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RESEARCH ARTICLE

Paeoniflorin increases the survival of *Pseudomonas aeruginosa* infected *Caenorhabditis elegans* at the immunosuppression stage by activating PMK-1, BAR-1, and EGL-1 signals

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Abstract

Paeoniflorin is the major active compound of total glycoside of paeony in *Paeonia lactiflora* Pall. Although several aspects of beneficial effects of paeoniflorin have been described, whether the paeoniflorin treatment is helpful for inhibiting the pathogen infection-induced immunosuppression remains largely unclear. Using the immunosuppression model in *Caenorhabditis elegans* induced by *Pseudomonas aeruginosa* infection, we here examined the beneficial effect of paeoniflorin treatment against the immunosuppression induced by bacterial pathogen infection. In this immunosuppression model, we observed that the survival rate of *P. aeruginosa* infected nematodes at the immunosuppression stage could be significantly increased by 25–100 mg/L paeoniflorin treatment. *P. aeruginosa* accumulation in intestinal lumen of nematodes at the immunosuppression stage was reduced by paeoniflorin treatment. Paeoniflorin could activate the expressions of antimicrobial genes (*lys-1* and *lys-8*) in nematodes at the immunosuppression stage. Moreover, at the immunosuppression stage, paeoniflorin treatment increased the expressions of *bar-1*, *pmk-1*, and *egl-1* required for the control of innate immunity against bacterial infection. Meanwhile, RNAi of *bar-1*, *pmk-1*, and *egl-1* inhibited the beneficial effect of paeoniflorin treatment in increasing the survival, reducing the *P. aeruginosa* accumulation in intestinal lumen, and activating the expressions of antimicrobial genes (*lys-1* and *lys-8*) in nematodes at the immunosuppression stage. Therefore, paeoniflorin treatment could effectively inhibit the immunosuppression induced by bacterial pathogen infection in the hosts.

Keywords Immunosuppression · Bacterial infection · Paeoniflorin · *C. elegans*

Introduction

The immunosuppression is associated with occurrence and treatment of many diseases (Dickler and Albright 1994; Córneo et al. 2021). For example, the immunosuppression is usually induced by infectious diseases (McGrath et al. 2020; Córneo et al. 2021). Among those diseases, the sepsis is caused by a severe life-threatening pathogen infection, and considered as a race to the death between host immune system and pathogens at the immunosuppression stage (Hotchkiss et al. 2013; Bouras et al. 2018). In the clinical, some drugs have been shown to have the immunosuppressive activity (Efferth and Oesch 2021). Besides synthetic compounds, identification of natural compounds having the immunosuppressive activity is also important for the clinical treatment of immunosuppression associated diseases (Coutinho and Chapman 2011; Syafni et al. 2021).

Increasing evidence has proven that the nematode *Caenorhabditis elegans* is a powerful animal model to determine the host-pathogen interactions (Mylonakis et al. 2003; Martineau et al. 2021), because *C. elegans* will meet both bacterial and fungal microbes in the natural habitat (Kim and Ausubel 2005; Kim and Ewbank 2018). *C. elegans* is highly sensitive to various environmental exposures, including the pathogen infection (Tan et al. 1999; Hua et al. 2023e; Shao et al. 2023; Wang et al. 2023c). Infection with bacterial pathogens (such as *Pseudomonas aeruginosa*) or fungal pathogens (such as *Candida albicans*) can cause some toxic effects on nematodes, such as the enhancement in lifespan reduction and the accumulation of pathogens within intestinal lumen (Tan et al. 1999; Irazoqui et al. 2010; Sun et al.

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2016). In order to reproduce and survive long enough, innate immunity will be activated within the intestine of nematodes by secreting antimicrobial proteins to detect and to kill the pathogens (Aballay and Ausubel 2002; Mallo et al. 2002; Alper et al. 2007; Taffoni and Pujol 2015). This innate immunity is under the control of some important signaling pathways, including p38 MAPK, insulin, TGF-β, and Wnt signaling pathways (Kurz and Tan 2004; Evans et al. 2008; Irazoqui et al. 2008; Elliott et al. 2011; Yu et al. 2018). *C. elegans* is helpful for the pharmacological identification of compounds against the toxicity of bacterial or fungal pathogen infection (Kirienko et al. 2016; Kim et al. 2018). Considering the cost-effective option, *C. elegans* has been shown the great potential of large-scale pharmacological screening (Kwok et al. 2006; Lehner et al. 2006).

Paeoniflorin is one active nitrogen glycoside compound in Paeoniae Radix, the roots of *Paeonia lactiflora* Pall. Paeoniflorin has been shown to have some beneficial effects, such as neuroprotection and anti-oxidation (Zhao et al. 2013; Zhang and Wei 2020; Zhou et al. 2020; Tang et al. 2021). Besides these, paeoniflorin treatment also has the function of anti-inflammation by lowering the immune response (Chen et al. 2016; Ji et al. 2018). The aim of this study was to further determine the possible effect of paeoniflorin treatment in inhibiting the immunosuppression induced by bacterial pathogen infection and the underlying mechanism in *C. elegans*. Infection with *P. aeruginosa*, a Gram-negative opportunistic bacterial pathogen, is associated with the occurrence of sepsis (Cheluvappa et al. 2009). Thus, *P. aeruginosa* was selected as the bacterial pathogen. Our results demonstrated the beneficial effect of paeoniflorin treatment in inhibiting the bacterial pathogen infection-induced immunosuppression and the important role of PMK-1, BAR-1, and EGL-1 signals in forming this beneficial effect in nematodes.

Materials and methods

C. elegans maintenance

The used *C. elegans* was wild-type N2. Nematodes were normally cultured on nematode growth medium (NGM) petri dish (Brenner 1974). On NGM plates, *Escherichia coli* OP50 is seeded as the food source for nematodes. The synchronized young adults were prepared as described (Xu et al. 2022a). After the larval development, the young adult means the developmental stage at which usually no eggs are formed in the body of nematodes. The adult nematodes were lysed with bleaching solution (2% HOCl, 0.45 M NaOH) (He et al. 2023), and then centrifugated at 3000 rpm for 3 min to collect the released eggs. The eggs were transferred onto new NGM plates fed with OP50 to allow them develop into the young adults.

Bacterial infection

PA14 is a common laboratory reference strain for *P. aeruginosa* (Grace et al. 2022). PA14::GFP is a green fluorescent protein tagged strain of *P. aeruginosa*. *P. aeruginosa* PA14 and PA14::GFP were cultured in Luria broth, and seeded on modified NGM killing plate containing 0.35% peptone. To prepare the killing plates, the full lawn PA14 or PA14::GFP was fed on NGM plates. Before the infection, full lawn PA14 or PA14::GFP killing plates were incubated for 24 h at 37 °C and further for 24 h at 25 °C. To identify the immunosuppression stage, young adults were transferred onto the killing plates to perform the *P. aeruginosa* infection at 25 °C for different times (from adult day-1 to adult day-7) (Zhi et al. 2017a). Fifty animals were added to each killing plate.

