

# SCIENTIFIC REPORTS



OPEN

## Haemocoel injection of PirA<sub>1</sub>B<sub>1</sub> to *Galleria mellonella* larvae leads to disruption of the haemocyte immune functions

Received: 05 July 2016  
Accepted: 22 September 2016  
Published: 13 October 2016

Gongqing Wu<sup>1,2</sup> & Yunhong Yi<sup>1</sup>

The bacterium *Photorhabdus luminescens* produces a number of insecticidal proteins to kill its larval prey. In this study, we cloned the gene coding for a binary toxin PirA<sub>1</sub>B<sub>1</sub> and purified the recombinant protein using affinity chromatography combined with desalination technology. Furthermore, the cytotoxicity of the recombinant protein against the haemocytes of *Galleria mellonella* larvae was investigated. We found that the protein had haemocoel insecticidal activity against *G. mellonella* with an LD50 of 131.5 ng/larva. Intrahaemocoelic injection of PirA<sub>1</sub>B<sub>1</sub> into *G. mellonella* resulted in significant decreases in haemocyte number and phagocytic ability. In *in vitro* experiments, PirA<sub>1</sub>B<sub>1</sub> inhibited the spreading behaviour of the haemocytes of *G. mellonella* larvae and even caused haemocyte degeneration. Fluorescence microscope analysis and visualization of haemocyte F-actin stained with phalloidin-FITC showed that the PirA<sub>1</sub>B<sub>1</sub> toxin disrupted the organization of the haemocyte cytoskeleton. Our results demonstrated that the PirA<sub>1</sub>B<sub>1</sub> toxin disarmed the insect cellular immune system.

*Photorhabdus luminescens*, a Gram-negative bacterium, resides as a symbiont in the gut of entomopathogenic nematodes (EPNs) of the genus *Heterorhabditis*<sup>1</sup>. Upon entering an insect host, EPNs release the symbiotic bacteria directly into the insect haemocoel. To infect its host and survive, bacteria must be capable of producing a wide range of proteins, including toxins<sup>2</sup>. To date, four primary classes of toxins are characterized in *P. luminescens*. The first class, toxin complexes (Tcs), shows both oral and injectable activity against the *Colorado potato beetle*<sup>3</sup>. The second class is *Photorhabdus* Virulence Cassettes (PVCs), and the injection of PVCs destroys insect haemocytes, which undergo dramatic actin cytoskeleton condensation<sup>4</sup>. Making caterpillars floppy (Mcf), the third class of toxins promotes apoptosis in the midgut and produces a characteristic “floppy” phenotype in the infected insect<sup>5</sup>. The fourth class, *Photorhabdus* insect-related toxins (PirAB), are binary toxins that exhibit injectable and oral toxicity against mosquitos and lepidopterans<sup>6</sup>.

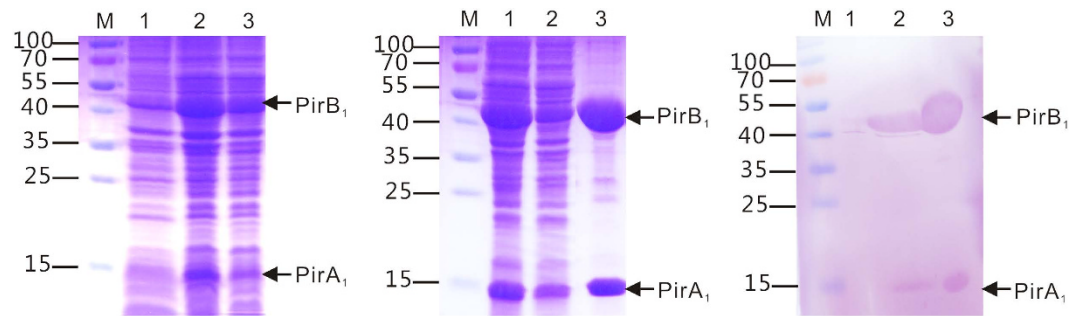
To combat infection, insects rely on multiple immune responses that encompass both humoral and cellular defence reactions. Humoral reactions include the production of antimicrobial peptides (AMPs), reactive oxygen and nitrogen species, and the prophenoloxidase (proPO) activating system that regulates coagulation or melanization of haemolymph<sup>7–9</sup>. Cellular responses include the phagocytosis of small pathogens such as bacteria and fungi and the encapsulation of parasites such as parasitoids and nematodes by haemocytes<sup>10–11</sup>. The symbiotic bacteria of entomopathogenic nematodes that enter the insect haemocoel must fight with the haemocytes and AMPs. Many toxic proteins produced by symbiotic bacteria are reported to target haemocytes, which affects the host immune system by reducing vitality<sup>12</sup> and by inducing apoptosis and cytolysis<sup>13–14</sup>. Moreover, in a recent study, Tc toxins inhibited the phagocytic activity of haemocytes from *Galleria mellonella*, and the biologically active components of the Tc toxins were characterized as adenosine diphosphate (ADP)-ribosyltransferases, which modify unusual amino acids and cause actin polymerization<sup>15</sup>.

