RESEARCH PAPER

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Pre-exposure of *Galleria mellonella* larvae to different doses of *Aspergillus fumigatus* conidia causes differential activation of cellular and humoral immune responses

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Larvae of *Galleria mellonella* are useful models for studying the virulence of microbial pathogens or for evaluating the potency of antimicrobial agents. In this work we demonstrated that prior exposure of larvae to non-lethal doses of *Aspergillus fumigatus* conidia increases the resistance of larvae to a lethal dose (1×10^7 20 μl^{-1}) 24 h later. Exposure of larvae to a conidia concentration of 1×10^4 20 μl^{-1} leads to an increase in hemocyte density but an inoculum of 1×10^5 conidia leads to enhanced expression of antimicrobial peptides, increased binding of proteins (e.g., arylophorin, prophenoloxidase, apolipophorin) to conidia and elevated hemocyte density. These results suggest that a low dose of conidia (1×10^4) predominantly activates the cellular immune response but that a higher dose (1×10^5) that is still not lethal activates a humoral immune response to the greatest extent. While insects have an immune system analogous to the innate immune response of mammals these results suggest that it is capable of assessing the extent of the microbial challenge and mounting a 'proportionate' immune response which may have important survival advantages.

Introduction

Insects are an extremely successful group of animals and inhabit all ecological niches on the Earth with the exception of the oceans.[1] Part of the success of insects in colonizing such a wide variety of habitats is their rapid rates of reproduction, which allow them quickly replace members that die due to disease, starvation or predators. Insects also possess cellular and humoral immune responses that allow them respond rapidly and effectively to infection and minimize mortality. The cellular immune responses are mediated by hemocytes, which engulf and kill pathogens[2] while the humoral immune response is mediated by hemocytes and internal organs (e.g., fat body, digestive tract) and involves the production of a wide range of antimicrobial peptides that can arrest and kill pathogens that evade the cellular immune response.[3]

The insect immune response demonstrates a number of strong structural and functional similarities to the innate immune response of mammals[4] and, in particular, insect hemocytes and mammalian neutrophils have been shown to phagocytose and kill pathogens in a similar manner.[5,6] As a result of these similarities insects (e.g., *Galleria mellonella*, *Drosophila melanogaster*) are now widely employed as models for studying the virulence of human microbial pathogens or for the in vivo screening of the efficacy of anti-microbial drugs.[4] Other similarities between the two immune responses include the Toll pathway which is similar to the Toll-like receptor (TLR) pathway of mammals[7] and the immunodeficiency (IMD) pathway, which is similar in function to the TNFα pathway of mammals.[8] Insects do not have an immune system analogous to the adaptive immune response of mammals in terms of generation of antibodies but do have the capacity to mount an immune response in anticipation of a subsequent infection that shows some elements of similarity to the function of the adaptive immune response of mammals. Prior exposure of larvae of *Galleria mellonella* to non-lethal doses of yeast cells provokes an immune response, which increases their resistance to a subsequent lethal inoculum.[9] Administration of microbial cell wall components[10] or stress (physical or thermal)[11,12] induce a similar protective response. The elevated immune response subsequent to the initial challenge is mediated by an increase in the hemocyte density and the elevated expression of antimicrobial peptides. This usually reaches a peak 24 h after the initial priming event and thereafter declines. Previous work has demonstrated that the magnitude of the immune response is proportional to the size of the microbial challenge thus indicating that the insect immune response can distinguish between a low level, potentially non-lethal infection and one that threatens the survival of the individual.[10]

Due to the strong structural and functional similarities between the insect immune response and the innate immune

