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Regulation of apoptosis-related genes during interactions between oyster hemocytes and the alveolate parasite *Perkinsus marinus*

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ABSTRACT

The alveolate *Perkinsus marinus* is the most devastating parasite of the eastern oyster *Crassostrea virginica*. The parasite is readily phagocytosed by oyster hemocytes, but instead of intracellular killing and digestion, *P. marinus* can survive phagocytosis and divide in host cells. This intracellular parasitism is accompanied by a regulation of host cell apoptosis. This study was designed to gain a better understanding of the molecular mechanisms of apoptosis regulation in oyster hemocytes following exposure to *P. marinus*. Regulation of apoptosis-related genes in *C. virginica*, and apoptosis-regulatory genes in *P. marinus*, were investigated via qPCR to assess the possible pathways involved during these interactions. *In vitro* experiments were also carried out to evaluate the effect of chemical inhibitors of *P. marinus* antioxidant processes on hemocyte apoptosis. Results indicate the involvement of the mitochondrial pathway (Bcl-2, anamorsin) of apoptosis in *C. virginica* exposed to *P. marinus*. In parallel, the antioxidants peroxiredoxin and superoxide dismutase were regulated in *P. marinus* exposed to *C. virginica* hemocytes suggesting that apoptosis regulation in infected oysters may be mediated by anti-oxidative processes. Chemical inhibition of *P. marinus* superoxide dismutase resulted in a marked increase of reactive oxygen species production and apoptosis in infected hemocytes. The implication of oxygen-dependent apoptosis during *P. marinus* infection and disease development in *C. virginica* is discussed.

1. Introduction

Perkinsus marinus is a protozoan parasite of the eastern oyster *Crassostrea virginica* and causes Perkinsiosis, an infection commonly known as Dermo disease. *P. marinus* cells are readily phagocytosed by *C. virginica* hemocytes; however, intracellular lytic processes are impeded and the parasite flourishes inside infected host cells [1,2]. Previous studies reported significantly higher phagocytosis of *P. marinus* by hemocytes from *C. virginica* compared to the less Dermo-susceptible *Crassostrea gigas* [3]. In addition, there is no increase in reactive oxygen species (ROS) production following phagocytosis, and *P. marinus* has been shown to manipulate apoptosis of host hemocytes to favor infection establishment [2,4–6]. Combined with the lack of intracellular lysis of *P. marinus* in hemocytes, the regulation of hemocyte apoptosis appears to be playing a role in the development of the disease.

Recent investigations have suggested that the acquisition of *P. marinus* is mediated, at least in part, by oyster hemocytes that uptake waterborne parasite cells at mucosal surfaces before migrating back to underlying tissues allowing the initiation of the infection following a Trojan horse strategy [7–9]. Therefore, suppressing apoptosis could confer a major advantage for *P. marinus* to be masked within a hemocyte and transported across mucosal epithelia thereby establishing infection. This is not surprising given the number of pathogens that manipulate host cell apoptosis as part of their infectious process. Protozoan parasites such as *Toxoplasma gondii*, *Cryptosporidium parvum*, *Trypanosoma cruzi*, *Leishmania* species, *Theileria* species, and *Plasmodium* species, have been well studied for their ability to regulate host-cell apoptosis [10]. *T. gondii*, the causative agent of toxoplasmosis, is a particularly well studied protozoan that has developed multiple tactics to suppress cell apoptosis. For instance, it has been shown to inhibit apoptosome activity and the release of cytochrome-C, thereby inhibiting caspase-9 activity [10,11]. *T. gondii* has also been observed to upregulate pro-survival B-cell lymphoma 2 (Bcl-2) [12], and stimulate inhibitor of apoptosis proteins (IAPs) [10,13]. In this context, a more detailed understanding of the regulation of the apoptosis pathway by *P. marinus* in oyster hemocytes is vital for characterizing the mechanisms of *P. marinus* pathogenesis.

Both external and internal signals can trigger apoptosis in vertebrates and invertebrates. The extrinsic pathway involves transmembrane receptors-mediated interactions. The intrinsic, also known as the mitochondrial pathway, is driven by intracellular signals [14]. Interestingly, the intrinsic pathway can be activated by ROS disruption of

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the mitochondrial membrane potential leading to the release of cytochrome-C and subsequent apoptosis [15,16]. Both the intrinsic and extrinsic pathways lead to the execution pathway characterized by caspase-induced apoptosis [14]. In addition to the execution pathway, caspase-independent apoptosis occurs through death effector molecules such as apoptosis-inducing factor (AIF), endonuclease G, and granzyme A/B amongst others [14,17].

Compared to model mammalian species [14], there is very limited information on the mechanisms underlying regulation of apoptosis in mollusks in general and during host-parasite interactions (e.g. *C. virginica-P. marinus*) in particular [17]. Previous studies in oysters (*C. gigas* and *C. virginica*) have identified a number of genes involved in apoptosis regulation including caspase-3, AIF, Tumor necrosis factor (TNF) receptor, Bcl-2, c-Jun, IAP, anamorsin and mitogen-activated protein kinase 14 (MAPK14) [17–22]. Similarly, different apoptosis-related genes have also been identified in *P. marinus* including programmed cell death 3981 (PCD 3981), Fas apoptotic inhibitory protein (FAIM), programmed cell death (PCD), apoptosis inducing factor 19150 (AIF 19150), peroxiredoxin (Prx), and superoxide dismutase (SOD) [23]. Interestingly, these authors showed a marked upregulation of anti-apoptotic genes in virulent cultures of *P. marinus* as compared to avirulent cultures and suggested that anti-apoptotic factors may be produced by the parasite to reduce host cell apoptosis and favor infection [23,24].

This study investigated the molecular interactions between *P. marinus* and *C. virginica* hemocytes with the aim of identifying the mechanisms used by the parasite to regulate host cell apoptosis. Experiments were designed to evaluate change in the expression of apoptosis-related genes in *C. virginica* hemocytes and *P. marinus* cells during *in vitro* infection studies. Functional experiments were performed to assess the role of anti-oxidant molecules produced by the parasite in the regulation of host cell apoptosis. Results are discussed with a focus on the possible role of ROS in hemocyte apoptosis and the resulting implications on *P. marinus* infection and disease development in *C. virginica*.

2. Methods

2.1. Oysters

Adult eastern oysters, *C. virginica*, were obtained from Frank M. Flower and Sons (Oyster Bay, NY). Oysters were stripped of debris and fouling organisms and acclimated in aerated UV-filtered seawater (28–30ppt, 23 °C) for 7–10 days prior to the experiment. They were fed daily with a commercial diet (DT's Live Marine Phytoplankton, Sycamore, Illinois, USA).

2.2. Perkinsus marinus

Two 650 ml (for *C. virginica* gene expression experiments) and six 120 ml (for *P. marinus* gene expression experiments) cultures of *P. marinus* (ATCC 50439) were grown in DME/F12-3 media [25]. Cultures were grown in an Ambi-Hi-Lo chamber (Lab-Line Instruments Inc.) at 23 °C. Log phase *P. marinus* cultures (approximately 10 days old) were collected via centrifugation (1200 g, 10 min, 23 °C), washed and resuspended in filtered artificial seawater (FSW, Instant Ocean, 28ppt).