PirAB toxin proteins, PirA<sub>1</sub>B<sub>1</sub> and PirA<sub>2</sub>B<sub>2</sub>, are encoded at two distinct loci, *plu4093* to *plu4092* and *plu4437* to *plu4436*, in the TT01 genome, respectively, and these toxins show similarity to both  $\delta$ -endotoxins from *Bacillus thuringiensis* and a developmentally regulated protein from the *Colorado potato beetle*<sup>6,16</sup>. PirA<sub>1</sub>B<sub>1</sub> has

<sup>1</sup>School of Chemistry and Chemical Engineering, Guangdong Pharmaceutical University, Zhongshan, 528458, China.

<sup>2</sup>Guangdong Cosmetics Engineering & Technology Research Centre, Zhongshan, 528458, China. Correspondence and requests for materials should be addressed to Y. Yi. (email: yunhongyi@163.com)





**Figure 1.** (A) SDS-PAGE analysis of PirA<sub>1</sub>B<sub>1</sub> expression in *E. coli* M15 cells (pQE-pirA<sub>1</sub>B<sub>1</sub>). Lane M, markers (kDa); lane 1, uninduced pQE-pirA<sub>1</sub>B<sub>1</sub>; lanes 2 and 3, pQE-pirA<sub>1</sub>B<sub>1</sub> induced with IPTG. (B) SDS-PAGE analysis of PirA<sub>1</sub>B<sub>1</sub> expressed in *E. coli* M15 (pQE-pirA<sub>1</sub>B<sub>1</sub>) as soluble proteins followed by the purification and desalination protocols described. Lane M, markers (kDa); lanes 1 and 2, unpurified PirA<sub>1</sub>B<sub>1</sub>; lane 3, purified and desalinated PirA<sub>1</sub>B<sub>1</sub>. (C) Western blot analysis of expressed protein PirA<sub>1</sub>B<sub>1</sub> in *E. coli* M15 cells (pQE-pirA<sub>1</sub>B<sub>1</sub>). Lane M, markers (kDa); lane 1, uninduced pQE-pirA<sub>1</sub>B<sub>1</sub>; lanes 2 and 3, pQE-pirA<sub>1</sub>B<sub>1</sub> induced with IPTG.

F-actin staining with phalloidin-FITC was performed according to previous study. Briefly, haemolymph was collected from the larvae injected with PirA<sub>1</sub>B<sub>1</sub> (131.5 ng per larva), BSA (131.5 ng per larva) or PBS at 6 h after injection. An aliquot of 50 µl of diluted haemolymph (20 µl of haemolymph diluted in 30 µl of PBS) from each treatment group was applied to a microscope slide. The slides were placed in a moist chamber for 30 min for haemocytes to attach to the glass surface and then were fixed for 5 min in 3.7% (w/v) formaldehyde in PBS, washed three times in PBS, and then permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. After washing the slides with PBS, haemocyte monolayers were overlaid with phalloidin-FITC (Sigma, USA) at a concentration of 0.05 mg/ml in PBS containing 1% dimethyl sulfoxide for 40 min at room temperature in a humidified chamber. Then, the slides were washed with PBS several times and overlaid with a mixture of 30% glycerol and 70% PBS (v/v) and a cover slip. Haemocytes were examined using a fluorescence microscope.

**In vitro toxicity experiments of PirA<sub>1</sub>B<sub>1</sub> on haemocytes.** The larvae were chilled, surface sterilized and bled into prechilled GIM, and the haemocyte density was adjusted accordingly to  $1 \times 10^7$  cells/ml. The haemocyte suspension was exposed to 10 µl of PBS containing 131.5 ng of PirA<sub>1</sub>B<sub>1</sub> toxins, BSA (positive control) or PBS alone (negative control). Haemocytes were then incubated in GIM and maintained at 28 °C. After incubation for the designated time (6 and 12 h), cell morphological changes were analysed using an inverted light microscope.