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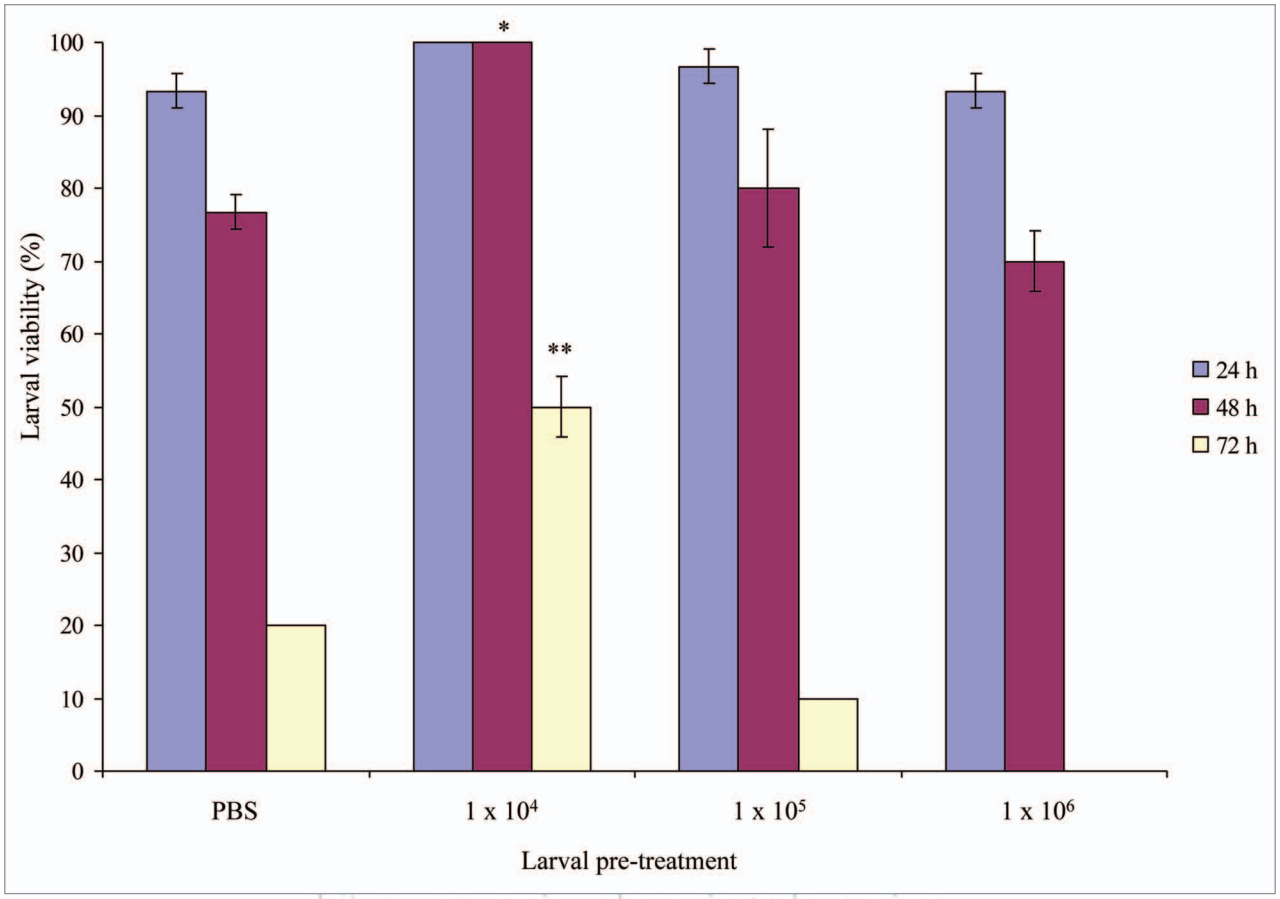


Figure 1. Determination of immuno-protection after 24 h priming with *A. fumigatus* conidia. Larvae were inoculated with PBS, 1 x 10^4, 1 x 10^5 or 1 x 10^6 *A. fumigatus* ATCC 26933 conidia. Following 24 h incubation at 30°C larvae were injected with a lethal (1 x 10^7) dose of conidia and viability was recorded at 24, 48 and 72 h. The data shown are the mean of three independent experiments with standard error included. *$p < 0.05$ relative to PBS injection; **$p < 0.012$ relative to PBS injection.

response of mammals[4] insects have been used as alternatives to mammals for the screening of microbial pathogens[13-15] or for evaluating the potency of antimicrobial agents.[16,17] Understanding the mechanisms employed by insects to withstand infection is critical to the successful use of them as models.

The aim of the work presented here was to analyze the response of the immune system of *G. mellonella* larvae to challenge with spores of *Aspergillus fumigatus* and to establish whether the immune response could distinguish between different levels of pathogen in terms of whether the cellular and/or humoral immune responses were activated. It was hoped to establish how the cellular and humoral responses are affected by different concentrations of the same pathogen (*A. fumigatus* conidia) and to determine whether a differential activation of one response, compared with the other, occurred.

Results

Prior exposure to low doses of *A. fumigatus* conidia decreases susceptibility of larvae to a lethal dose. Larvae were injected with 1 x 10^4, 1 x 10^5 or 1 x 10^6 *A. fumigatus* conidia as described, incubated at 30°C and subsequently injected with a lethal dose of conidia (1 x 10^7 20 μl^{-1}) at 24 h (**Fig. 1**). Larvae that received a dose of 1 x 10^4 conidia 24 h prior to the lethal dose displayed 100% viability 48 h after the administration of the lethal dose compared with 78.3 ± 3.1% viability in the control ($p = 0.05$) and 45 ± 4.3% viability at 72 h compared with 13.3 ± 3.3% in the control ($p = 0.012$) (**Fig. 1**). Larvae that received inocula of 1 x 10^5 or 1 x 10^6 conidia 24 h prior to the administration of the lethal dose (1 x 10^7) displayed a small, or no, significant difference to the survival of the control larvae at 48 h.

These results demonstrate that pre-exposure of *G. mellonella* larvae to non-lethal doses (1 x 10^4 or 1 x 10^5) of *A. fumigatus* conidia leads to increased survival when a lethal dose (1 x 10^7) is administered 24 h later.

Effect of low doses of *A. fumigatus* conidia on hemocyte density. Fluctuations in the density of circulating hemocytes have previously been demonstrated following administration of low doses of pathogen or exposure of larvae to stress.[9-12] *G. mellonella* larvae were injected with sub-lethal doses of *A. fumigatus* conidia or sterile PBS and incubated for 24 h at 30°C. Larvae were bled and the hemocyte density was ascertained as described.