2.3. Hemocyte gene expression

Hemolymph was collected from *C. virginica* adductor muscle via 5 ml syringe into ten 60 ml pools at approximately 2.5×10^6 hemocytes/ml (~20–25 oysters per pool) stored on ice. Hemolymph samples were centrifuged (800 g, 10 min, 4°C), supernatant discarded, and remaining hemocytes were resuspended in 30 ml of chilled 0.22 μm sterile filtered seawater (FSW) at 28 ppt for a final concentration of approximately 5×10^6 hemocytes/ml. The collected *P. marinus* culture was resuspended to a concentration of 1×10^7 cells/ml in FSW one day prior to experiment. Half of the collected culture was incubated at 100 °C for 15 min to yield heat-killed parasite cells. Each hemocytes pool was aliquoted into one of 4 different treatments (3 ml/treatment) that were added with one of the following: 3 ml FSW (negative control), 3 ml *P. marinus* suspension, 3 ml suspension of heat killed *P. marinus*, or 3 ml suspension of 8 μm latex beads (to account for gene expression associated with phagocytosis of particles). A 1:1 hemocyte:*P.marinus* or bead ratio was targeted and each treatment was duplicated to allow for collection at two separate time points except for FSW, which was in triplicate to account for a time 0 collection. Five replicate pools were completed per day over a two-day period. Hemocytes with FSW alone were collected immediately at time 0, at 6 h, and at 24 h. All other treatments were collected at 6 h and 24 h post-exposure. Samples were centrifuged (1200g, 10 min, 4 °C), resuspended and homogenized in Trizol (Molecular Research Center, Inc. Cincinnati, OH) and stored at −80 °C for RNA extraction no greater than 36 h post-collection.

2.4. P. marinus gene expression

P. marinus cultures (6 cultures, 120 ml/culture) collected via centrifugation were washed and resuspended in FSW to a concentration of 1×10^7 cells/ml. Hemolymph was collected from approximately 60 adult oysters for a total of 300 ml of hemolymph (~2.5×10^6 hemocytes/ml). Hemolymph was then divided to 2 aliquots. The first aliquot was centrifuged (800g, 20 min, 4 °C) and resuspended in cold FSW for a final concentration of 1×10^7 hemocytes/ml. The second aliquot (150 ml) of hemolymph was incubated at 70 °C for 15 min to yield heat-killed hemocytes before undergoing centrifugation and resuspension in FSW (1×10^7 hemocytes/ml) as described above. Two ml of *P. marinus* from each culture were incubated with 2 ml of one of the following: FSW (negative control), heat-killed hemocytes, live hemocytes, or 8 μm beads (control for physical contact, also suspended in FSW). FSW treatments were immediately collected after set-up (time 0), at 6 h and at 24 h. The other treatments were collected at 6 h and at 24 h. Samples were centrifuged (1400g, 10 min, 4 °C), resuspended and homogenized in 1 ml Trizol (Molecular Research Center, Inc. Cincinnati, OH) and stored at −80 °C for RNA extraction no greater than 36 h post-collection.

2.5. RNA isolation and cDNA synthesis

Total RNA was extracted following the manufacturer's protocol with the addition of Proteinase K and an added step of ethanol wash with molecular grade glycogen (Thermo Scientific, Wilmington, Delaware, USA) to yield high quality RNA. Samples were analyzed with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) for quality and quantity. cDNA was reverse-transcribed from RNA samples (2.5 μg) utilizing Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV) (Promega, Madison, Wisconsin, USA) following the manufacturer's protocol.

2.6. Real-time PCR of relative expression of apoptosis-related genes

Primers were designed to amplify genes known to be involved in apoptosis regulation in oysters (8 genes: Bcl-2, Caspase-3, AIF, TNF, c-Jun, anamorsin, MAPK14, IAP) and *P. marinus* (6 genes: PCD 3981, Prx, SOD, FAIM, PCD, AIF 19150) based on previous studies and genomic information available at the NCBI database (Table 1) [17–23]. In addition, primers were also designed to amplify oyster NF-κB gene to evaluate whether apoptosis regulation during infection involves synthesis of this transcription factor as a proxy for *de novo* transcription of host genes. Each primer pair was tested for the optimal efficiency and amplification products were confirmed on gel electrophoresis. Preliminary assays were preformed to ensure amplification was specific to the organism of interest since treatment samples contained both *P.*

Table 1
Primers designed for assessing transcript levels of apoptosis-related genes in *C. virginica* and *P. marinus*.

Gene	Forward (5′-3′)	Reverse (3′-5′)
C. virginica		
Bcl-2	TGCTGATTGGTCTCTCCGTG	TTGGGTTTGGAGGGGCATTT
Caspase-3	TCCTGTTTGGATGGGTCAGTT	TCCAGTGCAAAGATGGACCC
Apoptosis inducing factor (AIF)	GCA CAT CCA CCC CAC TTT CT	GAAGCAGTGGAGGGGTCAAG
NF-κB	CATGGGCCTGGGGAGTAATG	CCGTCCACTGTTTTGTGCTG
TNF receptor	CTTGGAAGGCTGGTGTTGGA	ACAAACAGTGGCATCACCGA
c-Jun	TGGCGAAAACAATGGAAGCG	AAGTCAGGAGAAGCCAGCAA
Anamorsin	GAAGGCTGGCATACCCAAGT	ATGAACTGGAGGCAGAAGCC
MAPK14	ACAAACCCTCAGTGACGACC	TGTGTGGCGAGCTAAACCAA
IAP	CATGTTGCCGTGTGGACATC	ATGCGAGCCAAGTACGGACA
β-actin (housekeeping)	TTGGACTTCGAGCAGGAGATGGC	ACATGGCCTCTGGGCACCTGA
P. marinus		
Programmed cell death (3981)	GCTAAGAAGTCCCTTGTCGG	CTTCACTATGCAGCCACCT
Apoptosis-inducing factor 19150, putative	CGGCGCCTATAGGGTTTATC	CCGGGGAATCACTTTCTGTC
Peroxiredoxin	GAGTTCCCGGCGCA TTCA	TTCCCCAAGCTTGCATCAC
Superoxide dismutase (4 forms)	CTTCGGCAGTGGTTGGG	TAGGCATGCTCCCATACATC
Fas apoptotic inhibitory protein	GATGATTGGGCGATGGACTT	ACCCATAGAGGCGGTGAATA
Programmed cell death	GCACTAGAGGCCTACAAACGGA	CGAGGCAAACAATTCTGTCGAGC
Ferredoxin (housekeeping)	AGGCCAGATGGATAAGGGGT	AGTTACTGCGGATGGTCG

marinus and *C. virginica* cDNA. Ferredoxin (for *P. marinus*) [26] and β-actin (for oysters) [27] were confirmed as reliable housekeeping genes during preliminary assays. Quantitative PCR reactions contained 5 ng cDNA template, 100 nM of forward and reverse primers and 5 µl of 2× Brilliant SYBR Green qPCR master mix (Agilent, Santa Clara, California, USA) in 10 µl total volume and were performed on an Eppendorf Mastercycler Realplex (Eppendorf, Hauppauge, New York, USA). The qPCR thermal profile was as follows for *P. marinus* and *C. virginica* samples: 95 °C for 10 min, 40 cycles of amplification with denaturation at 95 °C for 15 s, annealing and extension for 1 min (60 °C for *P. marinus* genes and 56 °C for *C. virginica* genes), and melting curve analysis. The relative expression of genes of interest was calculated based on the comparative CT method ($2^{-\Delta\Delta Ct}$) [28] using ferredoxin and β-actin as a reference gene for *P. marinus* and *C. virginica*, respectively.