Post-treatment with paeoniflorin

The paeoniflorin (purity, ≥ 98%) was purchased from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Based on the identified immunosuppression stage, the nematodes were treated with paeoniflorin after the PA14 infection for 5 days. Paeoniflorin treatment concentrations were 25, 50, and 100 mg/L, which were selected as described previously (Hua et al. 2023a).

Endpoints

To determine the *C. elegans* lifespan, the nematodess were scored every day (Tang et al. 2023). The nematodes were counted as dead if no response was detected after prodding using platinum wire. Fifty nematodes were tested for each group, and three replicates for lifespan assay were performed.

Locomotion behavior reflects the functional alteration in motor neurons of nematodes (Hua et al. 2023b, d). Locomotion behavior was assessed by endpoints of body bend and head thrash (Xu et al. 2022b). After the infection and the pharmacological treatment, the nematodes were washed with M9 buffer for three times. After the further recovery on NGM plate without OP50 feeding for 1 min, the locomotion behavior was assessed. The frequencies of body bend and head thrash were counted as the changes of direction for bending at the mid-body and the posterior bulb (y-axis), respectively, if the direction of swimming for nematodes was considered as the x-axis (Liu et al. 2022). Fifty nematodes were tested for each treatment, and three replicates for head thrash or body bend assay were performed.

Analysis of colony-forming unit (CFU)

The CFU of PA14 in the intestinal lumen was analyzed as described (Zhi et al. 2017b). After PA14 infection and

paeoniflorin treatment, the nematodes were first treated with 25 mM levamisole to block pharyngeal pumping. After that, the nematodes were transferred onto the NGM plate containing 1 mg/mL gentamicin and 1 mg/mL ampicillin to treat for 30 min in order to eliminate the PA14 on the surface of body. Fifty nematodes for each group were lysed using motorized pestle and transferred on Luria-Bertani (LB) plate containing 100 μg/mL rifampicin. After incubation overnight at 37 °C, the PA14 colony number was counted. Five replicates for CFU assay were performed.

To analyze the PA14 accumulation in intestinal lumen of nematodes, we also evaluated the PA14::GFP accumulation, which was reflected by relative fluorescence intensity of PA14::GFP in intestinal lumen after normalization to the intestinal autofluorescence. Fifty animals were analyzed for each treatment. Three replicates for assay of PA14::GFP accumulation were performed.

Analysis of transcriptional expression

Total RNAs of *C. elegans* after PA14 infection and paeoniflorin treatment were extracted using RNeasy Mini Kit (Qiagen). The quality of prepared RNAs was assessed by the ratio of OD260/280 in the Nanodrop One. The cDNAs were synthesized using M-MuLV reverse transcriptase. The quantitative real-time polymerase chain reaction (qRT-PCR) was carried out in an ABI 7500 real-time PCR system using SYBR Green master mix. Comparative cycle threshold method was used for analyzing the transcriptional alterations of examined genes. Transcriptional expression of examined genes was normalized to the expression of internal reference gene of *tba-1* encoding a tubulin protein (Zhao et al. 2022). Three replicates for analysis of transcriptional expression were performed. Designed primers are shown in Table S1.

RNA interference (RNAi)

To inhibit expression of certain gene, double-stranded RNAs (dsRNA) of target genes were cloned into plasmid L4440 (empty vector) after double enzyme digestion, and then the recombinant plasmid was transferred into *E. coli* HT115. HT115 is a *E. coli* strain commonly used to induce the RNAi response in nematodes (Wang et al. 2023b). The transferred HT115 was screened in Luria-Bertani (LB) agar with ampicillin and tetracycline. The HT115 containing dsRNA was amplified overnight and incubated with 0.4 mM IPTG for 4 h. RNAi was carried out by feeding nematodes with HT115 expressing certain gene as described (Zhao et al. 2023). After bacterial infection, the RNAi experiments were carried out. Meanwhile, paeoniflorin posttreatment was performed on the RNAi plates. Feeding with HT115 expressing L4440 was employed as the control (Hua et al. 2023c). RNAi efficiency of the examined genes was shown in Fig. S1.

Data analysis

Data are presented as means ± standard derivation (SD). SPSS 12.0 software (IBM, USA) was used for statistical analysis. Differences between different groups were analyzed by analysis of variance (ANOVA). A probability level of 0.01 was considered statistically significant.

Results

Toxic effects of *P. aeruginosa* infection on lifespan and locomotion

In nematodes, after PA14 infection, the survival curve decreased sharply from the day-1 to the day-7 (Fig. S2A). All the examined nematodes died at the day-7 after PA14 infection (Fig. S2A).

Two endpoints, head thrash and body bend, were used to reflect the locomotory ability. After the PA14 infection, both head thrash frequency and body bend frequency were decreased from the day-1 to the day-6 (Fig. S2B). The decrease in locomotion behavior was time dependent in PA14 infected nematodes (Fig. S2B).

P. aeruginosa accumulation in intestinal lumen after the infection

Both PA14::GFP and PA14 were used to evaluate the *P. aeruginosa* accumulation in intestinal lumen of nematodes. Using the strain of PA14::GFP, we observed the pronounced increase in fluorescence intensity of PA14::GFP in intestinal lumen after the infection (Fig. S3A). Meanwhile, using the strain of PA14, we also detected the significant increase in CFU of PA14 after the infection (Fig. S3B). In *P. aeruginosa* infected nematodes, both the increase in fluorescence intensity of PA14::GFP and the increase in CFU of PA14 were time dependent from the day-1 to the day-6 after the infection (Fig. S3A and S3B).

Antimicrobial gene expression in *P. aeruginosa* infected nematodes

The innate immune response to bacterial infection can be reflected by the expressions of antimicrobial genes in *C. elegans* (Couillault et al. 2004; Alegado and Tan 2008). The *lys-1* and *lys-8* were used as antimicrobial genes in response to *P. aeruginosa* infection as described previously (Zhang et al. 2022). After the PA14 infection, from the day-1 to the day-2, expressions of both *lys-1* and *lys-8* were significantly

increased compared with those at the day-0 (Fig. S4). From the day-3 to the day-6, expressions of both *lys-1* and *lys-8* were significantly decreased compared with those at the day-2 after the PA14 infection (Fig. S4). Nevertheless, the expressions of *lys-1* and *lys-8* at the day 3 and the day-4 were still higher than those at the day-0 after the PA14 infection (Fig. S4). Different from this, the expressions of *lys-1* and *lys-8* at the day-5 and the day-6 were lower than those at the day-0 after the PA14 infection (Fig. S4).

