The *Aspergillus fumigatus* toxin fumagillin suppresses the immune response of *Galleria mellonella* larvae by inhibiting the action of haemocytes

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Larvae of *Galleria mellonella* are widely used to evaluate microbial virulence and to assess the *in vivo* efficacy of antimicrobial agents. The aim of this work was to examine the ability of an *Aspergillus fumigatus* toxin, fumagillin, to suppress the immune response of larvae. Administration of fumagillin to larvae increased their susceptibility to subsequent infection with *A. fumigatus* conidia ($P=0.0052$). It was demonstrated that a dose of 2 μg fumagillin ml^{-1} reduced the ability of insect immune cells (haemocytes) to kill opsonized cells of *Candida albicans* ($P=0.039$) and to phagocytose *A. fumigatus* conidia ($P=0.016$). Fumagillin reduced the oxygen uptake of haemocytes and decreased the translocation of a p47 protein which is homologous to p47^{phox}, a protein essential for the formation of a functional NADPH oxidase complex required for superoxide production. In addition, toxin-treated haemocytes showed reduced levels of degranulation as measured by the release of a protein showing reactivity to an anti-myeloperoxidase antibody ($P<0.049$) that was subsequently identified by liquid chromatography-MS analysis as prophenoloxidase. This work demonstrates that fumagillin suppresses the immune response of *G. mellonella* larvae by inhibiting the action of haemocytes and thus renders the larvae susceptible to infection. During growth of the fungus in the larvae, this toxin, along with others, may facilitate growth by suppressing the cellular immune response.

INTRODUCTION

The fungus *Aspergillus fumigatus* is widely distributed in the environment and is capable of inducing disease in those with pre-existing pulmonary malfunction (e.g. asthma, cystic fibrosis), disease (e.g. tuberculosis, lung cancer) or undergoing immunosuppressive therapy prior to organ transplantation (Vonberg & Gastmeier, 2006). Colonies of *A. fumigatus* produce conidia, and due to their small size (2–4 μm) these are inhaled deep into the alveoli of the lung and can initiate infection if the immune system is deficient or weakened. Three forms of aspergillosis are recognized clinically: saprophytic, allergic and invasive. Invasive aspergillosis (IA) is the most serious form of disease as it involves the invasion of viable tissue and may produce a mortality rate of 80–95 % (Brakhage & Langfelder, 2002; Rementeria *et al.*, 2005). IA has emerged as an important disease in recent decades due to the use of aggressive immunosuppressive therapy that causes prolonged neutropenia in the treatment of cancer and leukaemia. Vandewoude *et al.* (2004) have estimated that mortality attributable to IA is 19 % in intensive care units. Furthermore the incidence of IA in solid organ transplant patients may be up to 15 %, with mortality ranging from 65 to 92 % (Singh *et al.*, 2009).

A. fumigatus produces a range of hydrolytic enzymes and mycotoxins, such as gliotoxin, helvolic acid and fumagillin, which may facilitate its growth and persistence in the lung (Tekaia & Latgé, 2005). Gliotoxin possesses a number of powerful bioactivities, including inhibition of activation of the NADPH oxidase of human neutrophils (Tsunawaki *et al.*, 2004). It has been detected in tissue samples from animals (Richard & DeBey, 1995; Richard *et al.*, 1996) and humans (Lewis *et al.*, 2005), where it may facilitate fungal persistence and colonization of tissue. In addition, gliotoxin has been implicated in the destruction of lung parenchyma in IA (Sutton *et al.*, 1996) and the penetration of blood vessels in angio-invasive aspergillosis (Fraser, 1993).

Abbreviations: IA, invasive aspergillosis; LC-MS, liquid chromatography-MS; MPO, myeloperoxidase; PMA, phorbol-12-myristate-13-acetate; PPO, prophenoloxidase; ROS, reactive oxygen species.