2.7. Role of P. marinus superoxide dismutase in apoptosis regulation

Based on gene expression results, a series of experiments was designed to evaluate the role of *P. marinus* superoxide dismutase (SOD) in the regulation of hemocyte apoptosis. A first experiment was performed to evaluate a protocol for the inhibition of SOD production in *P. marinus* using the chemical inhibitor Diethyldithiocarbamate (DDC; Spectrum Chemical, New Brunswick, NJ; catalog number S1270). Five replicate cultures of exponentially-growing *P. marinus* were centrifuged (200g for 10 min at 4 °C), resuspended in FSW and divided into 2 aliquots each. DDC was dissolved in DMSO and added to 1 aliquot of resuspended parasite cells at a 10 mM final concentration (control aliquots added with DMSO). Following incubation (90 min at room temperature), parasite cells were washed by centrifugation and resuspended at 10^6 cell ml^{-1} in FSW. SOD activity in control and DDC-added *P. marinus* cells was then measured using a commercial kit following the manufacturer's protocol (Cayman Chemical, Ann Arbor, MI, catalog number 706002).

The second set of experiments evaluated changes in reactive oxygen species (ROS) production in hemocytes exposed to *P. marinus* cells added or not with DDC. Exponentially-growing *P. marinus* cells were centrifuged (200g for 10 min at 4 °C), resuspended in FSW (10^7 cells ml^{-1}) and stained with the cell tracker 2 µM DDAO (7-Hydroxy-9H-1,3-Dichloro-9,9-Dimethylacridin-2-One; Life Technology, Carlsbad, CA; catalog number 34553). After incubation (5 min in the dark at room temperature), cells were retrieved by centrifugation, resuspended in FSW and divided into 2 aliquots with one aliquot remaining untreated and the other added with DDC as described above. Hemolymph from 12 oysters (1.5 ml/oyster) was individually withdrawn into syringes prefilled with 1.5 ml cold FSW, then stained with 2 µM of the cell tracker carboxyfluorescein diacetate succinimidyl ester (CFSE; Life Technologies Catalog #C34554). Following incubation (5 min in the dark), hemocytes were washed by centrifugation and resuspended in FSW at 10^6 cells ml^{-1}. Hemocytes from each oyster were divided into 3 aliquots and then added with labeled *P. marinus* cells (either pretreated with DDC or not, 5:1 *P. marinus*:hemocyte ratio) or with FSW and incubated in the dark for 6 h before the addition of the ROS substrate dihydrorhodamine 123 (0.5 µg ml^{-1} final concentration). The samples were then incubated for 30 min in the dark before analyzed with a 4-color BD FACScalibur flow cytometer. Data were acquired for a minimum of 10,000 hemocytes. In this experimental setting, the flow cytometer allows the measurement of ROS production (rhodamine 123 fluorescence intensity in the red channel) in uninfected hemocytes (green-fluorescent hemocytes labeled with CFSE) and infected hemocytes (green hemocytes that also display far red fluorescence generated by internalized *P. marinus* -although this assay does not allow discrimination between truly infected hemocytes or hemocytes that have *P. marinus* attached to the cell surface).

The last set of experiments measured changes in the percent of apoptotic hemocytes following exposure to *P. marinus* pretreated or not with DDC. Here, a 4-color experiment following the general protocol described above was performed. Hemocytes were individually collected from a total of 12 oysters, labeled with CFSE, then added with DDAO-labeled *P. marinus* pre-incubated or not with DDC (or added with DMSO for negative controls). Following a 6-h incubation in the dark, hemocyte apoptosis was assessed using Annexin V (PE Annexin V solution; Biolegend, San Diego, CA; catalog number 640908) and 7-amino-actinomycin D (7-AAD solution; Biolegend catalog number 420404) to label apoptotic (orange) and dead (red) cells, respectively. For staining, cells (hemocytes, free *P. marinus*, and hemocytes infected with *P. marinus*) were pelleted by centrifugation (200 g for 10 min at 4 °C) then resuspended in 50 µL FSW and 50 µL Annexin binding buffer provided by the manufacturer. Then, 2.5 µL Annexin-PE and 3 µL 7-AAD (containing 125 and 150 ng, respectively) were added and preparations were incubated for 15 min in the dark, after which 200 µL FSW were added to bring the volume up for the flow cytometer measurements. Data from a minimum of 10,000 hemocytes were acquired to allow the assessment of the proportion of apoptotic and dead cells in infected and uninfected hemocytes.