Paeoniflorin treatment increased survival rate and locomotion behavior of nematodes at the immunosuppression stage after *P. aeruginosa* infection

We next selected the day-5 as the immunosuppression stage to further investigate the possible beneficial effect of paeoniflorin treatment on nematodes at the immunosuppression stage after PA14 infection. After treatment for 1 day, 25–100 mg/L paeoniflorin all could significantly increase both survival rate and locomotion behavior compared with those in PA14 infected nematodes (Fig. 1A and B). The increase in both survival rate and locomotion behavior by paeoniflorin treatment was concentration dependent in PA14 infected nematodes (Fig. 1A and B). Compared with the no survival of PA14 infected nematodes, we observed both the alive nematodes and the locomotory ability after 25–100 mg/L paeoniflorin treatment for 2 days (Fig. 1A and B). In PA14 infected nematodes, we could further observe the alive nematodes and the locomotory ability after 100 mg/L paeoniflorin treatment for 3 days or 4 days (Fig. 1A and B).

Paeoniflorin treatment inhibited *P. aeruginosa* accumulation in intestinal lumen of nematodes at the immunosuppression stage

To determine the underlying mechanisms for the role of paeoniflorin treatment against toxic effects of *P. aeruginosa* infection on nematodes at the immunosuppression

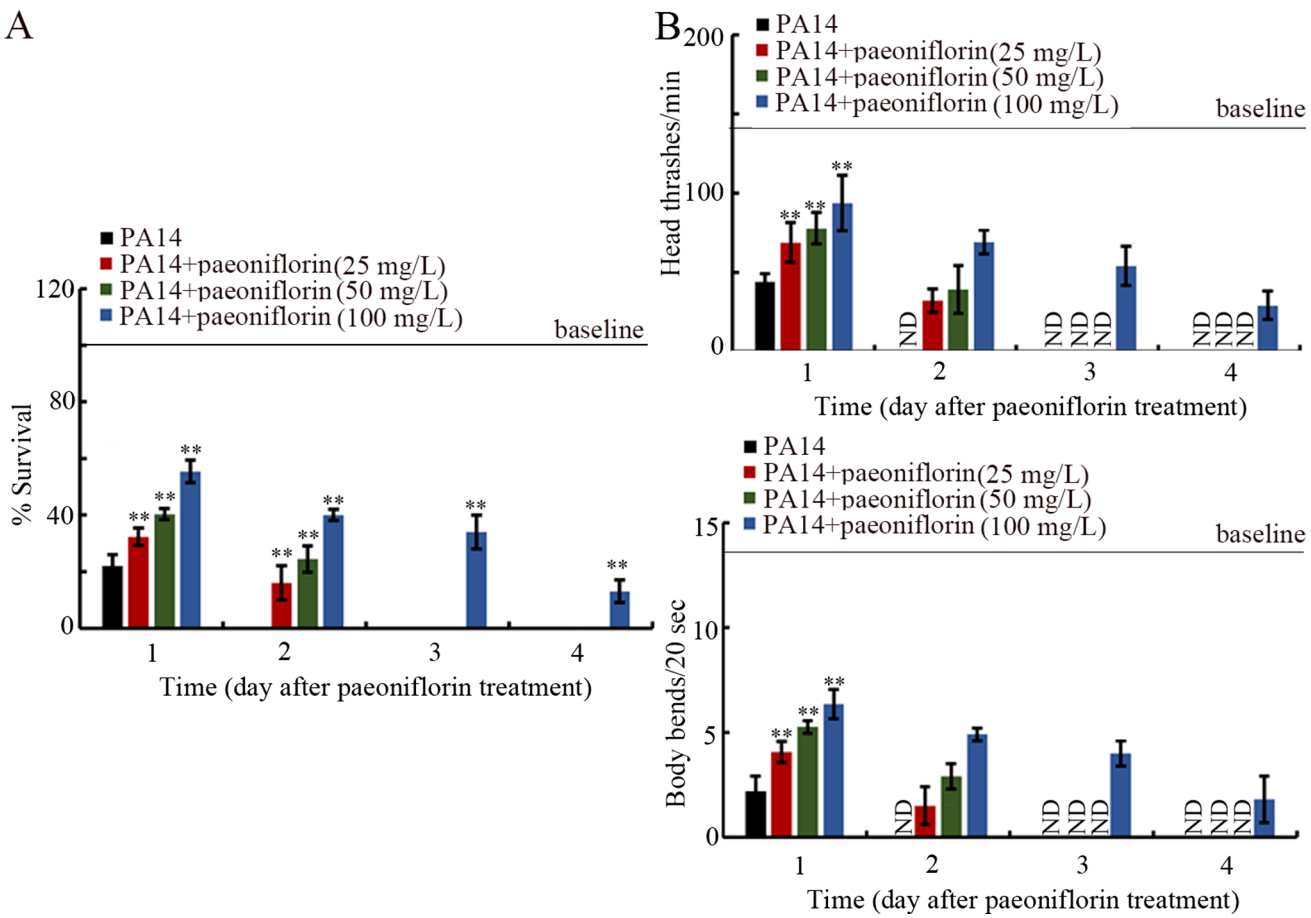

Fig. 1 Effect of paeoniflorin treatment on survival **A** and locomotion behavior **B** in nematodes at the immunosuppression stage (day-5) after *P. aeruginosa* infection. $^{**}P < 0.01$ vs. PA14. ND, not done

stage, we investigated the *P. aeruginosa* accumulation in intestinal lumen of nematodes after the paeoniflorin treatment. At the day-1 after treatment, 100 mg/L paeoniflorin could already noticeably decrease the PA14::GFP accumulation in intestinal lumen and the CFU of PA14 (Fig. 2A and B). At the day-2, the day-3, and the day-4 after treatment, 100 mg/L paeoniflorin caused the more obvious decrease in PA14::GFP accumulation in intestinal lumen and the CFU of PA14 in PA14 infected nematodes (Fig. 2A and B).