**Statistical analyses.** All results are expressed as the mean and standard deviation. Data were subjected to analysis of variance (ANOVA), and when the effects of ANOVA were significant, the factors that contributed to the significant differences were determined by means of least significant difference (LSD) tests using the DPS statistical software package. Significant differences were set at  $P < 0.05$ .

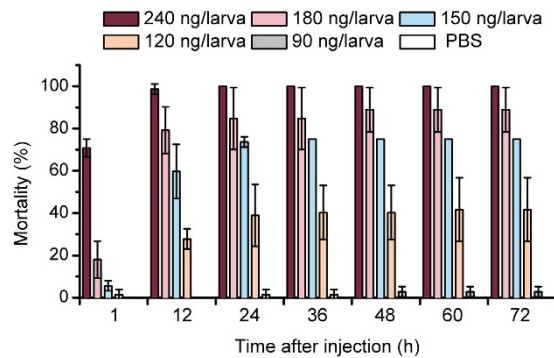
## Results

**Recombinant expression of PirA<sub>1</sub>B<sub>1</sub> in *E. coli*.** The recombinant plasmid pQE-pirA<sub>1</sub>B<sub>1</sub> was transformed and expressed in *E. coli* M15. After IPTG induction for 4 h, the whole cell lysate analysed by SDS-PAGE revealed two distinct bands with molecular weights of 45 kDa (PirA<sub>1</sub>) and 14 kDa (PirB<sub>1</sub>) (Fig. 1A), which were consistent with the predicted molecular masses. Products were not found in either the uninduced cultures or in the control (lysate of cells transfected with empty vector pQE, data not shown). PirA<sub>1</sub>B<sub>1</sub> was successfully purified using the protocols described (Fig. 1B). The objective band of recombinant PirA<sub>1</sub>B<sub>1</sub> was confirmed by western blot analysis with anti-His6 monoclonal antibody. For the control, no visible reaction band was detected in the group of cell lysates without IPTG induction (Fig. 1C).

**Insecticidal activity of PirA<sub>1</sub>B<sub>1</sub>.** PirA<sub>1</sub>B<sub>1</sub> effectively killed *G. mellonella* larvae, and the mortality rate was dose-dependent based on protein concentrations. The LD<sub>50</sub> of PirA<sub>1</sub>B<sub>1</sub> for *G. mellonella* was 131.5 ng per larva. The larvae developed to a deep brown body colour and showed swelling symptoms after injection with PirA<sub>1</sub>B<sub>1</sub>. Larvae of *G. mellonella* injected with a high dose of PirA<sub>1</sub>B<sub>1</sub> (240 ng per larva) died primarily during the post injection period of 1 to 12 h, with a mortality rate of 100% at 24 h after injection. No dead larvae or external symptoms were observed in the control (injected with PBS; Fig. 2).

**Injection of PirA<sub>1</sub>B<sub>1</sub> proteins into the larval haemocoel led to decreases in numbers and phagocytic ability of haemocytes.** As shown in Fig. 3, only internalized bacteria retained their fluorescence (bright green) after quenching with trypan blue. The phagocytic rate of haemocytes in the group injected with PirA<sub>1</sub>B<sub>1</sub> protein decreased significantly compared with that in the PBS control group ( $P < 0.05$ ), whereas a significant increase was detected in the BSA treatment group at 6 h after injection ( $P < 0.05$ ; Figs 3 and 4B).

There was no significant difference in the total haemocyte counts (THC) between the PBS and BSA treatment groups. However, after injecting PirA<sub>1</sub>B<sub>1</sub> protein into the *G. mellonella* haemocoel, THC decreased to 58% of the original values after 6 h and ultimately, to 54% of the initial numbers after 12 h (Fig. 4A).



**Figure 2.** Mortality of *Galleria mellonella* larvae after injection with different doses of PirA<sub>1</sub>B<sub>1</sub> protein.

### Effect of PirA<sub>1</sub>B<sub>1</sub> protein on the cytoskeletons of the haemocytes of *G. mellonella* larvae.

Staining of haemocytes from PirA<sub>1</sub>B<sub>1</sub>-injected larvae with phalloidin-FITC showed distinct differences in actin polymerization compared with the actin-mediated assembly of the cytoskeleton in haemocytes of larvae injected with PBS and BSA. Plasmatocytes in the PBS and BSA control larvae extended their filopodia over the surface of the slide. The granular cells spread to form rounder shapes, and the actin cytoskeleton was largely organized around the periphery of these cells. However, for both plasmatocytes and granular cells in the PirA<sub>1</sub>B<sub>1</sub>-injected larvae, the cytoskeleton appeared diffuse and not organized into bundles or stress fibres (Fig. 5).