There was a significant increase in the density of circulating hemocytes at 24 h in those larvae that received the initial doses of

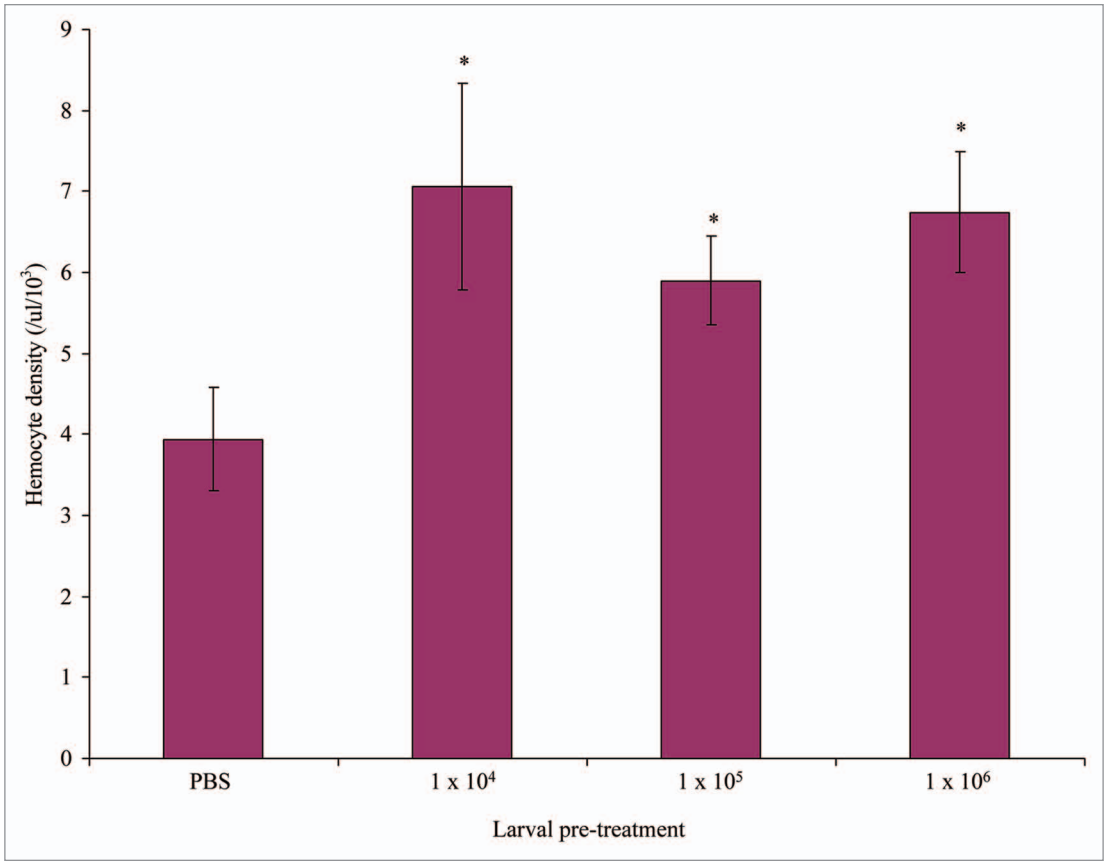
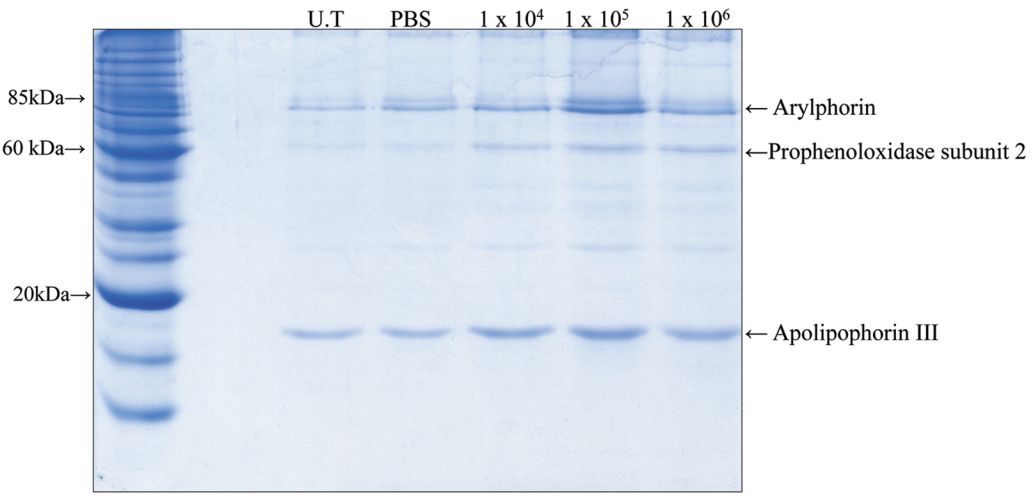


Figure 2. Larval hemocyte densities in response to *A. fumigatus* conidia infection. Larvae were injected with PBS or non-lethal doses of *A. fumigatus* ATCC 26933 and incubated at 30°C. Larvae were subsequently bled 24 h post-infection and hemocyte densities were ascertained by hemocytometry. The data shown are the mean of three independent experiments with standard error included. *$p \leq 0.05$ relative to PBS injection; **$p \leq 0.028$ relative to PBS injection.

conidia (**Fig. 2**). The hemocyte density in the control larvae was recorded as $3.94 \pm 0.64 \times 10^3$ cells μl^{-1}; however the density of hemocytes was $7.06 \pm 1.27 \times 10^3$ cells μl^{-1}, $5.9 \pm 0.55 \times 10^3$ cells μl^{-1} and $6.74 \pm 0.74 \times 10^3$ cells μl^{-1} in those larvae that were inoculated with 1×10^4, 1×10^5 or 1×10^6 conidia, respectively ($p \leq 0.028$). These results demonstrate that exposure to low doses of conidia leads to an elevation in the hemocyte density.