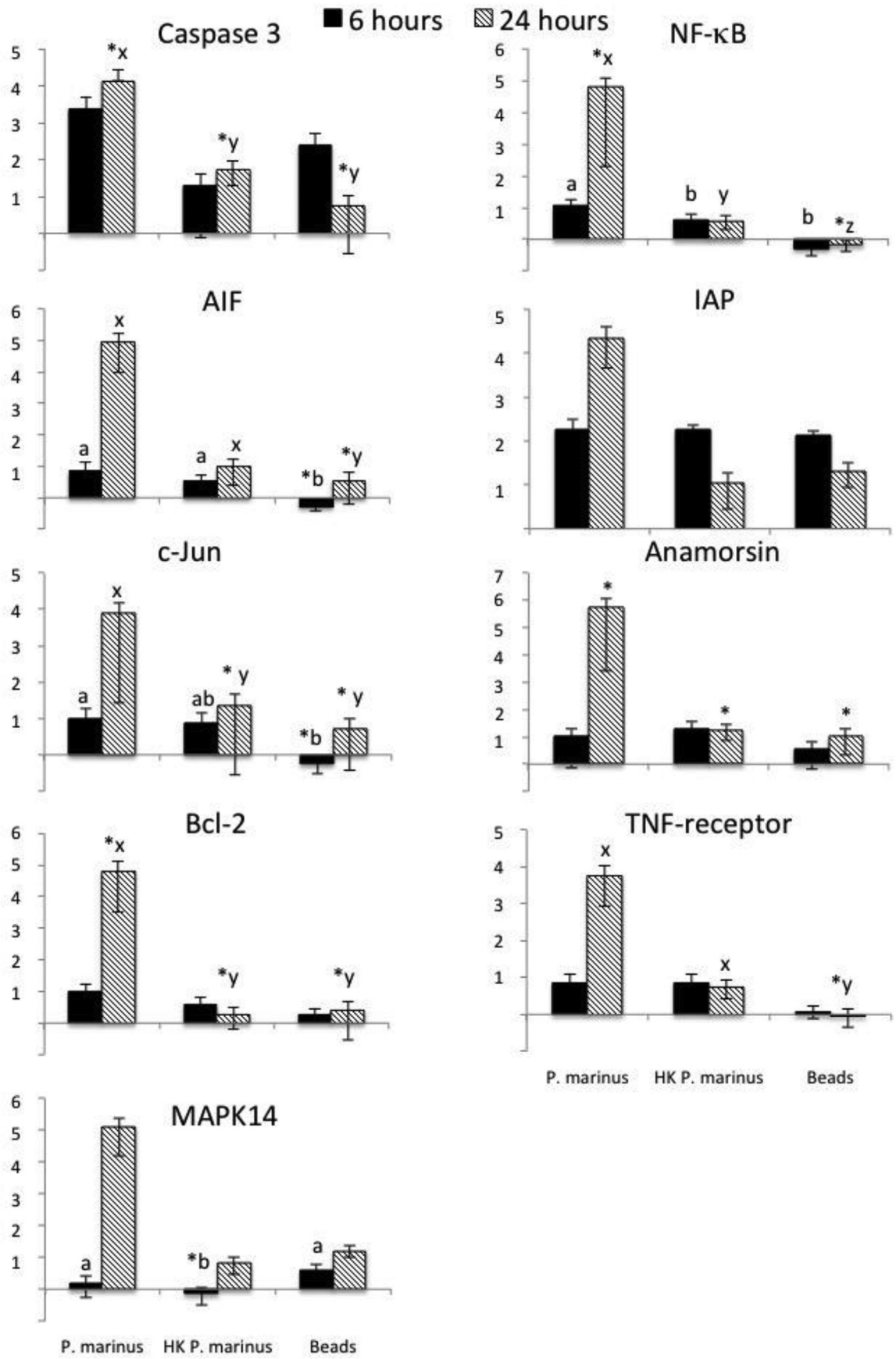

Fig. 1. Fold change (Log10, mean ± SE) of apoptosis-related genes in *C. virginica* hemocytes at 6 and 24 h after exposure to *P. marinus*, heat-killed (HK) *P. marinus*, or beads (mean ± SE). * Denotes significant difference from untreated control hemocytes (represented by the x-axis). Different letters (6 h: a and b; 24 h: x, y and z) indicate significant difference between treatments within each sampling time (SNK post-hoc test, $p < 0.05$, $n = 10$).

2.8. Data analysis

ΔCt data derived from the gene expression experiments and flow cytometry data were submitted to repeated measures analyses of variance followed with Student-Newman-Keuls post-hoc tests as appropriate. Discriminant analysis (DA) and principal components analysis (PCA) were used to evaluate the overall effect of each treatment on the expression of genes associated with apoptosis at both 6 h and 24 h post-exposure. Correlation analysis was conducted on gene expression data. SPSS and SigmaStat were used to perform all statistical analyses, which were considered significant at $p < 0.05$.

3. Results

3.1. Hemocyte gene expression

At 6 h post-exposure, AIF expression was significantly downregulated in hemocytes exposed to beads compared to all other treatments including the control (hemocytes alone, represented by the x-axis in Fig. 1). Similar trends were also detected for c-Jun expression where significant downregulation was measured in hemocytes exposed to beads although difference with those exposed to heat-killed *P. marinus* was not significant. MAPK14 expression was significantly downregulated in hemocytes exposed to heat-killed *P. marinus* compared to all other treatments including the control. Finally, significantly higher expression was measured for NF-κB in hemocytes exposed to live *P. marinus* as compared to hemocytes exposed to heat-killed *P. marinus* or beads.

At 24 h post-exposure, caspase-3 and Bcl-2 expression was significantly upregulated in hemocytes exposed to live *P. marinus* compared to all other treatments, including the control (Fig. 1). In parallel, AIF and TNF-receptor expression was significantly greater in hemocytes exposed to *P. marinus* or heat-killed *P. marinus* compared to hemocytes exposed to beads. C-Jun expression was significantly upregulated in hemocytes exposed to live *P. marinus* compared to hemocytes exposed to heat-killed *P. marinus* or beads. NF-κB expression was significantly different across all treatments with greater expression in hemocytes exposed to live *P. marinus* as compared to all other treatments, followed by hemocytes exposed heat-killed *P. marinus*. Anamorsin expression was significantly upregulated in all treatments compared to the control (Fig. 1).

Discriminant analysis (DA) for apoptosis-related gene expression levels in hemocytes exposed to the various treatments (hemocytes alone, *P. marinus*, heat-killed *P. marinus*, and beads) were distinctly separated (Fig. 2). Interestingly, temporal dynamics in gene expression were obvious from the DA profiles. For instance, hemocytes exposed to live and heat-killed *P. marinus* clustered together along Function 1 (x-axis) but were separated along Function 2, and were well separated from control hemocytes and the bead treatment at 6 h post treatment. At 24 h, hemocytes exposed to heat-killed *P. marinus* shifted away and were visible between controls and hemocytes exposed to beads. It should be noted that hemocytes exposed to live *P. marinus* were diametrically opposed to those exposed to beads at both sampling times, suggesting that changes in gene expression were not a mere response to hemocyte phagocytic activity. Overall, the clustering of the different treatments was strongly significant along function 1 (at 6 h, 76.3% variance explained, Eigenvalue = 6.033, Lambda = 0.04, $P < 0.0001$; at 24 h, 70.7% variance explained, Eigenvalue = 7.549, Wilks Lambda = 0.018, $P < 0.0001$). PCA analysis of apoptosis-related gene expression indicates that at 6 h, MAPK14, Bcl-2 and caspase-3 were each separated away from the other genes (Supplementary Fig. 1). At 24 h, most genes clustered together, while caspase-3 segregated apart, and anamorsin and MAPK14 clustered separately from the other genes. Correlation analysis supported the clustering obtained with the PCA (Supplementary Table S1).

3.2. P. marinus gene expression

At 6 h post-exposure, *P. marinus* exposed to live hemocytes exhibited significant upregulation of Prx and FAIM compared to *P. marinus* exposed to heat-killed hemocytes (Fig. 3). SOD was also significantly upregulated in *P. marinus* exposed to hemocytes compared to *P. marinus* exposed to heat-killed hemocytes or untreated *P. marinus*. Changes in the expression of the other genes were not significant.

At 24 h post-exposure, *P. marinus* exposed to hemocytes continued to exhibit higher expression of Prx compared to *P. marinus* exposed to heat-killed hemocytes. *P. marinus* exposed to beads displayed a significant upregulation of SOD and FAIM as compared to *P. marinus* exposed to heat-killed hemocytes. Significantly higher expression of PCD 3981 and PCD was also measured in *P. marinus* exposed to beads as compared to parasite cells exposed to live or heat-killed hemocytes (Fig. 3).

Discriminant analysis (DA) for gene expression levels in *P. marinus* exposed to the various treatments indicates that *P. marinus* exposed to live hemocytes or beads had the most segregated expression profiles (Fig. 4). At 6 h post-exposure, separation of *P. marinus* exposed to live hemocytes and *P. marinus* exposed to beads was observed along function 1 and function 2, respectively (66% variance explained by function 1, Eigenvalue = 1.1512, Wilks Lamda = 2.19, $P < 0.073$). At 24 h, *P. marinus* exposed to live hemocytes and *P. marinus* exposed to beads were both still separated from the remaining treatments, but *P. marinus* exposed to beads was positioned farther along function 1 (68% variance explained by function 1, Eigenvalue = 2.742, Wilks Lambda = 0.103, $P < 0.002$). Principal components analysis (PCA) of all treatments combined showed a clustering of FAIM, SOD and Prx at 6 h and SOD and PCD3981 at 24 h (Supplementary Fig. 2). Correlation analysis of gene transcript levels supported the trends obtained with the PCA (Supplementary Table S2).