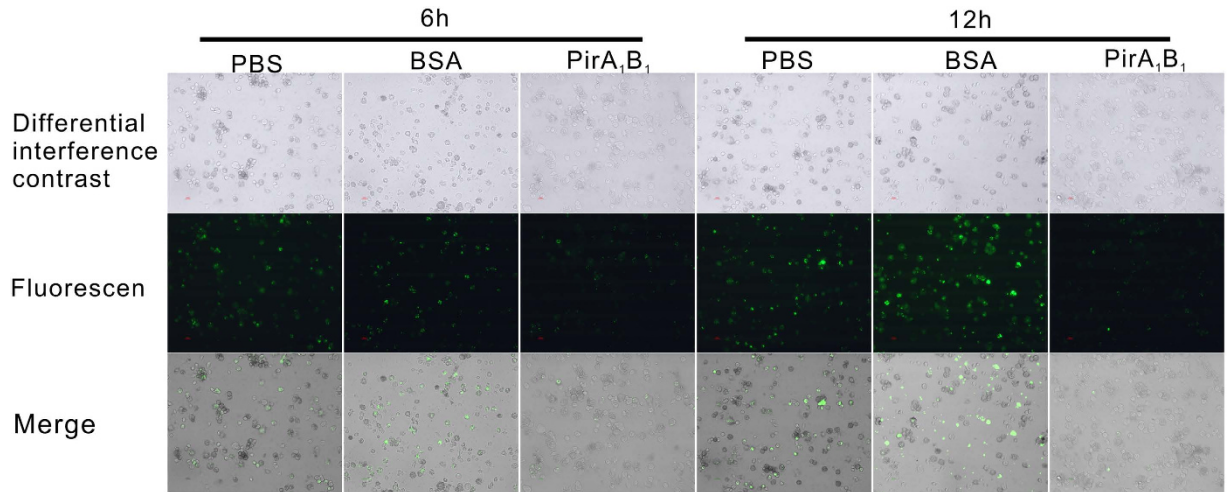
**Toxic effect of PirA<sub>1</sub>B<sub>1</sub> protein on haemocytes of *G. mellonella* larvae *in vitro*.** The haemocytes treated with PBS and BSA clearly spread normally and all the plasmatocytes were spindle-shaped. By contrast, most PirA<sub>1</sub>B<sub>1</sub>-treated haemocytes were small and failed to spread. After incubation for 12 h, the cytotoxic effects of PirA<sub>1</sub>B<sub>1</sub> toxin on haemocytes were more severe, with the dead cells degenerating to form debris in the culture (Fig. 6).

### Discussion

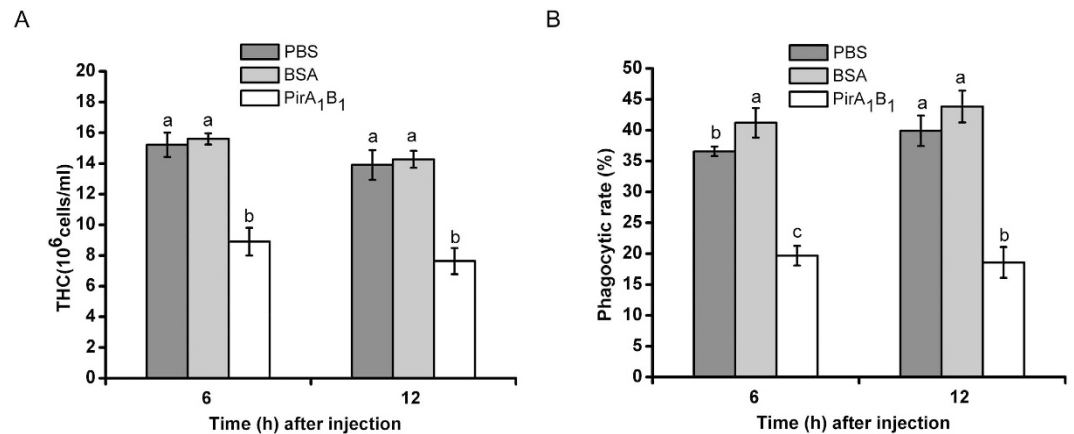
In this study, the gene coding for the binary toxin PirA<sub>1</sub>B<sub>1</sub> was cloned from *P. luminescens* and expressed in *E. coli* M15. The recombinant protein was purified from *E. coli* M15 using nickel affinity chromatography. The toxin protein had injection activity against *G. mellonella* with an LD50 of 131.5 ng/larva. In a previous study, the protein PirA<sub>2</sub>B<sub>2</sub> encoded by the loci *plu4437* to *plu4436* within *P. luminescens* TT01 had haemocoel insecticidal activity against the fifth instar larvae of both *G. mellonella* and *Spodopteralitura*, with an LD50 of 4.0 and 2.8 µg/larva, respectively<sup>20</sup>. Compared with PirA<sub>2</sub>B<sub>2</sub>, PirA<sub>1</sub>B<sub>1</sub> had stronger toxicity against *G. mellonella* larvae. The causes of the difference in toxicity between PirA<sub>2</sub>B<sub>2</sub> and PirA<sub>1</sub>B<sub>1</sub> are not clear yet. In addition to the unique biological properties of these two toxin proteins, the purification process, which can affect the structure of toxin proteins, was also an important factor worth consideration.