Paeoniflorin treatment modulated innate immune response to *P. aeruginosa* infection in nematodes at the immunosuppression stage

To determine the underlying mechanisms for the role of paeoniflorin treatment against toxic effects of *P. aeruginosa* infection on nematodes at the immunosuppression stage, we also examined the innate immune response to *P. aeruginosa* infection after paeoniflorin treatment. At the day-1 after treatment, 100 mg/L paeoniflorin significantly increased

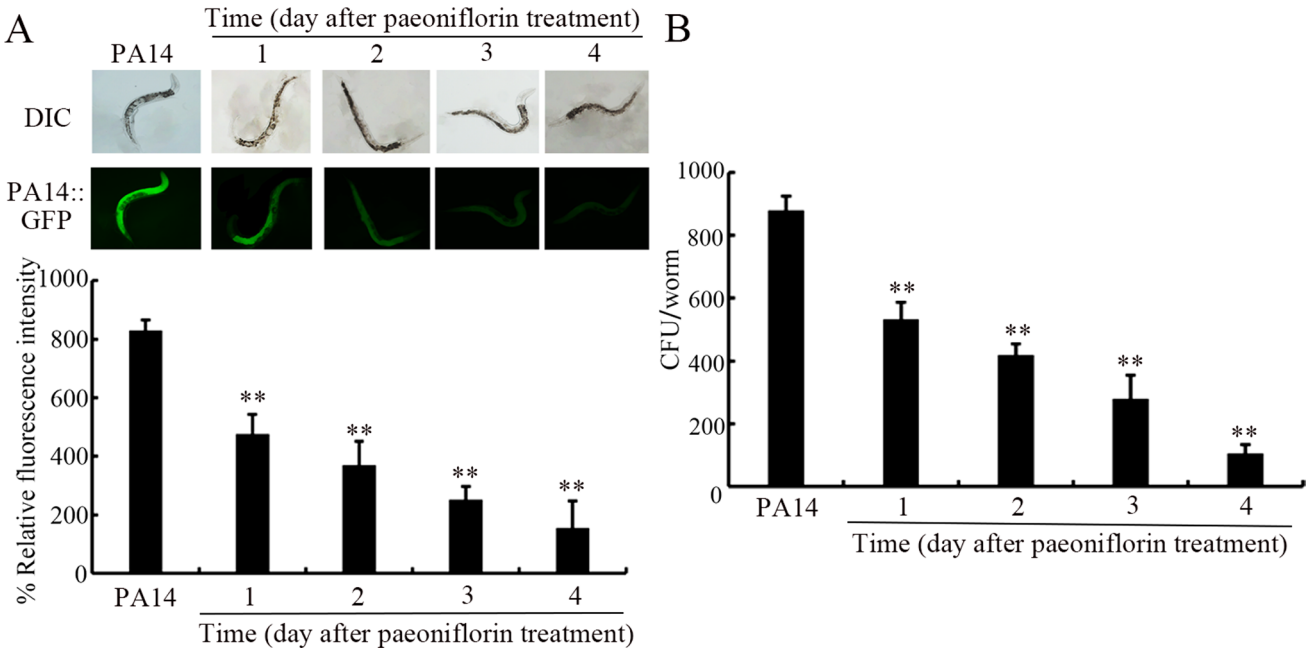

Fig. 2 Effect of 100 mg/L paeoniflorin treatment on PA14::GFP accumulation in intestinal lumen **A** and CFU of PA14 **B** in nematodes at the immunosuppression stage (day-5) after *P. aeruginosa* infection. $^{**}P < 0.01$ vs. PA14

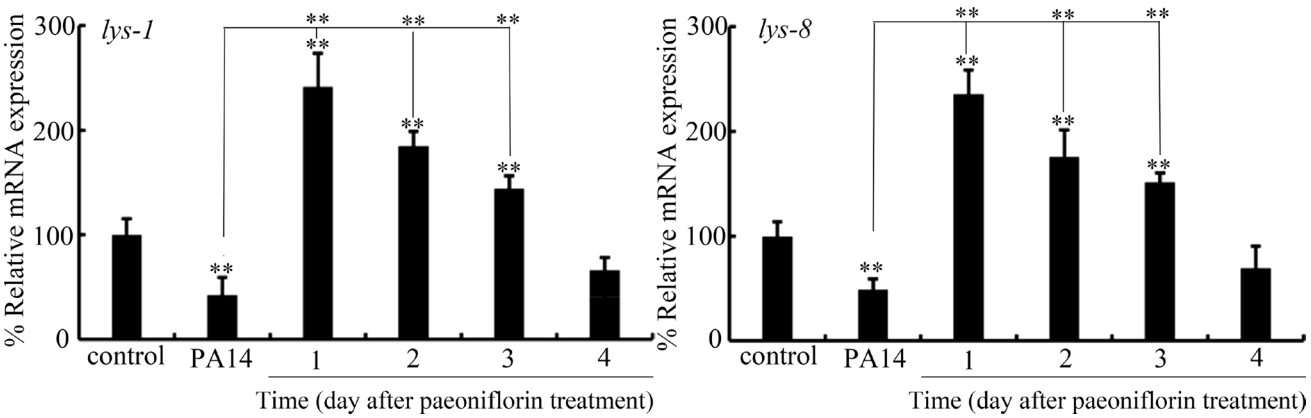

Fig. 3 Effect of 100 mg/L paeoniflorin treatment on expression of antimicrobial genes (*lys-1* and *lys-8*) in nematodes at the immunosuppression stage (day-5) after *P. aeruginosa* infection. $^{**}P < 0.01$ vs. control (if not specially indicated)

the expressions of *lys-1* and *lys-8* compared with those in PA14 infected nematodes (Fig. 3). At the day-1, the day-2, and the day-3, the expression levels of *lys-1* and *lys-8* in 100 mg/L paeoniflorin treated nematodes were higher than those in control nematodes (Fig. 3), suggesting the increase in innate immune response. Different from this, at the day-4, the expressions of *lys-1* and *lys-8* in 100 mg/L paeoniflorin treated nematodes were not higher than those in control nematodes (Fig. 3).

Paeoniflorin treatment increased the expressions of *bar-1*, *pmk-1*, and *egl-1* in nematodes at the immunosuppression stage after *P. aeruginosa* infection

In *C. elegans*, several signals (such as insulin, Wnt, ELT-2, TGF-β, p38 MAPK, and PCD related signals) regulate the bacterial infection and innate immune response (Kim et al. 2002; Irazoqui et al. 2008; Roberts et al. 2010; Arvanitis et al. 2013; Zou et al. 2013; Head et al. 2017). Among the genes involved in these signaling pathways, at the immunosuppression stage, PA14 infection caused the significant decrease in expressions of *daf-16*, *bar-1*, *elt-2*, *dbl-1*, *pmk-1*, and *egl-1* (Fig. 4). Among these genes, treatment with 100 mg/L paeoniflorin could obviously reverse the decrease in expressions of *bar-1*, *pmk-1*, and *egl-1* in PA14 infected nematodes (Fig. 4).

RNAi of *bar-1*, *pmk-1*, and *egl-1* suppressed the function of paeoniflorin treatment in increasing survival rate at the immunosuppression stage after *P. aeruginosa* infection

To determine the role of *bar-1*, *pmk-1*, and *egl-1* in regulating the function of paeoniflorin treatment against PA14 infection, we performed the RNAi of *bar-1*, *pmk-1*, and *egl-1* together with the paeoniflorin treatment. Without the RNAi of these genes, the survival rate in PA14 infected nematodes at the immunosuppression stage could be increased by 100 mg/L paeoniflorin treatment (Fig. 5). However, after RNAi of *bar-1*, *pmk-1*, and *egl-1*, this increase in survival rate by paeoniflorin treatment at the immunosuppression stage was significantly inhibited (Fig. 5). Therefore, BAR-1, PMK-1, and EGL-1 were required for the beneficial function of paeoniflorin treatment against the PA14 infection in nematodes at the immunosuppression stage.