3.3. Role of P. marinus superoxide dismutase in apoptosis regulation

Diethyldithiocarbamate (DDC) caused a significant (68%) reduction in superoxide dismutase (SOD) activity in *P. marinus* (Fig. 5A). Therefore, it was used in downstream experiments to evaluate the role of parasite's SOD in regulating ROS and apoptosis activity in hemocytes. ROS production was not significantly regulated in uninfected hemocytes exposed to either DDC-treated or untreated *P. marinus*, but contrasting trends were noted among infected hemocytes (Fig. 5B). For instance, ROS activity markedly decreased (42%) in infected hemocytes that have internalized untreated *P. marinus* as compared to controls, but was highest in infected hemocytes that have internalized DDC-treated parasite cells (57% increase compared to controls and 172% increase as compared to hemocytes that are infected with untreated *P. marinus*). Strikingly, a similar pattern was noted in the percent of apoptotic cells that displayed a 421% increase among infected hemocytes that have internalized DDC-treated as compared to untreated parasite cells (Fig. 5C).

4. Discussion

This study investigated changes in the expression of apoptosis-related genes in both the host and the parasite during interactions between *C. virginica* hemocytes and *P. marinus in vitro*. The effects of *P. marinus* on hemocyte apoptosis in the intrinsic (Bcl-2, IAP, anamorsin), extrinsic (TNF-receptor, c-Jun, MAPK14), and caspase-independent (AIF) pathways were examined to gain a better understanding of the mechanisms used by the parasite to regulate host apoptosis. Apoptosis of hemocytes exposed to *P. marinus* has been previously shown to be dynamic with a rapid increase (within 45 min and lasting for a few hours) after exposure followed by a suppression of apoptosis at longer incubation periods [5]. For this reason, our study focused on the evaluation of gene regulation at 2 key sampling times: 6 and 24 h. The

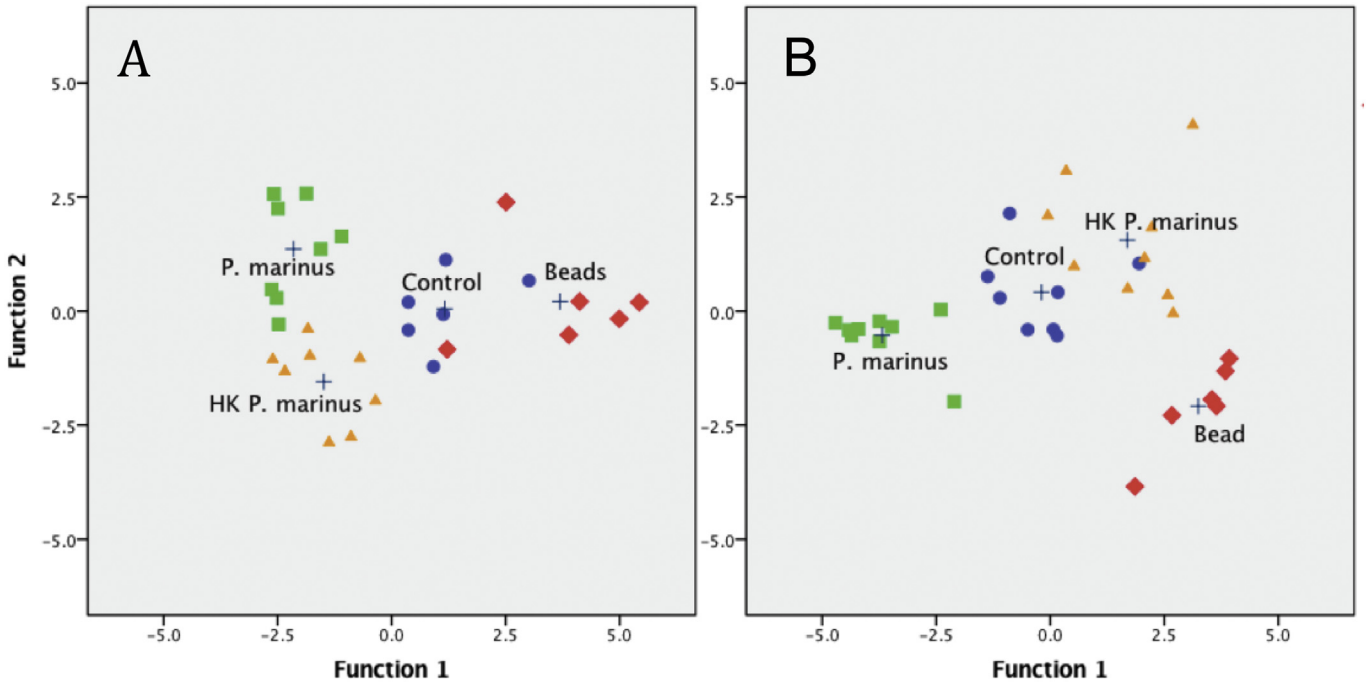

Fig. 2. Discriminant analysis of apoptosis-related gene expression in hemocytes at 6 (A) and 24 (B) hours post-exposure. Different treatment groups are indicated by different symbols and the position of the group centroids are indicated by the cross symbols.