Fumagillin ($C_{26}H_{34}O_7$, 458.6 Da) is also produced by *A. fumigatus* during hyphal development (Mitchell *et al.*, 1997). The toxin has well-established amoebicidal properties (McCowen *et al.*, 1951) and has been identified as an inhibitor of angiogenesis through a covalent interaction with methionine aminopeptidase-2 (MetAP-2) (Ingber *et al.*, 1990). Fumagillin can retard the ciliary beat frequency of pulmonary epithelial cells and thus may facilitate the continued persistence of *A. fumigatus* conidia in the lung (Amitani *et al.*, 1995). Fumagillin inhibits the growth of colorectal cancer cells and retards metastasis in mice; however, the molecular mechanism of the various activities of fumagillin is ill defined (Hou *et al.*, 2009).

The insect immune response and the innate immune response of mammals demonstrate many structural and functional similarities, and as a consequence insects have been utilized to evaluate the virulence of a variety of microbial pathogens and to model the innate immune response without the requirement for mammals (Kavanagh & Reeves, 2004; Fuchs & Mylonakis, 2006; Lionakis & Kontoyiannis, 2005). Insect haemocytes and human neutrophils demonstrate a number of similarities in terms of their ability to phagocytose and kill pathogens. We previously demonstrated that reactive oxygen species (ROS) production in haemocytes of *Galleria mellonella* is mediated by an NADPH oxidase complex that contains proteins homologous to the p40^{phox}, p47^{phox}, p67^{phox} and gp91^{phox} of human neutrophils (Bergin *et al.*, 2005). In addition, the homologous cytosolic proteins (p40, p47 and p67) translocate from the cytosol to the membrane fraction in stimulated haemocytes (Renwick *et al.*, 2007). Insects have been employed to assess the virulence of bacteria (Mansfield *et al.*, 2003), fungi (Cotter *et al.*, 2000) and parasites (Boulanger *et al.*, 2001), and positive correlations with results from murine studies have been demonstrated for *Candida albicans* (Brennan *et al.*, 2002) and *Pseudomonas aeruginosa* (Jander *et al.*, 2000). *G. mellonella* larvae have been employed to measure the virulence of clinical isolates of *A. fumigatus* (Reeves *et al.*, 2004; Jackson *et al.*, 2009).

While insects are now widely used to measure the virulence of fungal pathogens (Mylonakis *et al.*, 2005; Fuchs & Mylonakis, 2006) and *A. fumigatus* isolates in particular (Reeves *et al.*, 2004; Lionakis *et al.*, 2005; Lionakis & Kontoyiannis, 2005), the ability of *A. fumigatus* to grow within the haemocoel of *G. mellonella* larvae and the insect immune response to the fungus are ill defined. The aim of the work presented here was to establish whether fumagillin alters the cellular immune response of larvae and so facilitates the growth of *A. fumigatus*. Characterizing the interaction of this toxin with the insect immune response could give insights into how the toxin interacts with the immune system of mammals and establish its role in facilitating fungal growth *in vivo*.

METHODS

Chemicals and reagents. All chemicals were of the highest purity and were purchased from Sigma-Aldrich, unless otherwise stated.

Larval inoculation. Sixth instar larvae of the Greater Wax Moth, *G. mellonella* (The Mealworm Company Ltd), were stored in the dark at 15 °C. Larvae (0.2–0.4 g) were selected at random and all experiments were performed independently on three occasions. Larvae were inoculated with 1×10^7 conidia in 20 µl through the last left pro-leg into the haemocoel with a Myjector U-100 insulin syringe (Terumo Europe). Larvae were inoculated with 20 µl of 0.1, 1 or 2 µg fumagillin ml^{-1}. Fumagillin was dissolved in methanol and serially diluted so that the 2 µg fumagillin ml^{-1} dose was in 10 %, v/v, methanol. A solvent (10 %, v/v, methanol) control was used in all experiments.

Fungal cultures. *A. fumigatus* ATCC 26933 was grown on malt extract agar plates at 37 °C for 7 days, and conidia were harvested by rinsing with PBS supplemented with 0.01 % (v/v) Tween-80. Following centrifugation at 500 *g* for 5 min, conidia were washed in sterile PBS and enumerated using a haemocytometer. *C. albicans* MEN was maintained on YEPD agar and grown for approximately 24 h in YEPD broth [2 % (w/v) glucose, 2 % (w/v) bactopeptone (Difco Laboratories), 1 % (w/v) yeast extract (Oxoid)] to the stationary phase. *C. albicans* cells were washed twice in sterile PBS and enumerated by haemocytometry.