RNAi of *bar-1*, *pmk-1*, and *egl-1* suppressed the function of paeoniflorin treatment in inhibiting *P. aeruginosa* accumulation in intestinal lumen of nematodes

After the RNAi of *bar-1*, *pmk-1*, and *egl-1*, we also investigated their effect on the beneficial effect of paeoniflorin treatment in reducing *P. aeruginosa* accumulation in

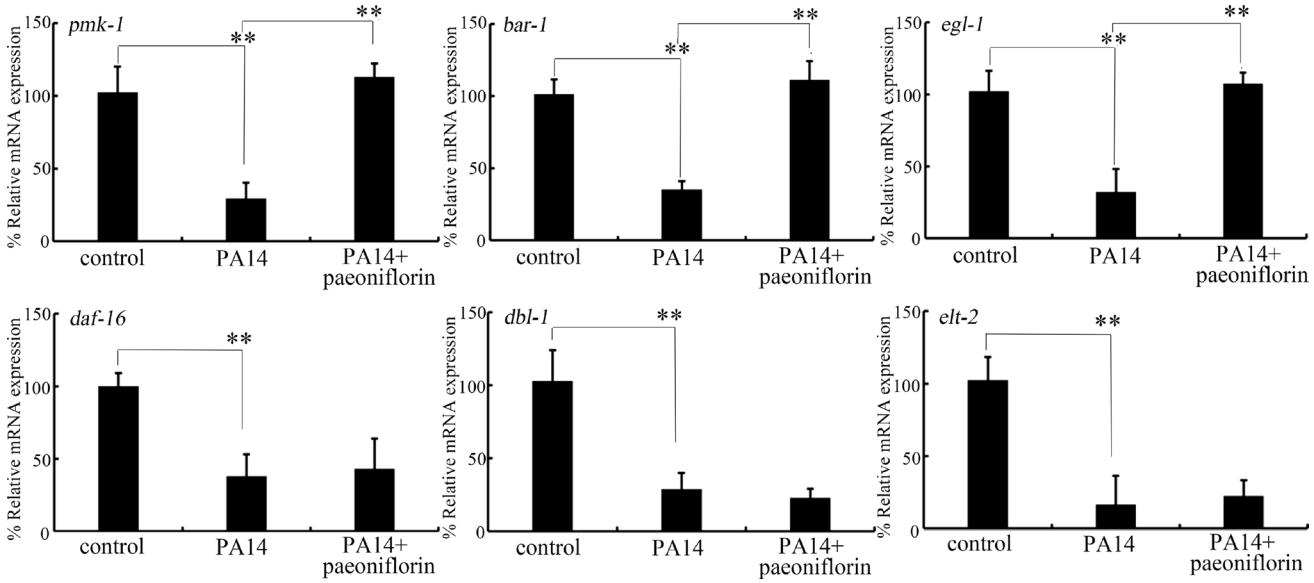

Fig. 4 Effect of 100 mg/L paeoniflorin treatment on expressions of *daf-16*, *bar-1*, *elt-2*, *dbl-1*, *pmk-1*, and *egl-1* in nematodes at the immunosuppression stage (day-5) after *P. aeruginosa* infection. $^{**}P < 0.01$

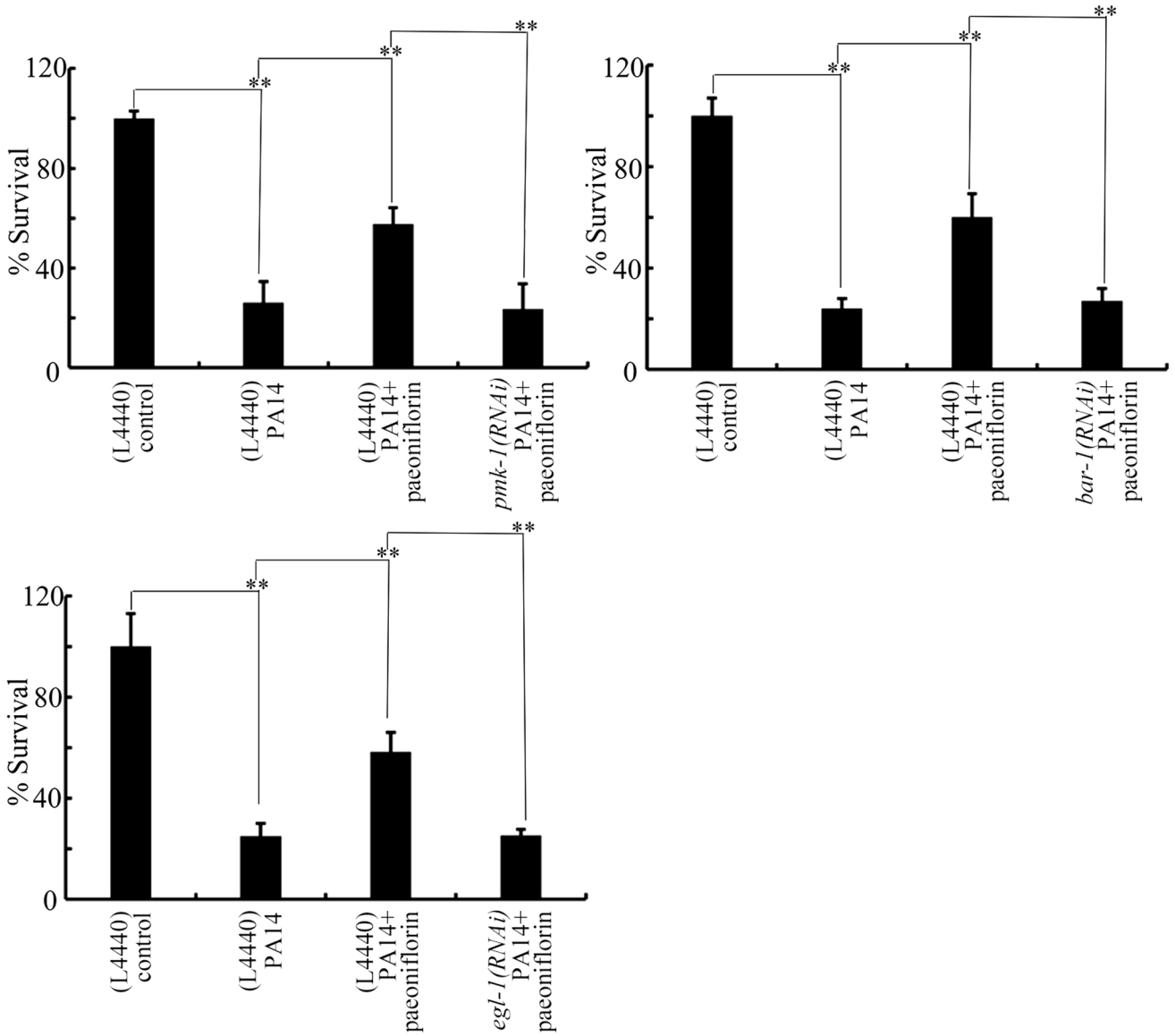

Fig. 5 RNAi of *bar-1*, *pmk-1*, and *egl-1* inhibited the effect of 100 mg/L paeoniflorin treatment for 1 day in increasing survival of nematodes at the immunosuppression stage (day-5) after *P. aeruginosa* infection. Feeding with HT115 expressing L4440 (empty vector) was employed as the control. $^{**}P < 0.01$

