expression levels were analyzed using the Duncan multiple range test. All the analyses were performed using the 'R' statistical packages and a p value less than 0.05 was accepted as significant difference.

3. Results

3.1. Impacts of $nTiO_2$ exposure on the total counts of haemocytes

As shown in Fig. 2, the average total counts of haemocytes (THC) of *T. granosa* were significantly (Table 2, $p < 0.01$) affected by $nTiO_2$ exposure. After been exposed to 10 and 100 μg/L $nTiO_2$ for 30 days, the THC of *T. granosa* were approximately 79.76% and 70.98% of that of the control group, respectively.

3.2. Impacts of $nTiO_2$ exposure on the cell type composition of haemocytes

According to the results obtained (Fig. 3), the cell type composition of haemocytes was significantly altered by $nTiO_2$ exposure as well. Compared to that of the control, blood clams reared in $nTiO_2$ contaminated seawater had significantly fewer proportions of red granulocytes (Table 2, $p < 0.01$) but higher proportions of basophil

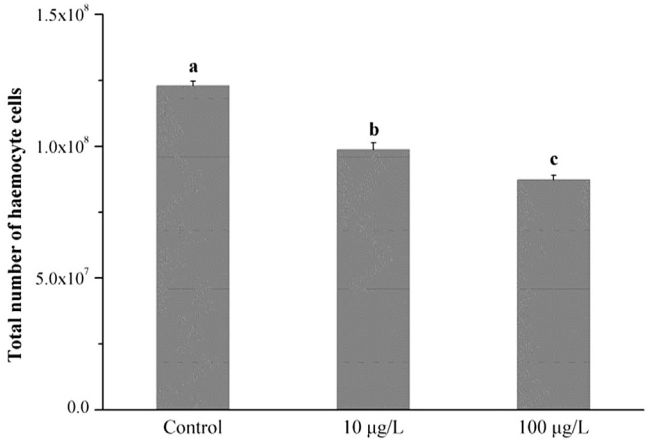

Fig. 2. The total counts of haemocytes (THC) of *T. granosa* after 30 days' exposure to 0, 10 and 100 μg/L $nTiO_2$, respectively. Mean values that do not share the same superscript were significantly different.

Table 2
ANOVAs showing the effects of $nTiO_2$ concentrations (0, 10 and 100 μg/L) on various haemocyte parameters of *T. granosa*. "*"and "**" indicated significant difference compared to that of control at $p < 0.05$ and $p < 0.01$, respectively.

Haemocyte parameters	Mean square	*F*	*p*
THC	9.76×10^{14}	75.11	$5.67 \times 10^{-5**}$
Phagocytosis (%)	0.02	26.08	$1.1 \times 10^{-3**}$
Percentage (%) of the			
Red granulocyte	8.52×10^{-3}	14.86	$4.74 \times 10^{-3**}$
Basophil granulocyte	8.91×10^{-3}	10.45	0.01*
Hyalinocyte	1.06×10^{-4}	1.91	0.23

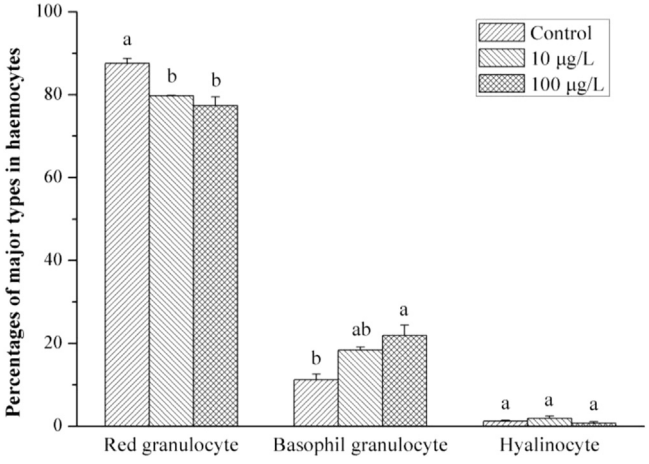

Fig. 3. Percentages of three major types of haemocytes after 30 days' exposure to 0, 10 and 100 μg/L $nTiO_2$, respectively. Mean values that do not share the same superscript were significantly different.

granulocytes (Table 2, $p < 0.05$).