Assessment of hemolymph protein binding to conidia. The conidial binding abilities of proteins from sera of larvae that were previously exposed to low inocula of *A. fumigatus* conidia were assessed as described. Larvae (10) were inoculated with conidia (1×10^4, 1×10^5 or 1×10^6), or PBS and were incubated at 30°C for 24 h. The binding of hemolymph proteins to the surface of conidia added to hemolymph from larvae previously exposed to conidia was analyzed by SDS-PAGE. Analysis of the resulting gel showed variations in the level of sera proteins that had bound to the conidial surfaces. Protein A (**Fig. 3**) was identified as arylphorin (Mass: 83.7 kDa, gi:159078, score: 271, peptides matched: 6, coverage: 10%). Arylphorin has been characterized as an amino acid storage and transport protein[18] however the first 20 amino acids of this protein have been suggested to function in the insect antimicrobial response and has been shown to have sequence homology with the N-terminus of gallysin-1. Gallysin-1 in the presence of lysozyme has been shown to elicit cytotoxicity in the presence of lysozyme[19] therefore it is possible that such a binding event may enhance antimicrobial potential of hemolymph. The binding of arylphorin was significantly greater in sera from larvae that received 1×10^4 or 1×10^5 conidia initially relative to that evident in sera from control larvae ($p < 0.008$) (**Fig. 4**). Protein B was identified as prophenoloxidase subunit 2 (gi:34556399, mass: 59.5 kDa, score: 167, peptides matched: 3, coverage: 4%) (**Fig. 3**). The activation of phenoloxidase cascade has been shown to be dependent upon the serine protease cascade which results in the activation of the prophenoloxidase activating enzyme which catalyzes the formation of cytotoxic quinones which can be toxic to host tissue if not regulated to the site of infection.[20] The sera from larvae that received an inoculation of 1×10^5 conidia showed greatest binding of PPO to conidia relative to sera from control larvae however all pre-treatments resulted in higher levels of binding of this protein ($p < 0.001$) (**Fig. 4**). Protein C was identified as showing homology to apolipophorin III (gi:5915688, mass: 20.499 kDa, score: 704, peptides matched: 15, coverage: 71%) (**Fig. 3**). Whitten et al. identified a role for apoLp III as a pathogen recognition receptor of β1,3-glucan, a key component of the fungal cell wall. Densitometric analysis of this band revealed that hemolymph from larvae that received



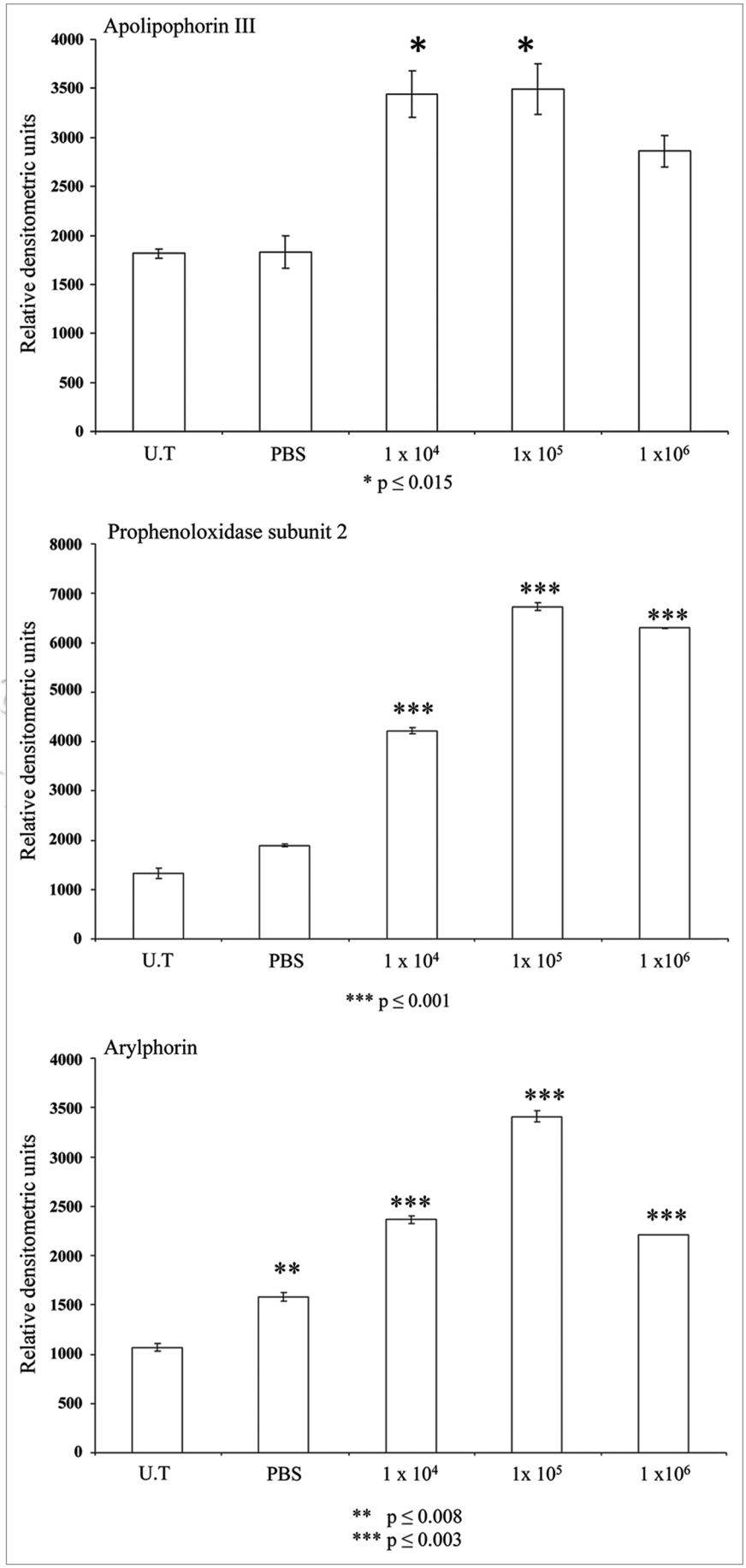
Protein I.D	gi.	Score	Peptides matched	Coverage
Arylphorin	159078	271	6	10%
Prophenoloxidase subunit 2	34556399	167	3	4%
Apolipophorin III	5915688	704	15	71%