Haemocyte-mediated immunity is activated immediately after the insect haemocoel is penetrated by entomopathogenic nematodes. However, *P. luminescens* released from the nematode escape the insect cellular response and proliferate successfully in the haemolymph before the insect dies. A successful outcome is primarily attributed to the toxin proteins secreted by the *P. luminescens*, which fight against the haemocyte-mediated cellular immunity and protect the bacteria from the insect immune responses<sup>21–22</sup>. Recombinant *E. coli* clones carrying *plu4093–plu4092* (PirA<sub>1</sub>B<sub>1</sub>) of TT01 genes show oral activity against both mosquito larvae and the larvae of the moth *P. xylostella*<sup>16</sup>. In a recent study, PirAB-fusion protein encoded by *plu4093* and *plu4092* from *P. luminescens* TT01 also exhibited injectable insecticidal activity against *Spodoptera exigua* larvae and had cytotoxicity against insect midgut CF-203 cells<sup>23</sup>. However, whether the binary toxin PirA<sub>1</sub>B<sub>1</sub> can destroy the immune function of the insect haemocytes is unknown.

Our results showed that injection of PirA<sub>1</sub>B<sub>1</sub> into the haemocoel of *G. mellonella* larvae significantly decreased the number of circulating haemocytes and ability for phagocytosis. The phagocytic rate and total number of haemocytes decreased to 47% and 54% of their original values at 12 h after the toxin injection, respectively. In a previous study, we also demonstrated that another toxin, PirA<sub>2</sub>B<sub>2</sub>, had similar effects on the cellular immunity of *G. mellonella* larvae<sup>18</sup>; however, the phagocytic rate decreased to 71% of the PBS control at 12 h after injection and did not change afterwards, which indicated higher toxicity of the toxin PirA<sub>1</sub>B<sub>1</sub> against *G. mellonella* larvae. Haemocytes play important roles in phagocytosis and capsule formation<sup>11,24</sup>, and with more haemocytes, insect have greater ability to clear invading pathogens<sup>25</sup>. Therefore, with the decrease in haemocytes, we believe that the immune strength and immune system coordination were weakened directly. The mechanism by which PirA<sub>1</sub>B<sub>1</sub> causes this reduction in circulating haemocyte number is currently unknown. One possibility is the reduction in haemocyte number due to death and disintegration of haemocytes and/or reduced proliferation of haemocytes<sup>26</sup>. We performed *in vitro* experiments and directly treated haemocytes with PirA<sub>1</sub>B<sub>1</sub>, and the results showed that the toxin disrupted the spreading behaviour of haemocytes at 6 h after treatment. Furthermore, after incubation for another 12 h, the PirA<sub>1</sub>B<sub>1</sub> toxin caused the haemocytes to lyse, which degenerated to form debris in the culture. We also observed that treatment with the PirA<sub>1</sub>B<sub>1</sub> toxin caused a reduction in haemocyte pseudopod formation.



**Figure 3.** Differential interference contrast and corresponding fluorescence microscope of *Galleria mellonella* larvae haemocytes with phagocytised FITC-labelled *E. coli* cells *in vitro*. Haemocytes collected from larvae injected with PBS, BSA or PirA<sub>1</sub>B<sub>1</sub> at 6 and 12 h after injection.