3.3. Impacts of $nTiO_2$ exposure on the phagocytosis of haemocytes

Similarly, the phagocytosis of haemocytes was significantly hampered by 30 days' $nTiO_2$ exposure (Fig. 4 and Table 2, $p < 0.01$), which decreased to approximately 84.21% and 63.16% of that of the control, respectively.

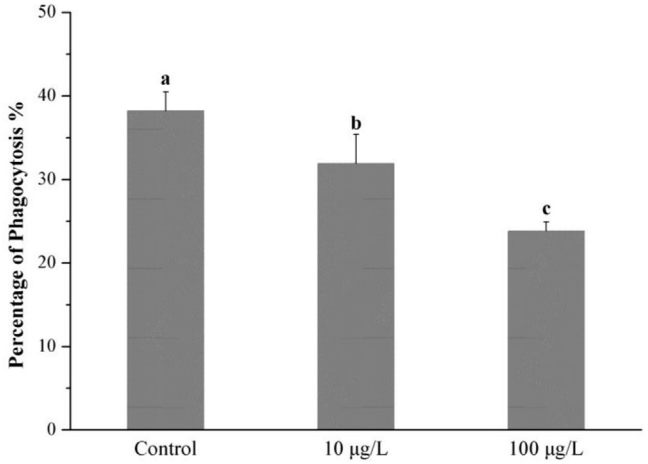

Fig. 4. The phagocytosis of haemocytes in *T. granosa* after 30 days' exposure to 0, 10 and 100 μg/L $nTiO_2$, respectively. Mean values that do not share the same superscript were significantly different.

3.4. Impacts of $nTiO_2$ exposure on the expression of genes encoding PRRs and downstream responding molecules

The expressions of genes encoding PRRs and downstream responding molecules after 30 days' exposure to various doses of $nTiO_2$ (0, 10, and 100 μg/L) were shown in Fig. 5 and Fig. 6, respectively. Compared to that of the control, the expressions of all PRRs encoding genes (TLR1, TLR2, TLR4, TLR5, TLR6, and RIG1) investigated were significantly suppressed by 30 days' $nTiO_2$ exposure. Though no significant down-regulation of TRAF2, NFκβ1 and TRIM58 was detected, exposure to $nTiO_2$ contaminated seawater at the concentration of 10 μg/L significantly suppressed the expressions of all the other PRRs downstream genes tested. In addition, when blood clams were exposed to $nTiO_2$ at a higher experimental concentration at 100 μg/L, the expressions of all the PRRs downstream genes (IRAK4, TRAF2, TRAF3, TRAF6, NFκβ1, and TRIM58) were significantly down-regulated.

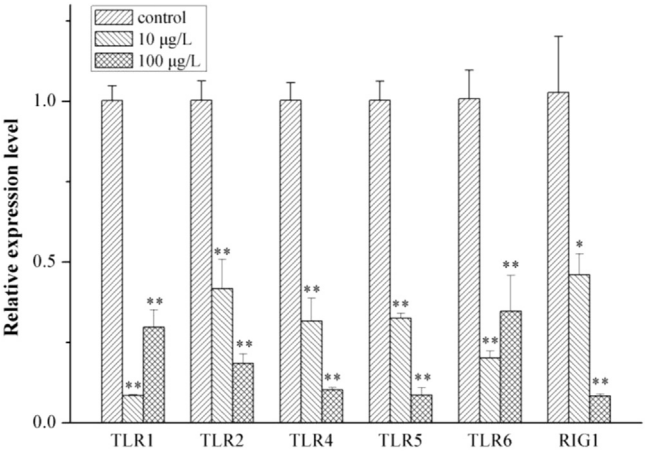

Fig. 5. Expressions of genes encoding PRRs TLR1, TLR2, TLR4, TLR5, TLR6, and RIG1 after 30 days' exposure to 0, 10, and 100 μg/L $nTiO_2$, respectively. All data was presented as means ± SE (n = 3). "*"and "**" indicated significant difference compared to that of control at $p < 0.05$ and $p < 0.01$, respectively.