Haemocyte isolation. Larvae were bled through the anterior region, and the resulting haemolymph was collected in sterile insect physiological saline (IPS; 150 mM NaCl, 5 mM KCl, 100 mM Tris/HCl, 10 mM EDTA, 30 mM sodium citrate, pH 6.9). Following haemolymph collection, haemocytes were collected by centrifugation at 500 *g* for 5 min and subsequently washed twice in IPS to remove excess haemolymph protein. Cells were collected by centrifugation and resuspended in PBS supplemented with 5 mM glucose. The concentration of cells was determined by haemocytometry, and cellular viability was assessed using the Trypan Blue exclusion assay.

In vitro phagocytosis and killing of pathogenic targets by haemocytes. Isolated haemocytes (5×10^6 ml^{-1}) were incubated in the presence of 2 µg fumagillin ml^{-1} for 25 min at 37 °C. *A. fumigatus* conidia were opsonized using cell-free haemolymph for 40 min at 37 °C. Phagocytosis was measured by incubating pre-treated haemocytes (5×10^6) with opsonized *A. fumigatus* conidia (1×10^7) in a final volume of 1 ml in a stirred thermally controlled chamber at 37 °C. An aliquot was removed immediately after the addition of conidia to the cells ($t=0$), and after a further 30, 60 and 120 min of incubation. Three hundred haemocytes were examined and the number of cells containing phagocytosed conidia was ascertained by microscopy. The dark-green colour of *A. fumigatus* conidia facilitated the visualization of phagocytosed conidia. The mean ± SEM was calculated.

Assays to determine the effect of 2 µg fumagillin ml^{-1} on the microbicidal activity of haemocytes (5×10^6) employed haemolymph-opsonized *C. albicans* cells at a ratio of 2.5×10^6 to 5×10^6 haemocytes in a final volume of 1 ml. Aliquots were removed at $t=0$, 20, 40 and 60 min and serially diluted in ice-cold minimal essential medium (MEM) to quench phagocytosis, prior to plating on YEPD plates to ascertain viability. The percentage reduction in pathogen viability was calculated in relation to the initial viability of the *C. albicans* cells. Cells of *C. albicans* were chosen for use in the microbicidal assay as the kinetics of their killing by haemocytes is well characterized (Bergin *et al.*, 2005).

Measurement of oxygen consumption. Oxygen consumption was measured in a thermostatically controlled (37 °C) chamber attached to a Clarke-type oxygen electrode. The rate of oxygen consumption was calculated based on the fact that the oxygen content of PBS at 37 °C is 230 nmol ml^{-1} (Segal & Coade, 1978). Haemocytes were pre-treated with 2 µg fumagillin ml^{-1} at 37 °C for 25 min, and

5×10^7 cells were placed in a thermally controlled stirred chamber and stimulated with phorbol-12-myristate-13-acetate (PMA; 1 µg ml^{-1}).

Subcellular fractionation of haemocytes. Haemocytes were fractionated using the method described by Renwick *et al.* (2007). In brief, haemocytes (1×10^8 ml^{-1}) were incubated in the presence of 2 µg fumagillin ml^{-1} for 25 min, harvested, washed and exposed to 1 µg PMA ml^{-1} for 6 min. Following treatment, cells were harvested by centrifugation and resuspended in 500 µl break buffer (10 mM KCl, 3 mM NaCl, 4 mM MgCl$_2$, 10 mM PIPES, pH 7.2) containing protease inhibitors [10 µg ml^{-1} leupeptin, pepstatin A, aprotinin and *N*-α-*p*-tosyl-L-lysine chloromethylketone hydrochloride (TLCK)], sonicated (Bandelin Sonopuls) three times for 5 s at 20 % power, and centrifuged at 200 *g* for 10 min at 4 °C. The post-nuclear supernatant (PNS) was centrifuged at 100 000 *g* for 1 h at 4 °C in a Beckman SW 60 Ti rotor. The membrane pellet was solubilized in break buffer supplemented with 36 mM CHAPS by mixing for 1 h. The membrane fraction was sonicated for five 1 min intervals with cooling on ice in between. The solubilized fraction was centrifuged at 7000 *g* to remove any membrane debris. Bradford assay reagent (Bio-Rad Laboratories) was used to assay the amount of protein in each fraction, using BSA as standard.