Figure 3. Hemolymph protein binding profile from larvae primed with *A. fumigatus* conidia. SDS-PAGE and LC/MS identification of hemolymph proteins demonstrating differential binding to *A. fumigatus* conidia from larvae previously exposed to non-lethal doses of conidia. Larvae were inoculated with non-lethal inocula of conidia, incubated at 30°C for 24 h and the hemolymph protein binding assay was performed as described. Bound proteins were clarified using 12.5% SDS-PAGE and bands of interest were excised prior to trypsin in-gel digestion and identification by LC/MS.

1 x 10^4, 1 x 10^5 or 1 x 10^6 *A. fumigatus* conidia initially displayed a greater binding ability toward conidia than hemolymph from untreated or PBS inoculated larvae ($p \leq 0.028$, 0.048 and 0.015 respectively) (**Fig. 4**).

Analysis of alterations in protein expression in hemolymph of larvae that received non-lethal doses of *A. fumigatus* conidia. Larvae were inoculated with non-lethal doses of *A. fumigatus* conidia (1 x 10^4 or 1 x 10^5 20 μl^{-1}) and 24 h later hemolymph was extracted, hemocytes were removed and the serum was resolved by 2D SDS-PAGE. The resulting gels were stained and analyzed for the differential expression of peptides. Proteins showing alteration in expression were excised and identified by LC/MS as described. The results (**Figs. 5 and 6**) indicate that in larvae that received an inoculum of 1 x 10^5 conidia that the expression of hexamerin (spot 1, gi:347090, mass: 81.8 kDa, Score: 183, peptides matched: 5, coverage 8%) and hexamerin receptor (spot 2, gi:282722536, mass: 84 kDa, score: 96, peptides matched: 2, coverage: 2%) was increased by 4.38- and 7.3-fold, respectively. In contrast those larvae that received an inoculum of 1 x 10^4 showed reduced expression of these peptides. Hexamerin biosynthesis takes place in the fat body and is subsequently released into the hemolymph where it functions in macromolecule storage.[22,23] It has been demonstrated that hexamerins are endocytosed by fat body cells and act as storage proteins during larval feeding prior to metamorphosis.[24] The hexamerin receptor is classified as a member of the low density lipoprotein superfamily[22] and functions as a binding site for hexamerin in fat body cells.[25] The expression of the 32 kDa ferritin subunit (spot 3, gi:17901818, mass: 26.5 kDa score: 270, peptides matched: 7, coverage: 25%) was increased approximately 2.08- and 1.96-fold in the larvae that received 10^4 or 10^5 conidia (**Figs. 5 and 6**). Ferritin contributes to cellular iron homeostasis by sequestering free iron and has been demonstrated to show increased expression following bacterial expression in *Drosophila melanogaster*.[26] The antimicrobial protein apolipophorin (spot 4, gi:50404098, mass: 167 kDa, score 132, peptides matched: 3, coverage: 28%) showed a 1.7-fold increase in larvae that received an inoculum of 1 x 10^4 conidia. Apolipophorin plays a role in the insect immune response and has been shown to display functionality in the response to bacterial challenge where coagulation of apolipophorin takes place to form a globular structure resulting from oligomeric interactions with lipopolysaccharide thus neutralizing LPS from the surrounding environment. Gallerin (spot 5, gi:1146408, mass: 22.4 kDa, score: 169, peptides matched: 2, coverage: 12%) showed a small increase in expression in both groups of larvae. Gallerin is annotated as a member of the calycin family of immunologically relevant proteins that are implicated in the acute phase response to infection and regulation of immune processes.[27] The expression of juvenile binding hormone (spot 6, gi:158515746, mass: 27.2 kDa, score: 165, peptides matched: 8, coverage: 17%) remained relatively constant in all the treatments. JHBP has been previously demonstrated as a loading control in proteomic

Figure 4. Densitometric analysis of hemolymph proteins, from larvae previously exposed to non-lethal doses of conidia, showing differential binding to conidia. The binding of three identified protein of interest from conidia-challenged larvae was analyzed by densitometry. Arylphorin, prophenoloxidase subunit 2 and apolipophorin III from the hemolymph of *G. mellonella* larvae injected with PBS or non-lethal inocula of conidia demonstrated varying levels of binding to *A. fumigatus* conidia under standardized conditions. The effect of immune priming was compared with the binding exhibited by untreated larvae (U.T.) which had been incubated at 30°C only. *p ≤ 0.028, **p ≤ 0.008, ***p ≤ 0.003



studies of *G. mellonella* hemolymph.[10,17] The results presented here indicate that exposure of *G. mellonella* larvae to low doses of *A. fumigatus* conidia (10^4 or 10^5) leads to an increase in the expression of a range of antimicrobial peptides and immune proteins which is indicative of an elevated immune response that could counteract a subsequent infection.