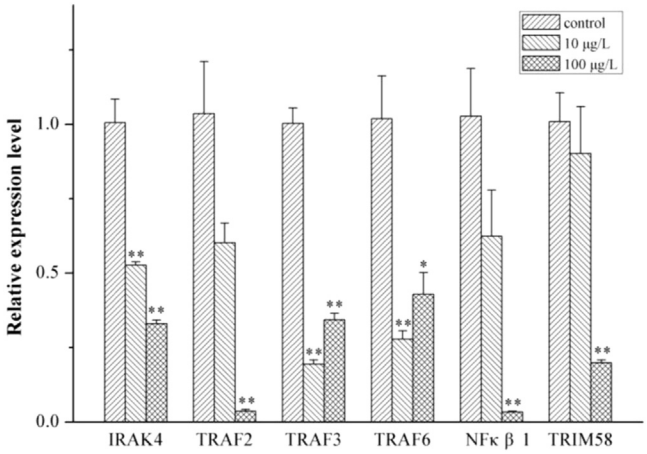

Fig. 6. Expressions of genes encoding PRRs downstream IRAK4, TRAF2, TRAF3, TRAF6, NFκβ1, and TRIM58 after 30 days' exposure to 0, 10, and 100 μg/L $nTiO_2$, respectively. All data was presented as means ± SE (n = 3). "*"and "**" indicated significant difference compared to that of control at $p < 0.05$ and $p < 0.01$, respectively.

4. Discussion

In reality, marine sessile bivalves are more likely to be challenged by chronic NPs pollutants at the realistic concentration (e.g. about 0.7–16 μg/L for $nTiO_2$) [38]. However, present knowledge of the immunotoxicity of NPs on marine bivalves is generally obtained from investigations conducted with acute NPs exposures (Table 3). Therefore, the impact of long-term NPs exposure at realistic concentration on the immunity of marine bivalves still remains poorly understood to date.

The results obtained in the present study showed that the THC, cell type composition, and phagocytosis of the haemocytes were significantly altered by 30 days' $nTiO_2$ exposure at realistic concentrations (10 and 100 μg/L), indicating a significant immunotoxicity of $nTiO_2$ to blood clams. Though the types, doses, and durations of NPs exposures varied among studies, the results obtained in the present study are comparable to those previously reported in other marine bivalve species [2,23,24]. For example, reduction in phagocytic activity of *M. galloprovincialis* hemocytes were observed after 96 h *in vivo* exposure to $nTiO_2$ (diameter at about 15–60 nm) at concentrations that ranged from 1 to 100 μg/L [2,23]. Similarly, acute exposure (21 h) to CdS/CdTe (1–10 nm) also reduced phagocytosis of the hemocytes of *M. edulis* at an exposure concentration of 2.7 μg/L [24]. Similar to these studies conducted in other bivalves, the results obtained in the present study indicate that $nTiO_2$ contamination may render blood clams more susceptible to pathogen challenges.

Interestingly, similar weakened immune responses in terms of reduced THC and phagocytosis have also been detected when marine bivalves are challenged with other pollutants and/or environmental stressors such as elevated pCO_2, heavy metals, and persistent organic pollutants (POPs) [43]. For instances, elevated pCO_2 or POPs (Benzo[*a*]pyrene, 5 μg/L) can reduce THC and hamper phagocytic activity of the haemocytes of *T. granosa* [32,41]. Similar hampered immune responses were also observed when bivalves such as *M. edulis* and *C. gigas* were exposed to heavy metal contaminations such as cadmium, chromium, and mercury [44,45]. It has been suggested that more energy of marine organisms would be allocated to critical life processes under environmental stress, leading to a reduction in the energy budget for immune responses [46]. In addition, since a series of fundamental biological process, including growth [47], metabolism [48], and energy availability [49] of both invertebrates and vertebrates were reported to be hampered by NPs' exposure, the weakened immune responses

Table 3
Summary of the immunotoxicological effects of nanoparticles (NPs) on marine bivalves.