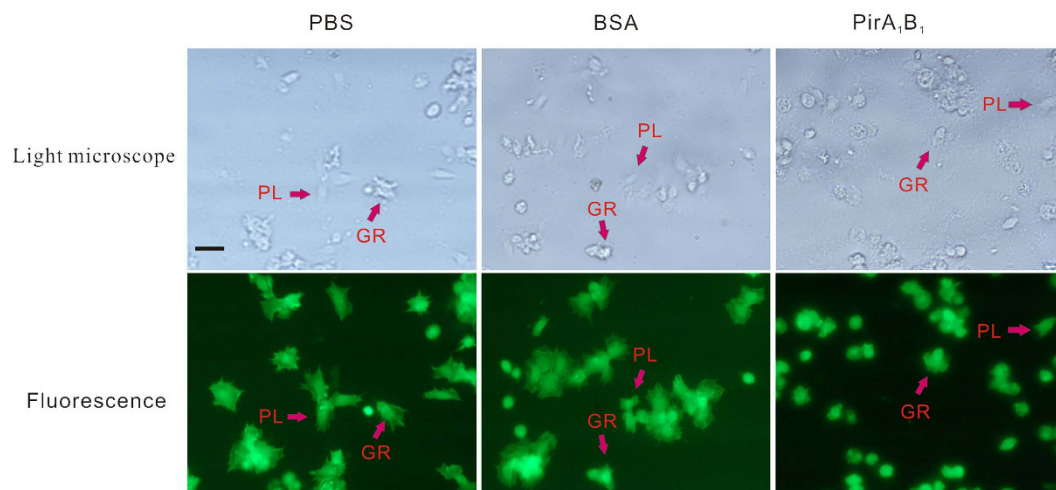


**Figure 4.** Changes in total haemocyte counts and phagocytic rates of *Galleria mellonella* larvae at 6 and 12 h after injection with PBS, BSA or PirA<sub>1</sub>B<sub>1</sub>. Values for different groups at the identical points in time followed by different letters are significantly different ( $P < 0.05$ ) according to ANOVA and LSD tests.

Therefore, based on our results, the binary toxin PirA<sub>1</sub>B<sub>1</sub> was cytotoxic against the haemocytes of *G. mellonella* larvae. These results were consistent with the *in vivo* experiments in which haemocoel injection of PirA<sub>1</sub>B<sub>1</sub> caused significant reductions in number of circulating haemocytes in *G. mellonella* larvae.

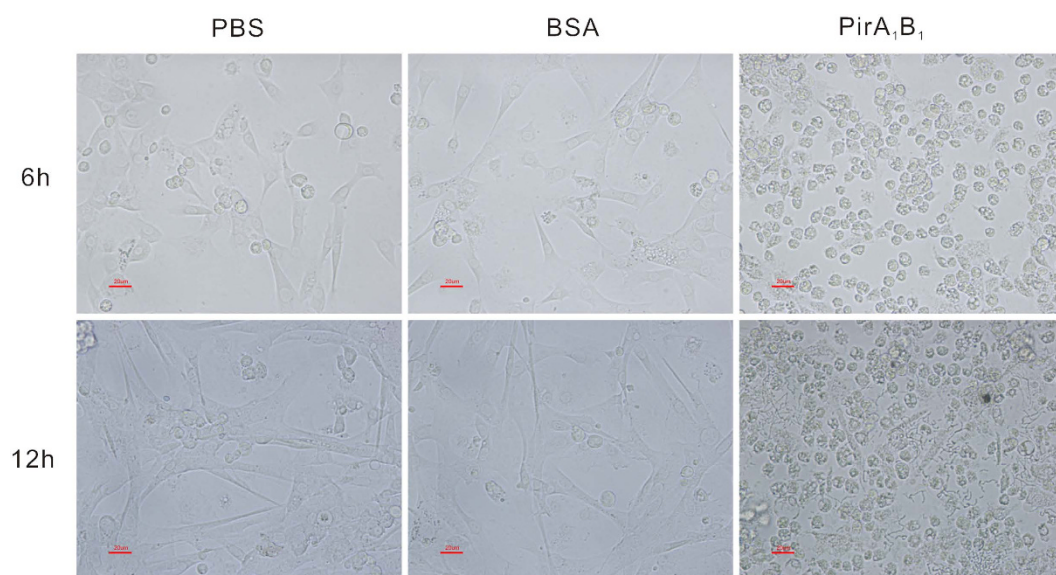
To investigate the mechanisms responsible for the inhibition of phagocytosis by haemocytes after PirA<sub>1</sub>B<sub>1</sub> treatment, we performed experiments to determine whether the toxin altered the haemocyte cytoskeleton. Staining the haemocytes with FITC-labelled phalloidin revealed major differences in the cytoskeletal architecture of cells following injection of the toxin protein compared with those in BSA or PBS control larvae. These assays indicated that PirA<sub>1</sub>B<sub>1</sub> adversely affected the cytoskeleton of *G. mellonella* haemocytes, causing it to become diffuse and disorganized, and this result also correlated with the reduction in formation of pseudopods by the cells. Therefore, we concluded that the toxin PirA<sub>1</sub>B<sub>1</sub> disrupted the cytoskeletons of haemocytes, which led to abnormal haemocyte spreading behaviour and pseudopod formation. Because haemocyte spreading behaviour and the ability to extend pseudopods are essential for phagocytosis<sup>27</sup>, with their disruption, phagocytosis was suppressed. Thus, PirA<sub>1</sub>B<sub>1</sub> inhibited phagocytosis by affecting the cytoskeleton of the immunocytes. Numerous bacterial toxins reorganize the actin cytoskeleton of target cells. Moreover, *Photobacterium* W14 bacterial supernatants cause marked changes in the actin cytoskeleton of specific haemocyte types<sup>22</sup>. Additionally, the injection of recombinant *E. coli* expressing *Photobacterium* virulence cassettes (PVC) containing cosmids from *Photobacterium* destroys insect haemocytes, which undergo dramatic actin cytoskeleton condensation<sup>4</sup>.