Discussion

The results presented here demonstrate that inoculation of *G. mellonella* larvae with non-lethal doses of *A. fumigatus* conidia conferred a significant protective response when larvae were subsequently inoculated with a lethal dose of conidia. Larvae injected with conidia also demonstrated a significant increase in the density of circulating hemocytes. Hemocytes mediate the cellular immune response of insects and play a key role in phagocytosing and killing pathogens or binding to larger structures and immobilizing them within the hemolymph. In the work presented here it was demonstrated that those larvae that received non-lethal doses of *A. fumigatus* conidia displayed increased hemocyte densities. Previous work demonstrated that the hemocyte density of larvae is altered by the presence of pathogenic stimuli or by components of the fungal cell wall.[9,10] Furthermore this alteration in hemocyte density was correlated with increased resistance to subsequent infection in larvae primed with 1 x 10^4 conidia.

Prior exposure of larvae to non-lethal doses of conidia increased the binding of selected hemolymph proteins to conidia. In particular the binding of arylphorin, PPO and apolipophorin III were significantly increased when conidia were exposed to sera from conidia-challenged larvae. This result indicates that prior exposure of larvae to conidia increases

the expression or binding activity of key immune related proteins in the sera that can bind to conidia. The increased binding of these proteins in sera of immunologically primed larvae indicates that the immune system is prepared for subsequent infection by a pathogen.

Proteomic analysis of protein expression in larvae that received non-lethal doses of conidia revealed increased expression of selected proteins. Spots identified as hexamerin, hexamerin receptor, 32 kDa ferritin subunit and gallerin showed increased expression 24 h post-infection in larvae inoculated with 1 x 10^5 conidia. Hexamerin is a storage protein which is endocytosed by fat body cells.[24] The 2.08- and 1.96-fold increase in expression of the 32 kDa ferritin subunit in larvae administered an inoculum of 1 x 10^4 and 1 x 10^5 conidia is interesting as it may indicate a means by which the larvae can limit the growth of the fungus within the hemocoel. This balancing of iron availability has been demonstrated by others to play a key role in the immune system/fungal iron sequesterization balance.[26,28] It is possible that enhanced synthesis of ferritin acts as a means of sequestering iron from Aspergillus siderophores and may play a role in protecting the host against the higher fungal inoculum by depleting the fungus of its iron requirement. The increased expression of apolipophorin (apo 1) is interesting in the light of work of Ma et al. where the oligomeric interactions with LPS initiate particle neutralization. The interaction of apo 1 and *A. fumigatus* conidia has yet to be fully elucidated however the finding that prior exposure of larvae to conidia leads to the increased expression of such an immunologically important protein is indicative of an increase in activity of the humoral immune response. Larvae inoculated with 1 x 10^4 and 1 x 10^5 *A. fumigatus* conidia displayed a 1.21- and 1.33-fold increase, respectively, in gallerin expression relative to untreated larvae. Gallerin is annotated as a member of the α-1-acid glycoprotein 1 related proteins which function in the acute phase response to infection and regulate immune system processes. This process is mediated in neutrophils by cytosolic Ca^{2+} through the binding of immunoglobulin-like lectins with the effect of inhibiting superoxide production.[27,30] The presence of a functionally similar protein in *G. mellonella* larvae is interesting as the precise role of gallerin in combating fungal infections is not fully characterized.