intestinal lumen of nematodes at the immunosuppression stage. The function of paeoniflorin treatment in inhibiting PA14 accumulation in intestinal lumen reflected by both PA14::GFP accumulation and CFU of PA14 at the immunosuppression stage was significantly suppressed by RNAi of *bar-1*, *pmk-1*, and *egl-1* (Fig. 6). Therefore, BAR-1, PMK-1, and EGL-1 were also involved in the beneficial effect of paeoniflorin treatment in reducing PA14 accumulation in nematodes at the immunosuppression stage.

RNAi of *bar-1*, *pmk-1*, and *egl-1* inhibited the activation of innate immune response by paeoniflorin treatment at the immunosuppression stage after *P. aeruginosa* infection

Finally, we investigated the effect of RNAi of *bar-1*, *pmk-1*, and *egl-1* on paeoniflorin-activated innate immune response at the immunosuppression stage. After PA14 infection,

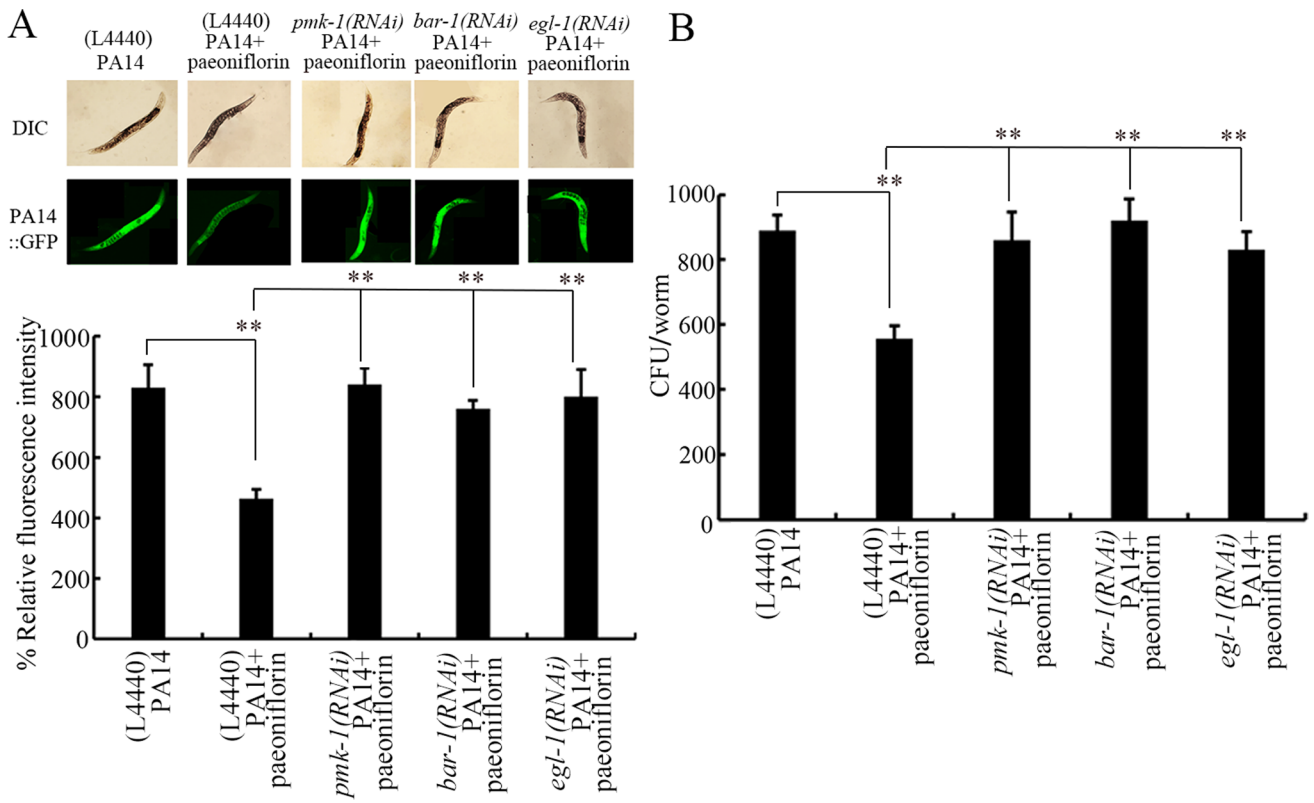

Fig. 6 RNAi of *bar-1*, *pmk-1*, and *egl-1* suppressed the effect of 100 mg/L paeoniflorin treatment in inhibiting PA14::GFP accumulation in intestinal lumen **A** and CFU of PA14 **B** in nematodes at the immunosuppression stage (day-5) after *P. aeruginosa* infection. Feeding with HT115 expressing L4440 (empty vector) was employed as the control. $^{**}P < 0.01$