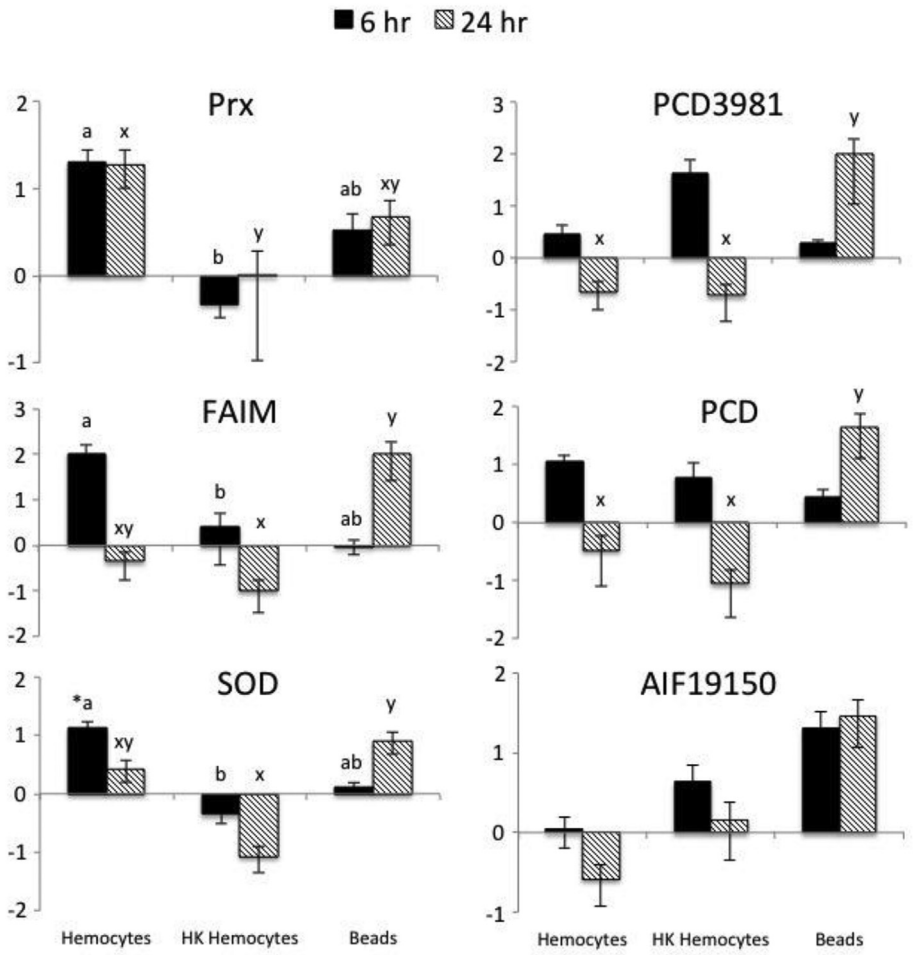

Fig. 3. Fold change (Log10, mean ± SE) of apoptosis-related genes in *P. marinus* at 6 and 24 h after exposure to *C. virginica* hemocytes, heat-killed hemocytes, or beads. * Denotes significant difference from untreated control *P. marinus* (represented by the x-axis). Different letters (6 h: a and b; 24 h: x and y) indicate significant difference between treatments within each sampling time (SNK post-hoc test, $p < 0.05$, $n = 6$).

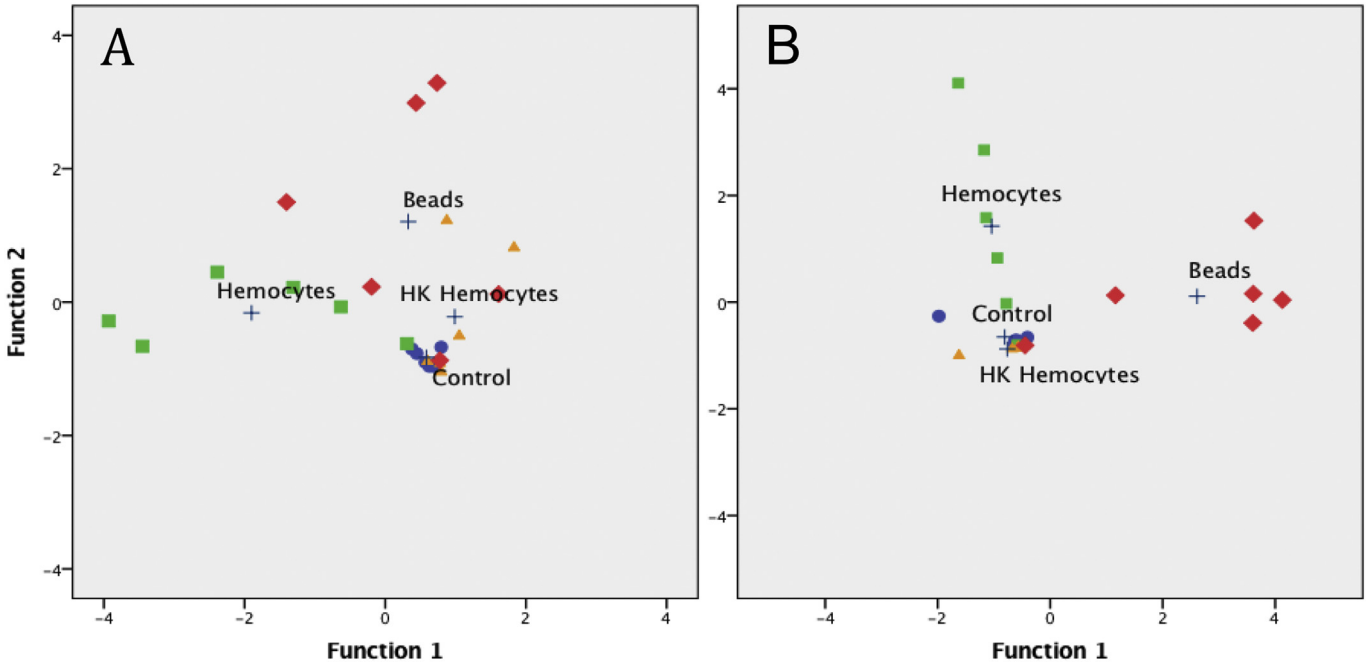

Fig. 4. Discriminant analysis of apoptosis-related gene expression in *P. marinus* at 6 (A) and 24 (B) hours post-exposure. Different treatment groups are indicated by different symbols and the positions of the group centroids are presented by the cross symbols.

expression of individual genes and integrative discriminant analyses indicated marked regulation of apoptosis-related genes in hemocytes exposed to *P. marinus* supporting previous findings of apoptosis modification by *P. marinus*. In parallel, we followed the expression of apoptosis-related genes in *P. marinus* and showed marked regulation of antioxidant-related gene expression. Regulation of apoptosis-related genes in both the host and parasite showed temporal dynamics with overall anti-apoptotic expression patterns at 6 h followed by pro-apoptotic expression patterns at 24 h and expression patterns of continued ROS suppression throughout.

Bcl-2 expression was significantly greater in hemocytes exposed to live *P. marinus* compared to heat-killed *P. marinus* and beads indicating regulation of the intrinsic apoptosis pathway. The delayed upregulation of Bcl-2 expression at 24 h suggests that feedback from infective *P. marinus* cells may be needed to induce upregulation of Bcl-2. Anti-apoptotic members of the Bcl-2 protein family such as Bcl-2, work antagonistically against their pro-apoptotic counterparts thereby suppressing mitochondrial apoptotic events driven by cytochrome *c* release [29–32]. Modification of Bcl-2 has been observed in *Toxoplasma gondii*, a protozoan parasite capable of inhibiting apoptosis, by inducing greater Bcl-2 production in human macrophage cells [12]. Release of cytochrome *c* is suggested to be predominately mediated by ROS activity [16,33] and Bcl-2 has also been found to assist in sequestration in the nucleus of glutathione, an antioxidant factor linked to reduction of apoptosis [34,35]. Therefore, the upregulation of Bcl-2 at 24 h may also contribute to the reduction of hydrogen peroxide potentially produced in exposed hemocytes. Anamorsin, an anti-apoptotic molecule that in mammals was suggested to regulate Bcl-2 activity [20,36,37], was significantly upregulated in all treatments compared to the control with the greatest expression in hemocytes exposed to *P. marinus* at 24 h. Interestingly, anamorsin has also been described to bind iron-sulfur clusters [38]. If this function is conserved in invertebrates, upregulation of anamorsin could also lead to iron sequestration in hemocytes. Iron has been described to affect pathogen virulence and propagation [39]. In *P. marinus*, iron chelators have been shown to decrease proliferation in cell cultures indicating iron is essential to *P. marinus* growth [40]. Iron is also needed to generate ROS in the fenton reaction and therefore, reduction in the available free iron could also affect ROS production by hemocytes [41]. Therefore, the temporal upregulation of both anamorsin and Bcl-2 expression may be a response to ROS production.