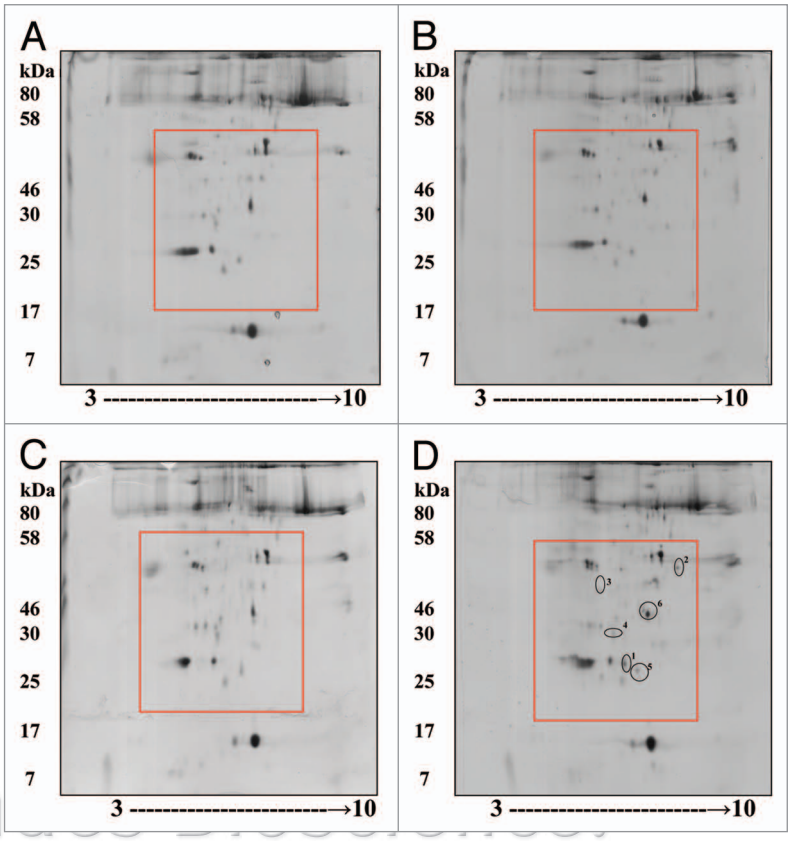


Figure 5. Larval hemolymph proteomic profile following challenge with non-lethal *A. fumigatus* conidia inocula. Untreated larvae (A) or larvae injected with PBS (B), 1 x 10^4 (C) or 1 x 10^5 (D) *A. fumigatus* ATCC26933 conidia were incubated at 30°C for 24 h. Protein was extracted and the proteomic profile was examined using 2-D electrophoresis as described. Alterations to relative protein expression following immune priming were compared.

The results presented here demonstrate that prior exposure of *G. mellonella* larvae to non-lethal doses of *A. fumigatus* conidia leads to enhanced protection against a lethal dose of conidia administered 24 h later. Interestingly, the maximum survival was seen in larvae given an initial inoculum of 1 x 10^4 conidia, which was also the dose which induced the highest hemocyte density. There was a similar level of hemocyte density in larvae injected with 1 x 10^6 conidia. The observed reduction in viability in this treatment relative to those injected with 1 x 10^4 conidia may be explained by the accumulated stress induced by the higher fungal burden and the possible secretion of extracellular proteases during the initial pathogen exposure therefore leaving the host more susceptible to subsequent infection. The greatest increase in protein binding to conidia was evident in the sera from larvae challenged with 1 x 10^5 conidia and these larvae also demonstrated the greatest increase in protein expression. This result suggests that an inoculum of 1 x 10^4 conidia leads to activation of the cellular immune response but that an inoculum of 1 x 10^5 leads to the increased expression and binding of immune related proteins, which are components of the humoral immune response, as well as increased hemocyte numbers. It is possible that a low level infection can be eradicated by increasing the hemocyte density but that larger inocula require elevated hemocyte densities as well as increased expression of proteins. Prior exposure of insects to low levels of pathogens,[9] microbial cell wall components[10] or stress[12] leads to increased protection against subsequent lethal infection. Elevated immunity following such challenge does offer protection to the insect and would have an obvious survival advantage but does have a cost in terms of utilization of resources. Upregulation of immune responses in bumble bees following exposure to low levels of pathogen, but in the absence of compensatory feeding can be fatal,[31] thus indicating that immune protection does have a cost for the insect. We have

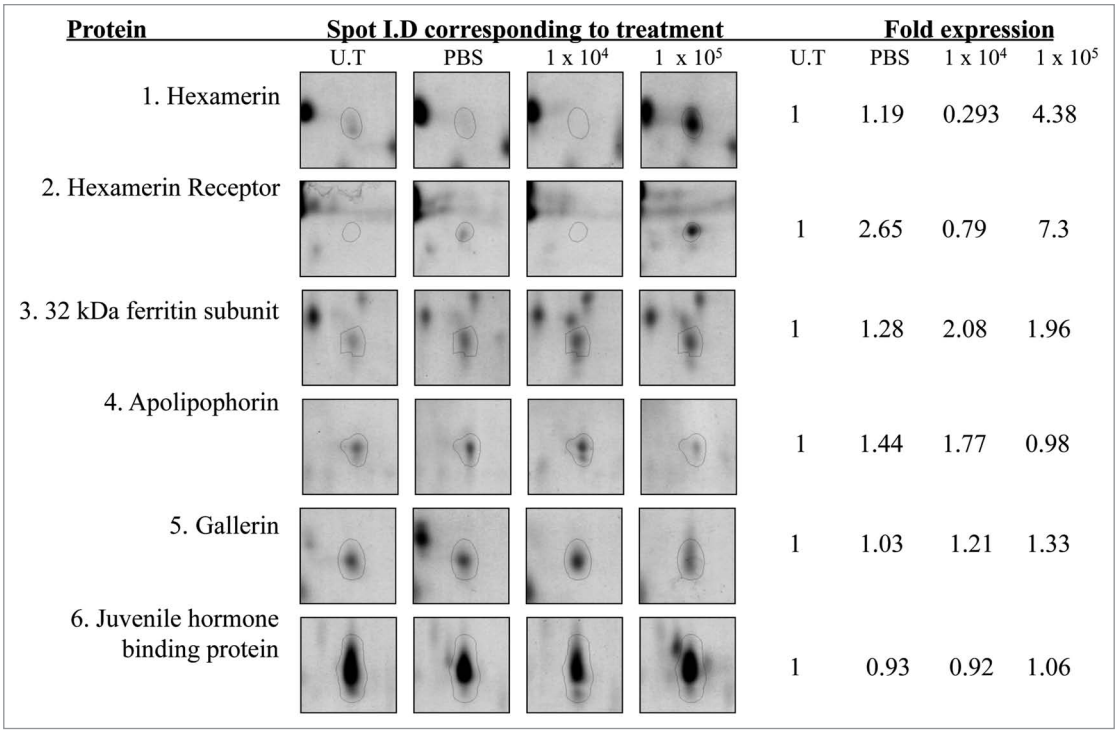


Figure 6. Analysis of differential protein expression in larvae challenged with *A. fumigatus* conidia. Gels were analyzed using Progenesis™ "Same spot" software. Spots which showed differential expression relative to untreated larvae ($p \leq 0.05$) were excised and identified using LC/MS analysis. Juvenile hormone binding protein (spot 6) did not change significantly in expression ($p = 0.1$) and is considered as a loading control.