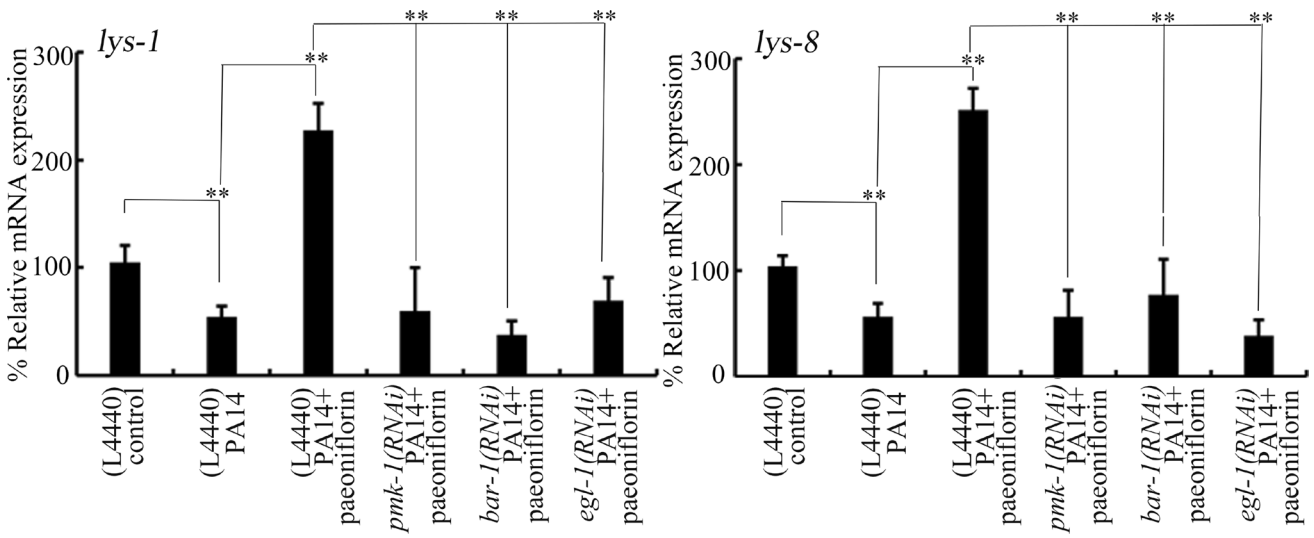

Fig. 7 RNAi of *bar-1*, *pmk-1*, and *egl-1* inhibited the effect of 100 mg/L paeoniflorin treatment in increasing the expression of antimicrobial genes (*lys-1* and *lys-8*) at the immunosuppression stage (day-5) after *P. aeruginosa* infection. Feeding with HT115 expressing L4440 (empty vector) was employed as the control. $^{**}P < 0.01$

paeoniflorin treatment-activated increase in *lys-1 e*xpression at the immunosuppression stage was inhibited by RNAi of *bar-1*, *pmk-1*, and *egl-1* (Fig. 7). Similarly, RNAi of *bar-1*, *pmk-1*, and *egl-1* also suppressed the paeoniflorin treatment-activated increase in *lys-8 e*xpression at the immunosuppression stage in PA14 infected nematodes (Fig. 7). Therefore,

BAR-1, PMK-1, and EGL-1 were further required for the beneficial role of paeoniflorin treatment in activating innate immune response in nematodes at the immunosuppression stage.

Discussion

In this study, using *P. aeruginosa* as a model bacterial pathogen, we established an immunosuppression animal model in *C. elegans* after bacterial infection. We established this immunosuppression model from three aspects in nematodes. In this immunosuppression animal model, the survival and the locomotion behavior were used as endpoints to reflect the physiological alterations at the immunosuppression stage. (Fig. S2A and S2B). The fluorescence intensity of PA14::GFP and the CFU of PA14 were used to reflect the amount of *P. aeruginosa* accumulated in intestinal lumen of nematodes (Fig. S3A and S3B). The expression of antimicrobial genes (such as *lys-1* and *lys-8*) was used to reflect the alteration in innate immune response at the immunosuppression stage after *P. aeruginosa* infection (Fig. S4).

In this immunosuppression animal model, we suggested the day-5 and the day-6 as the immunosuppression stage after PA14 infection. This is due to three aspects of reasons: (1) the survival rate and the locomotory ability of nematodes were decreased by PA14 infection, (2) the PA14 cells were severely accumulated in the intestinal lumen of nematodes, and (3) the expressions of antimicrobial genes (such as *lys-1* and *lys-8*) were already lower than those at day-0 after the PA14 infection. For this raised immunosuppression model, it can be potentially applied in several aspects. Firstly, it is useful to determine the mechanisms of interaction between the hosts and the microbe at the immunosuppression stage. Secondly, it will be helpful for the screen of candidate compounds or drugs against the immunosuppression induced by bacterial pathogen infection and the further examination of the underlying pharmacological mechanisms. Thirdly, it can provide a tool to analyze the different factors affecting the immunosuppression process after bacterial infection in the hosts. In addition, the data obtained in this raised immunosuppression model can provide the useful clues for investigating different aspects for immunosuppression after infection with fungal or other bacterial pathogens in *C. elegans*.

Using the established immunosuppression model in nematodes, we found that paeoniflorin treatment could effectively inhibit the toxic effect of PA14 infection at the immunosuppression stage. Firstly, paeoniflorin treatment could extend the survival time at the immunosuppression stage in PA14 infected nematodes. The survival time at the immunosuppression stage in PA14 infected nematodes could be extended by 25–100 mg/L paeoniflorin treatment for 1 day or 2 days and by 100 mg/L paeoniflorin treatment for 3 or 4 days (Fig. 1A). Secondly, paeoniflorin treatment could improve the health status at the immunosuppression stage in PA14 infected nematodes. Compared with the locomotion behavior in PA14 infected nematodes, at the day-1 or the day-2 after the treatment, 25–100 mg/L paeoniflorin could significantly increase the locomotory ability reflected by the endpoints of head thrash and body bend (Fig. 1B). Moreover, at the day-4 after the treatment, the 100 mg/L paeoniflorin treated nematodes still showed obvious locomotory ability (Fig. 1B). These observations suggested the potential of paeoniflorin treatment in reducing the fatality rate and in improving the health status in hosts at the immunosuppression stage. Besides this, the paeoniflorin treatment has been shown to have other aspects of beneficial effects in *C. elegans*, such as suppression in reproductive toxicity of nanoplastics and inhibition in glucose toxicity in reducing lifespan (Hua et al. 2023a; Liu et al. 2023). Previous studies have also suggested the usefulness of paeoniflorin in treating certain aspects of sepsis, such as the functions in attenuating cardiac dysfunction and in inhibiting systematic inflammation in sepsis (Jiang et al. 2009; Wang et al. 2021). Our results further suggest the potential of paeoniflorin treatment in inhibiting the immunosuppression in sepsis.

Recently, it was reported that the reduction of PA14 accumulation in intestinal lumen acted as one important mechanism for Xuebijing treatment against PA14 infection from young adults for 24 h (Zhang et al. 2022). Paeoniflorin is one of major compounds in the Xuebijing, a Traditional Chinese Medicine used for clinical treatment of sepsis (Fan et al. 2020). Our results further indicated that suppression in PA14 accumulation in intestinal lumen contributed to forming the function of paeoniflorin treatment against toxic effects of bacterial infection on nematodes at the immunosuppression stage. As shown in Fig. 2, treatment with 100 mg/L paeoniflorin reduced both the PA14::GFP accumulation in intestinal lumen and the CFU of PA14 at the immunosuppression stage. Meanwhile, we observed that the obvious decrease in both PA14::GFP accumulation in intestinal lumen and CFU of PA14 could be detected in PA14 infected nematodes after treatment with 100 mg/L paeoniflorin for 4 days (Fig. 2). Besides this, we recently further found that the biofilm formation of PA14 could be significantly inhibited by paeoniflorin treatment (Wang et al. 2023a). These suggested that the function of paeoniflorin treatment to extend the survival time of nematodes was attributable to both the inhibition in accumulation of bacterial pathogen in intestinal lumen of nematodes and the suppression in bacterial virulence.