In conclusion, based on our preliminary results, following the injection of PirA<sub>1</sub>B<sub>1</sub> toxin into the haemocoel of *G. mellonella* larvae, the host haemocyte number decreased and the ability of haemocyte phagocytosis was inhibited by the disruption of the cytoskeleton. Therefore, the toxin PirA<sub>1</sub>B<sub>1</sub> disarmed the insect cellular



**Figure 5. Light and corresponding fluorescence micrographs of monolayers of haemocytes from *Galleria mellonella* larvae showing localization of cytoskeletal actin stained with FITC-labelled phalloidin.**

Plasmotocytes (PL) and granular cells (GR) from the larvae injected with PBS or BSA showed pseudopods and an organized cytoskeleton; however, no pseudopods and spreading behaviour were observed in either plasmotocytes or granular cells from the larvae injected with PirA<sub>1</sub>B<sub>1</sub>.



**Figure 6. Morphology of *Galleria mellonella* larvae haemocytes treated with PBS, BSA or PirA<sub>1</sub>B<sub>1</sub> for 6 or 12 h.**

immune system. As a ubiquitous protein in entomopathogenic nematode symbiotic bacteria, uncovering the cellular immune responses of a host insect to the injection of PirA<sub>1</sub>B<sub>1</sub> helped to understand the interaction between nematode-symbiotic bacteria and their hosts.

## References

1. Forst, S., Dowds, B., Boemare, N. & Stackebrandt, E. Xenorhabdus and Photorhabdus spp.: bugs that kill bugs. *Annu Rev Microbiol.* **51**, 47–72 (1997).
2. ffrench-Constant, R. *et al.* Photorhabdus: towards a functional genomic analysis of a symbiont and pathogen. *FEMSMicrobiol Rev.* **26**, 433–456 (2003).
3. Blackburn, M. B., Domek, J. M., Gelman, D. B. & Hu, J. S. The broadly insecticidal Photorhabdus luminescens toxin complex a (Tca): activity against the Colorado potato beetle, *Leptinotarsa decemlineata*, and sweet potato whitefly, *Bemisia tabaci*. *J Insect Sci.* **5**, 32 (2005).
4. Yang, G., Dowling, A. J., Gerike, U., ffrench-Constant, R. H. & Waterfield, N. R. Photorhabdus virulence cassettes confer injectable insecticidal activity against the wax moth. *J Bacteriol.* **188**, 2254–2261 (2006).