Expression patterns of c-Jun, MAPK14, and TNF-receptor suggest the extrinsic pathway may be regulated both by heat-killed *P. marinus* and beads. Hemocytes exposed to beads and hemocytes exposed to heat-killed *P. marinus* resulted in c-Jun downregulation compared to the control or hemocytes exposed to live *P. marinus* at 24 h suggesting some apoptosis-related genes may be triggered by both heat-killed *P. marinus* and beads. TNF-receptor was significantly downregulated in hemocytes exposed to beads compared to hemocytes exposed to live or heat-killed *P. marinus* at 24 h. TNF binding to TNF receptor-1 is able to cause downstream activation of mitogen-activated kinase proteins (MAPKs) and the NF-κB pathway that are involved in the regulation of apoptosis [42]. MAPK14 was significantly downregulated in hemocytes exposed to heat-killed *P. marinus* compared to the control and hemocytes exposed to *P. marinus* or beads at 6 h suggesting that contact with *P. marinus* membranes may contribute to apoptosis gene regulation during the early infection process. Overall, gene expression patterns suggest that many of the apoptosis-related genes are differentially regulated by the various treatments; however, there may be some overlap in regulation in the extrinsic pathway.

Both the extrinsic and intrinsic apoptosis pathways can converge on the death protease caspase-3 leading to cell apoptosis [10,43]. The results from this study indicate there was significant upregulation of caspase-3 in hemocytes exposed to live *P. marinus* versus the control and hemocytes exposed to heat-killed *P. marinus* or beads 24 h post exposure. The upregulation of caspase-3 may be due to the progression of the infection since apoptosis levels have been previously observed to vary over time throughout the infection [5]. AIF, a caspase-independent apoptotic factor, induces apoptosis via translocation from the mitochondria to the nucleus [44]. AIF was significantly downregulated in hemocytes exposed to beads compared to the control and hemocytes exposed to live or heat-killed *P. marinus* at both time points indicating different apoptosis pathways may be regulated by abiotic versus biotic material.

NF-κB expression was significantly upregulated both at 6 and 24 h post-exposure in hemocytes exposed to live *P. marinus* compared to

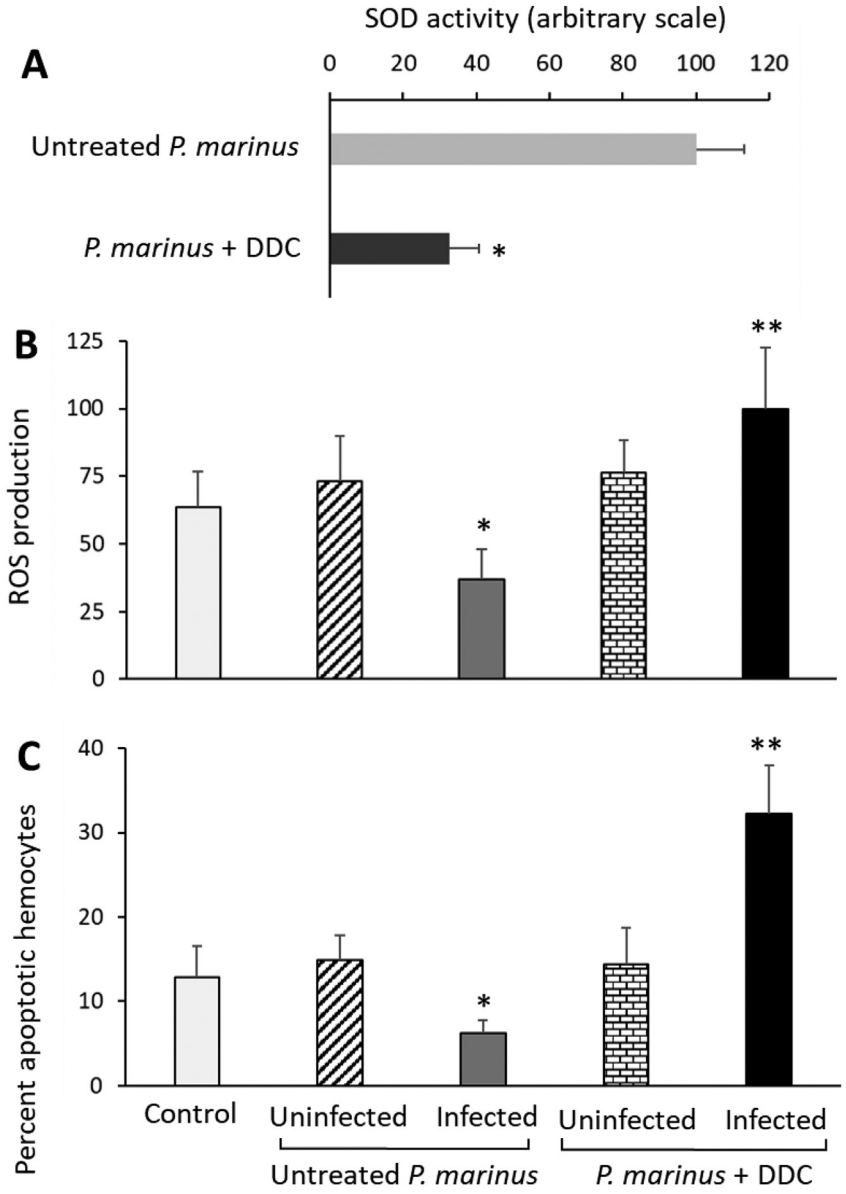

Fig. 5. Role of *P. marinus* SOD in apoptosis regulation. A: Effect of diethyldithiocarbamate (DDC) on SOD activity in *P. marinus*. B and C: regulation of reactive oxygen species production (B, arbitrary scale) and apoptosis (C) in uninfected and infected (having internalized *P. marinus*) hemocytes added with *P. marinus* pre-treated or not with DDC.

hemocytes exposed to heat-killed *P. marinus* or beads suggesting that *de novo* transcription is induced by *P. marinus* throughout the infection process. NF-κB was also significantly correlated to a number of apoptosis-related genes including caspase-3, AIF, TNF-receptor and c-Jun 6 h post-exposure and Bcl-2, caspase-3, AIF, TNF-receptor, c-Jun, anamorsin, MAPK14, and IAP 24 h post-exposure. This is not surprising given NF-κB can regulate a number of processes including the transcription of cytokines, adhesion molecules, and cell survival amongst others [45]. NF-κB has been shown to be regulated to manipulate host apoptosis in other host-protozoan parasite relationships [10,13,46,47].

Hemocyte gene expression results indicate that different apoptosis pathways may be regulated by the various treatments and while *P. marinus* membranes are able to induce a response in some apoptosis-related genes, additional regulation of apoptosis-related genes requires exposure to live *P. marinus* cells. Discriminant analysis also shows hemocytes exposed to heat-killed *P. marinus* shift from clustering closer to hemocytes exposed to *P. marinus* at 6 h to clustering closer to hemocytes exposed to beads at 24 h suggesting that the effects of *P. marinus* membrane may be transient. These results are consistent with previous observations by Hughes et al. [5] that *P. marinus* membranes are able to elicit an initial increase in apoptosis in hemocytes but at a lower level than live *P. marinus* cells. Phagocytosis-induced apoptosis has been observed in both vertebrate and invertebrate systems [48–50] and can be mediated by ROS [51,52]. Considering phagocytosis of different materials are suggested to occur through different pathways and at different rates [50,53], some of the difference in regulation of apoptosis related-genes may be both a reflection of the different pathways triggered as well as the temporal difference in phagocytosis and subsequent apoptosis processes.