More importantly, we found that the activation of innate immunity by paeoniflorin treatment played an important role in forming the beneficial effect of paeoniflorin treatment

against the toxic effects of PA14 infection on nematodes at the immunosuppression stage. As shown in Fig. 3, at the day-1, the day-2, and the day-3 after the treatment, the expressions of antimicrobial genes (*lys-1* and *lys-8*) were increased by 100 mg/L paeoniflorin and showed much higher levels compared with those in PA14 infected nematodes. This indicated that, on the one hand, paeoniflorin treatment could reverse the trend of immunosuppression caused by PA14 infection. On the other hand, paeoniflorin treatment could enhance the innate immune response to make the animals show a relatively healthy state. Nevertheless, compared with the antimicrobial gene expressions at the day-1 after paeoniflorin treatment, the expressions of *lys-1* and *lys-8* were gradually decreased from the day-2 to the day-4 after the treatment (Fig. 3). This may largely explain the inability to prolong the survival time of PA14 infected nematodes at the day-5 after the paeoniflorin treatment. Nevertheless, considering the difference in immune system between *C. elegans* and mammals or humans, the results obtained in *C. elegans* can only provide the possibility of pharmacological effect of paeoniflorin treatment against the immunosuppression caused by bacterial infection. More efforts for the possible pharmacological effect of paeoniflorin treatment against the suppression in both innate immunity and adaptive immunity are needed to be further performed in certain mice model, such as the sepsis model at the immunosuppression stage.

The insulin, Wnt, ELT-2, TGF-β, p38 MAPK, and PCD related signals participate in the control of bacterial infection in nematodes (Kurz and Tan 2004; Irazoqui et al. 2008; Arvanitis et al. 2013; Head et al. 2017; Zhi et al. 2017a; Harding and Ewbank 2021). In this study, we further provide evidence to show the requirement of PMK-1, BAR-1, and EGL-1 for forming the beneficial function of paeoniflorin treatment against the toxic effects of PA14 infection on nematodes at the immunosuppression stage, which raises the important molecular basis for the observed beneficial function of paeoniflorin treatment in PA14 infected nematodes. At the immunosuppression stage, 100 mg/L paeoniflorin treatment could inhibit the decreased expressions of *bar-1*, *pmk-1*, and *egl-1* caused by PA14 infection (Fig. 4). Meanwhile, RNAi of *bar-1*, *pmk-1*, and *egl-1* inhibited the beneficial effect of paeoniflorin treatment in increasing survival rate at the immunosuppression stage in PA14 infected nematodes (Fig. 5). In addition, the function of paeoniflorin treatment in reducing PA14::GFP accumulation in intestinal lumen and CFU of PA14 at the immunosuppression stage was also inhibited by RNAi of *bar-1*, *pmk-1*, and *egl-1* (Fig. 6). These results suggested that PMK-1, BAR-1, and EGL-1 were required for the function of paeoniflorin treatment in suppressing the toxic effects of PA14 infection and PA14 accumulation in intestinal lumen in nematodes at the immunosuppression stage. That is, paeoniflorin treatment exhibited the beneficial effects in inhibiting the toxic effects of PA14 infection and in reducing PA14 accumulation in intestinal lumen by activating BAR-1, PMK-1, and EGL-1. In *C. elegans*, PMK-1 is a p38 MAPK in the p38 MAPK signaling pathway, BAR-1 is the β-catenin transcriptional factor in the Wnt signaling pathway, and EGL-1 is a BH3 protein in the programed cell death (PCD) signaling pathway.

In *C. elegans*, BAR-1, PMK-1, and EGL-1 also regulate the innate immunity by modulating expressions of antimicrobial genes after bacterial infection (Irazoqui et al. 2008; Zhi et al. 2017b; Zhang et al. 2022). After paeoniflorin infection, the increase in expressions of antimicrobial genes (*lys-1* and *lys-8*) by 100 mg/L paeoniflorin treatment in nematodes at the immunosuppression stage was inhibited by RNAi of *bar-1*, *pmk-1*, and *egl-1* (Fig. 7). That is, these 3 molecular signals were required for the formation of beneficial effect of paeoniflorin treatment in activating innate immune response in nematodes at the immunosuppression stage after PA14 infection. This provides another aspect of molecular basis for the involvement of PMK-1, BAR-1, and EGL-1 in regulating the beneficial effect of paeoniflorin in inhibiting the immunosuppression caused by PA14 infection. This also provides important clues for further elucidating the underlying pharmacological mechanisms of paeoniflorin treatment in inhibiting the immunosuppression in the mice model of sepsis. In *C. elegans*, the p38 MAPK signaling cascade of NSY-1-SEK-1-PMK-1 regulates the bacterial infection and innate immunity by activating the downstream transcriptional factors of ATF-7 and SKN-1 (Shivers et al. 2010; van der Hoeven et al. 2011; Peterson et al. 2022). In nematodes, the BAR-1 regulates bacterial pathogen infection and immunity by acting its downstream homeobox protein EGL-5, and its function was activated by the transcriptional factor of HLH-26 (Irazoqui et al. 2008; Sang et al. 2022). During the induction of apoptosis, EGL-1 activates the downstream signaling cascade of CED-4-CED-3 (Zhao et al. 2016), and CED-4 and CED-3 were also involved in the control of bacterial pathogen infection (Aballay and Ausubel 2001). These backgrounds provide the important basis for the future elucidation of underling molecular mechanisms of PMK-1, BAR-1, and EGL-1 in regulating beneficial effect of paeoniflorin treatment in inhibiting immunosuppression caused by bacterial pathogen infection in *C. elegans*.

Together, in this study, we first established an immunosuppression model by assessing toxic effects, pathogen accumulation, and innate immune response in *C. elegans* after *P. aeruginosa* infection. Using this established immunosuppression model after bacterial infection, we observed that paeoniflorin treatment could effectively increase survival rate and locomotory ability and reduce *P. aeruginosa* accumulation in intestinal lumen at the immunosuppression stage after the *P. aeruginosa* infection. Meanwhile, the decrease in expression of antimicrobial genes caused

by *P. aeruginosa* infection at the immunosuppression stage could be inhibited by paeoniflorin treatment. Moreover, we found that PMK-1, BAR-1, and EGL-1 were required for forming the beneficial effect of paeoniflorin treatment against the immunosuppression induced by *P. aeruginosa* infection. Our results highlight the potential of paeoniflorin treatment in inhibiting the immunosuppression caused by bacterial infection in organisms.

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Declarations

Conflict of interest The authors declare no conflicts of interest.

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