5. Dowling, A. J. *et al.* The insecticidal toxin Makes caterpillars floppy (Mcf) promotes apoptosis in mammalian cells. *Cell Microbiol.* **6**, 345–353 (2004).
6. Waterfield, N., Kamita, S. G., Hammock, B. D. & french-Constant, R. The Photorhabdus Pir toxins are similar to a developmentally regulated insect protein but show no juvenile hormone esterase activity. *FemsMicrobiol Lett.* **245**, 47–52 (2005).
7. Lu, A. *et al.* Insect prophenoloxidase: the view beyond immunity. *Front Physiol.* **5**, 252 (2014).
8. Charroux, B. & Royet, J. Drosophila immune response: From systemic antimicrobial peptide production in fat body cells to local defense in the intestinal tract. *Fly (Austin)* **4**, 40–47 (2010).
9. Giglio, A. *et al.* Immune challenges trigger cellular and humoral responses in adults of *Pterostichus melas italicus* (Coleoptera, Carabidae). *Arthropod Struct Dev.* **44**, 209–217 (2015).
10. Williams, M. J. Drosophila hemopoiesis and cellular immunity. *J Immunol.* (2007).
11. Hillyer, J. F., Schmidt, S. L. & Christensen, B. M. Hemocyte-mediated phagocytosis and melanization in the mosquito *Armigeres subalbatus* following immune challenge by bacteria. *Cell Tissue Res.* **313**, 117–127 (2003).
12. Khandelwal, P. *et al.* Insecticidal pilin subunit from the insect pathogen *Xenorhabdus nematophila*. *J Bacteriol.* **186**, 6465–6476 (2004).
13. Ribeiro, C., Vignes, M. & Brehelin, M. *Xenorhabdus nematophila* (enterobacteriaceae) secretes a cation-selective calcium-independent porin which causes vacuolation of the rough endoplasmic reticulum and cell lysis. *J BiolChem.* **278**, 3030–3039 (2003).
14. Vigneux, F. *et al.* The xaxAB genes encoding a new apoptotic toxin from the insect pathogen *Xenorhabdus nematophila* are present in plant and human pathogens. *J BiolChem.* **282**, 9571–9580 (2007).
15. Lang, A. E. *et al.* Photorhabdus luminescens toxins ADP-ribosylate actin and RhoA to force actin clustering. *Science* **327**, 1139–1142 (2010).
16. Duchaud, E. *et al.* The genome sequence of the entomopathogenic bacterium *Photorhabdus luminescens*. *Nat Biotechnol.* **21**, 1307–1313 (2003).
17. Ahantarig, A., Chantawat, N., Waterfield, N. R., french-Constant, R. & Kittayapong, P. PirAB toxin from *Photorhabdus asymbiotica* as a larvicide against dengue vectors. *Appl Environ Microbiol.* **75**, 4627–4629 (2009).
18. Wu, G., Yi, Y., Sun, J., Li, M. & Qiu, L. No evidence for priming response in *Galleria mellonella* larvae exposed to toxin protein PirA2B2 from *Photorhabdus luminescens* TT01: An association with the inhibition of the host cellular immunity. *Vaccine* **33**, 6307–6313 (2015).
19. Wu, G. Q. & Yi, Y. H. Effects of dietary heavy metals on the immune and antioxidant systems of *Galleria mellonella* larvae. *Comp. Biochem. Phys. C* **167** (2015).
20. Sun, J., Liu, C. & Qiu, L. [Cloning, expression and insecticidal activity of the pirA2B2 gene from *Photorhabdus luminescens* TT01]. *Wei Sheng Wu Xue Bao.* **52**, 532–537 (2012).
21. Eleftherianos, I. *et al.* A single locus from the entomopathogenic bacterium *Photorhabdus luminescens* inhibits activated *Manduca sexta* phenoloxidase. *FemsMicrobiol Lett.* **293**, 170–176 (2009).
22. Au, C., Dean, P., Reynolds, S. E. & french-Constant, R. H. Effect of the insect pathogenic bacterium *Photorhabdus* on insect phagocytes. *Cell Microbiol.* **6**, 89–95 (2004).
23. Li, Y. *et al.* *Photorhabdus luminescens* PirAB-fusion protein exhibits both cytotoxicity and insecticidal activity. *FemsMicrobiol Lett.* **356**, 23–31 (2014).
24. Pech, L. L. & Strand, M. R. Plasmatocytes from the moth *Pseudoplusia includens* induce apoptosis of granular cells. *J Insect Physiol.* **46**, 1565–1573 (2000).
25. Bergin, D., Brennan, M. & Kavanagh, K. Fluctuations in haemocyte density and microbial load may be used as indicators of fungal pathogenicity in larvae of *Galleria mellonella*. *Microbes Infect.* **5**, 1389–1395 (2003).
26. Strand, M. R. The insect cellular immune response. *Insect Sci.* **15**, 1–14 (2008).
27. Goldenberg, P. Z., Huebner, E. & Greenberg, A. H. Activation of lobster hemocytes for phagocytosis. *J InvertebrPathol.* **43**, 77–88 (1984).

## Acknowledgements

The National Natural Science Foundation of Guangdong Province, China (2016A030310301), and the Medical Scientific Research Foundation of Guangdong Province, China (A2016607), supported this research.

## Author Contributions

G.W. performed the experiments and wrote the manuscript, and Y.Y. analysed the data and edited the manuscript. All authors have read and approved the manuscript for publication.

## Additional Information

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Wu, G. and Yi, Y. Haemocoel injection of PirA<sub>1</sub>B<sub>1</sub> to *Galleria mellonella* larvae leads to disruption of the haemocyte immune functions. *Sci. Rep.* **6**, 34996; doi: 10.1038/srep34996 (2016).



This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>

© The Author(s) 2016