previously demonstrated a dose-dependent immune response in *G. mellonella* larvae to β-glucan inoculation, which was postulated to allow the insect mount an immune response proportionate to the size of the inoculum as a means of reducing the immunological cost to the larva.[10] In the results presented here we have demonstrated a predominately cellular immune response to low levels of conidia (1 x 10^4) but the induction of cellular and humoral immune responses evidenced by increased binding of immunologically relevant proteins when a larger inoculum (1 x 10^5) is employed. These results indicate that the insect immune system is capable of sensing the extent of microbial challenge and mounting a 'proportionate' response in order to ensure survival but minimize the use of resources.

Methods

Chemicals and reagents. All chemicals were of high purity and were purchased from Sigma-Aldrich Chemical Company Ltd., unless otherwise stated.

Insect larvae injection and fungal cultures. Sixth instar larvae of the greater wax-moth *G. mellonella* (Livefoods Direct Ltd.), were stored in the dark at 15°C to prevent pupation. Larvae weighing 0.2–0.4 g were selected at random and all experiments were performed independently on three separate occasions. Larvae were inoculated with conidia through the last left proleg into the hemocoel with a Myjector U-100 insulin syringe, (Terumo Europe N.V.). Larvae were incubated at 30°C for all studies.

Conidia culture conditions and cellular enumeration. *Aspergillus fumigatus* ATCC 26933 was grown on Malt Extract Agar plates at 37°C for 7 d and conidia were harvested with PBS-Tween-80. Following centrifugation at 500x g for 5 min conidia were washed in sterile PBS to remove the excess Tween from the conidial surface.

Determination of hemocyte density. Larvae were inoculated with different doses of *A. fumigatus* conidia (1 x 10^4, 1 x 10^5 and 1 x 10^6 20 μl^{-1}) and incubated for 24 or 48 h. Hemocyte density was determined by bleeding three larvae into a pre-chilled micro-centrifuge tube to prevent melanization. The collected hemolymph was diluted in PBS supplemented with 0.37% (v/v) mercaptoethanol and cell density was assessed with a hemocytometer and expressed as hemocytes per microliter of hemolymph. All determinations were performed on three separate occasions.

Determination of protein binding to *A. fumigatus* conidia. Previously immune challenged larvae (10) were bled through the anterior region into a pre-chilled micro-centrifuge tube and centrifuged immediately at 800x g for 2 min at 4°C. Cell free hemolymph (100 μl) was diluted in ice-cold insect physiological saline[6] to give a concentration of 7.5 mg protein ml^{-1}, as confirmed by Bradford assay, and added to *A. fumigatus* conidia. The 5 x 10^8 conidia/hemolymph suspension was mixed gently for 40 min at 4°C and immediately centrifuged at 500x g for 5 min to pellet conidia. Conidia were washed three times with 1 ml sterile IPS to remove any non-specifically bound proteins and a 10 μl aliquot was withdrawn from all samples and enumerated by hemocytometry to ensure that no loss of conidia had occurred.

Protein was extracted from the conidial surface by the addition of 5X solubilization buffer (Glycerol; 52%, 10% (v/v) SDS; 10.5%, 1.5 M TRIS-HCl; 6.5%, Bromophenol Blue (0.5% w/v); 1.3%, 2-mercaptoethanol; 2.63%) and brief sonication in a water bath followed by boiling at 95°C for 5 min. The samples were centrifuged at 1,000x g for 5 min at 4°C and the protein supernatant was transferred to a pre-chilled micro-centrifuge tube and subjected to 12.5% 1-D SDS-PAGE and Colloidal Coomassie staining. In-gel digestion was performed on visualized bands of interest by the method described by Shevchenko et al. prior to LC/MS analysis on an Agilent 6340 Ion Trap. Peptide spots were identified using MASCOT search engine (www.matrixscience.com). Relative band intensity was ascertained using Image J software. Mascot scores greater than 65 are significant at $p < 0.05$.

Effect of conidial priming on the hemolymph protein profile. Larvae (10) were injected with PBS or conidia (1×10^4 or 1×10^5) and were incubated for 24 h at 30°C. Hemolymph was collected from larvae and the protein (300 μg) was separated by 2-D electrophoresis as described previously in reference 9. Resulting gels were visualized by colloidal Coomassie staining. Triplicate gel images were analyzed by Progenesis SameSpots™ software (Nonlinear Dynamics) in order to assess the fold change between the untreated control and the PBS injected and conidia primed larvae. Protein spots that demonstrated differential expression following priming were excised, washed and digested with trypsin as described in reference 32. Following peptide extraction identification was performed using an Agilent 6340 Ion Trap LC-MS calibrated using BSA. The resulting mass lists were BLAST searched using the MASCOT MS/MS ion search program available at www.matrixscience.com. Annotated function and protein information was ascertained through the UNIPROT database (www.uniprot.org).

Statistics. All experiments were performed on three independent occasions. Larval viability was analyzed using the Chi-square test. Densitometric and hemocyte density data were analyzed using a student t-test. Analysis of significant changes in protein expression was performed using ANOVA.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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