To understand the molecular crosstalk between *C. virginica* hemocytes and *P. marinus* cells, gene expression experiments also investigated the regulation of apoptosis-related genes in *P. marinus* after exposure to *C. virginica* hemocytes. Discriminant analysis showed separation of *P. marinus* exposed to live hemocytes and *P. marinus* exposed to beads from *P. marinus* alone suggesting hemocytes and beads are able to elicit a response, but in opposing directions of apoptosis-related genes in *P. marinus*. Consistent with the general lack of regulation of individual genes, *P. marinus* exposed to heat-killed hemocytes

clustered closely to untreated *P. marinus* implicating parasite contact with membranes or remnant cell content leachates of heat-killed hemocytes alone may not be enough to trigger apoptosis-related gene regulation in *P. marinus*.

P. marinus exposed to live hemocytes exhibited no change in pro-apoptotic genes AIF 19150, PCD, or PCD 3981; however, anti-apoptotic FAIM, and antioxidants SOD and Prx, were upregulated in the parasite suggesting an overall suppression of apoptosis at 6 h post-exposure. These results are consistent with our (Fig. 5) and previous findings of *P. marinus* apoptosis suppression in hemocytes [5,6]. Expression profiles show that FAIM, SOD and Prx are actively transcribed by the parasite in response to live hemocytes. The temporal scale suggests modification of FAIM, SOD and Prx occurs during initial interactions with hemocytes with continued Prx expression sustained. Inhibition of ROS from the oxidative burst response in *C. virginica* hemocytes infected by *P. marinus* has been previously reported and suggests that *P. marinus* may be scavenging and/or suppressing the production of ROS [2,54,55]. Our results support this hypothesis and show that superoxide dismutases produced by the parasite contribute to the inhibition of ROS in infected hemocytes (Fig. 5). SOD reduces ROS by dismutation of superoxide (O_2^-) to O_2 [56], while Prx is an antioxidant enzyme that can reduce hydroperoxides [57]. Since *P. marinus* has been observed to be resistant to superoxide and hydroperoxide [2,54,55], upregulation of the antioxidants SOD and Prx may reflect the activation of anti-ROS processes needed to help survivorship of the parasite in infected oyster cells. Correlation of Prx and SOD is not surprising given that both are involved in ROS scavenging activity. In addition to intracellular degradation, ROS are suggested to play a role in apoptosis [15,16,51]. Similar to the mammalian system, ROS-mediated apoptosis has been proposed in invertebrates, such as in the case of the snail *Lymnaea stagnalis* [58]. Interestingly, the inhibition of *P. marinus* SOD with DDC was associated with a restoration of apoptotic activity in hemocytes that contain internalized parasites supporting the involvement of ROS-mediated apoptosis in hemocytes. Altogether, these findings suggest that the upregulation of SOD and Prx in *P. marinus*, in conjunction with the upregulation of Bcl-2 and anamorsin in *C. virginica* hemocytes, may be a strategy employed by *P. marinus* to reduce ROS and subsequently delay apoptosis in the intrinsic pathway. The decrease in SOD expression at 24 h corresponds to upregulation of caspase-3 indicating superoxides may contribute to increased apoptosis, while continued Prx upregulation at 24 h may assist in maintaining reduced hydroperoxides therefore limiting oxidative degradation of *P. marinus*.

5. Conclusions

The overall apoptosis-related gene expression patterns in both *C. virginica* hemocytes and *P. marinus* suggest a shift from anti-apoptosis at 6 h to pro-apoptosis at 24 h. While interpreting gene expression results without further functional characterization of the biological roles of targeted genes is speculative, these findings likely reflect the time scale of apoptosis suppression and subsequent reversal in hemocytes exposed to *P. marinus* as previously suggested by Hughes et al. [5]. In addition, expression of anti-oxidant genes associated with scavenging both superoxides and hydroperoxides were upregulated at 6 h while only those associated with reducing hydroperoxides remained upregulated at 24 h, which may indicate hemocytes undergoing a physiological change. In humans, *Cryptosporidium parvum* causes activation of NF-κB and inhibition of apoptosis such that normal apoptosis response is delayed to afford more time for *C. parvum* to replicate within the infected cell prior to increasing apoptosis in its host cell to exit [10,46,47]. The temporal scale of anti-apoptotic gene expression in *P. marinus* and *C. virginica* hemocytes suggests *P. marinus* may employ a similar strategy to induce a temporary suppression of apoptosis to aid infection establishment and spread.

It should be noted that results of our functional assays (flow cytometry data), in conjunction with gene expression profiles, support previous observations that *P. marinus* is able to suppress ROS production in *C. virginica* hemocytes [2,54] and is resistant to superoxide and hydrogen peroxide [2,54,55]. Antioxidant activity by pathogens as a means of survival in their hosts has been reported in a number of protozoan parasites [59]. ROS, which are normally produced by host cells during phagocytosis of pathogens, are also suggested to play a role in apoptosis [51,52] by disrupting the mitochondrial membrane potential and causing the release of cytochrome *c* [15,16]. Although few studies have investigated the involvement of ROS in molluscan apoptosis, apoptosis in *C. gigas* hemocytes infected with *Planococcus citreus* was suppressed after treatment with antioxidants such as catalase [51]. This study supports the idea that ROS are involved in oyster hemocyte apoptosis. The regulation of anamorsin and Bcl-2 from the mitochondrial apoptosis pathway in hemocytes combined with the antioxidant activity of SOD and Prx in *P. marinus* suggest an overall decreased in ROS levels in hemocytes, which was confirmed in the flow cytometry experiment. Upregulation of caspase-3 at 24 h corresponds to lack of upregulation of SOD. One possibility is that lack of SOD upregulation at 24 h leads to accumulation of superoxide leading to increased chance of apoptosis, while Prx continues to decrease hydrogen peroxides to reduce oxidative degradation. Taken together, these results suggest that *P. marinus* uses its antioxidant arsenal (particularly SOD) to modulate host ROS production and, by extension, apoptosis of infected cells.

Recent studies suggest uptake of *P. marinus* by hemocytes associated with the mucosal surfaces of pallial organs (e.g. mantle) and subsequent migration of infected hemocytes through epithelial barriers may be a strategy employed by *P. marinus* to gain entry into its host [7] and [8,9]. Therefore, suppression of apoptosis in *C. virginica* hemocytes by *P. marinus* could confer a major advantage to *P. marinus* masked within a hemocyte by allowing the parasite enough time to be transported across mucosal epithelia thereby establishing infection. Given the major role of hemocytes in the acquisition and development of *P. marinus* in *C. virginica*, further studies are warranted to document the molecular crosstalk between *P. marinus* and *C. virginica* with a focus on the fine mechanisms of apoptosis regulation by ROS through the mitochondrial apoptosis pathway.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fsi.2018.09.006.

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