Species	NPs and size	Exposure		Effects	Reference
		Time	Concentration		
M. galloprovincialis	Polystyrene (50 nm)	0.5–4 h	1, 5, 50 mg/L	↑Extracellular reactive oxygen species (ROS) and nitric oxide (NO) production; induce apoptotic process.	[55]
	C60-fullerene (NA)	1 h	1, 10 mg/L	↓Lysosomal stability (LMS).	[12]
	C60 fullerene (0.7 nm) TiO_2 (22 nm) SiO_2 (12 nm)	0.5–4 h	1, 5, 10 mg/L	↑Lysozyme release; ↑ROS and NO production.	[13]
	CdS (5 nm)	24 h	0–5 mg/L	↓Phagocytosis.	[22]
	CdTe (6 nm)	14 days	10 μg/L	↓LMS; changes in hemocytes types.	[14]
	TiO_2 (15–20 nm) SiO_2 (5–30 nm) ZnO (10–2000 nm) CeO_2 (5–20 nm)	0.5–4 h	0, 1, 5, 10 mg/L	↓LMS; ↓phagocytosis.	[56]
	TiO_2 (15–60 nm)	96 h	1, 10, 100 μg/L	↓immune-related genes (*Mytilin B*, *Myticin B*, and *lysozyme*); ↓LMS and phagocytosis; ↑ROS production.	[2]
	TiO_2 (27 nm)	96 h	100 μg/L	↓LMS; ↓phagocytosis; ↑NO production and lysozyme release.	[23]
M. coruscus	TiO_2 (20–30 nm)	14 days	0, 2.5, 10 mg/L	Co-exposure (TiO_2 + low pH). ↓Total hemocyte count (THC); ↑HM and ROS production.	[57]
M. edulis	CdS/CdTe (25–100 nm)	24 h	0–2.7 mg/L	↓Phagocytosis.	[24]
Ruditapes philippinarum	TiO_2 (21 nm)	1 h	0, 1, 10 mg/L	↓Phagocytosis.	[58]
Perna viridis	TiO_2 (21 nm)	0–9 days	2.5 and 10 mg/L	↑HM; ↓phagocytosis; ↓ROS production; ↓Lysosomal content and THC.	[59]
C. virginica	Ag (26 nm) TiO_2 (70 nm)	2 h	1–400 μg/L	↓ Phagocytosis.	[25]

detected in the present study may be partially attributed to the stressful states of the clams induced by $nTiO_2$ exposure.

To date, the molecular mechanisms manifesting the immunotoxicity of NPs have been well elaborated in mammals [16]. Generally, it has been suggested that NPs will be recognized by the PRRs such as TLRs of the immune-related signaling pathways [50,51]. The activations of PRRs will then trigger downstream immune-related pathways and corresponding effective molecules, which subsequently lead to non-specific and specific immune responses such as oxidative stress, organ lesion, and inflammation [52–54]. However, to date, the molecular mechanism underneath the immunotoxicity of NPs still remains poorly understood in marine invertebrates.

In the present study, the gene expressions of all PRRs investigated (both the toll-like receptors TLR1, TLR2, TLR4, TLR5, TLR6 and the RIG1-like receptor) were significantly depressed after 30 days' exposure to $nTiO_2$. Though some studies demonstrated that acute exposure to NPs can result in increased expressions of Toll-like receptors genes in mammals [51], similar to the results of this study, suppressed expressions of TLRs have been reported in mice exposed to comparable long-term (28 days) NPs exposure [49]. Such reduced gene expression of toll-like receptor isoforms were also reported in the digestive gland of *M. galloprovincialis* after 96 h exposure to the mixture of $nTiO_2$ (100 μg/L) and Cd^{2+} (100 μg/L), though no alteration at mRNA level was detected when individuals were exposed to $nTiO_2$ alone [23]. Since PRRs are one of the most ancient and conserved components of the immune system (e.g. all TLRs have a toll-IL receptor domain in common) [51], the gene expression alterations of PRRs observed suggest that these PRRs, including RIG1, might be the molecules for NPs recognition in marine invertebrates as well. In addition, since each PRR specifically recognizes corresponding Pathogen-Associated Molecular Patterns (PAMPs), the suppressed expressions of PRRs detected indicate a reduced sensitivity of blood clam individuals to pathogen challenges. Furthermore, the expressions of downstream immune-related responding genes were also found to be significantly suppressed in this study, which suggests that $nTiO_2$ exposure renders the blood clams less effective to response to pathogen infections.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Acknowledgments

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