Assessment of degranulation in haemocytes. Haemocytes (5×10^7 ml^{-1}) were exposed to 2 µg fumagillin ml^{-1} for 25 min at 37 °C, and resuspended in PBS containing 5 mM glucose and protease inhibitors as described above. Following PMA (1 µg ml^{-1}) stimulation in a stirred chamber, an aliquot was removed at 6 min [previously established to be optimal for detecting degranulation (data not shown)]. Cells were removed by centrifugation (800 *g*). The protein content of the supernatant was precipitated and prepared for electrophoresis and immunoblotting.

Electrophoresis and immunoblotting. Samples were separated by SDS-PAGE (12.5 % acrylamide), and protein profiles were visualized by colloidal Coomassie staining. For Western blotting, the protein was transferred to a nitrocellulose membrane using wet blotting for 70 min at 100 V. Rabbit polyclonal antisera raised against human p47^{phox} were used at a dilution of 1 : 3000 overnight at 4 °C. This anti-p47^{phox} antibody had previously been employed to identify p47 in insect haemocytes (Bergin *et al.*, 2005; Renwick *et al.*, 2007). Myeloperoxidase (MPO) antiserum raised in rabbit (Genway) was used at a dilution of 1 : 650 overnight at 4 °C. Secondary antibody employed horseradish peroxidase-linked rabbit antisera at a dilution of 1 : 2000. Enhanced chemiluminescence (Pierce Biotechnology) was employed to develop reactive protein bands for MPO-like reactivity. 3,3′-Diaminobenzidine (DAB) staining was utilized for p47^{phox}-like staining and quantified by densitometry using ImageJ software. The data from four blots were used to determine the statistical significance between treatments.

Liquid chromatography-MS (LC-MS) analysis. In-gel digestion of the immunoreactive band was performed according to the method of Shevchenko *et al.* (2006). Briefly, the protein band corresponding to the MPO reactive band was excised and washed for approximately 60 min in 150 µl 100 mM ammonium bicarbonate/acetonitrile (1 : 1, v/v). Following removal of the wash buffer, gel pieces were incubated in 500 µl acetonitrile until the gel pieces became white and shrank. Following removal of acetonitrile, the excised bands were digested overnight in 13 ng trypsin µl^{-1}. Peptides were extracted and vacuum-dried prior to LC-MS analysis on an Agilent 6340 ion trap mass spectrometer. The resulting data were analysed using the Mascot search engine (http://www.matrixscience.com). The mass error tolerance was 1 Da, allowing for a maximum of no more than two missed cleavages. Verification of protein sequences was confirmed by BLAST analysis of the protein sequence on the UniProt (http://www.

uniprot.org) and National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) websites.

Statistics. All values are the mean ± SD of at least three independent determinations. Experimental data were tested for statistical significance at $P<0.05$ using a Student's *t* test (Minitab 14 package). Larval viability data (Fig. 1) were analysed using the log rank test.

RESULTS

Effect of fumagillin on susceptibility of *G. mellonella* larvae to *A. fumigatus* infection

In order to evaluate the ability of fumagillin to alter the immune response of *G. mellonella*, larvae were administered the toxin and their response to infection with conidia of *A. fumigatus* was monitored. The administration of fumagillin had no adverse effect on the viability of larvae over 72 h (data not shown). However, larvae administered 2 µg fumagillin ml^{-1} for 4 h and subsequently infected with 1×10^7 *A. fumigatus* conidia in a 20 µl volume demonstrated increased mortality compared with those infected with *A. fumigatus* conidia without prior exposure to the toxin (Fig. 1). At 72 h, 80 ± 5.7 % of larvae that had received 2 µg fumagillin ml^{-1} had died compared with 53.3 ± 12.0 % of those that had not received the toxin prior to fungal inoculation ($P=0.0052$). In contrast, administration of 0.1 or 1.0 µg fumagillin ml^{-1} to larvae only resulted in small alterations in the susceptibility of larvae to
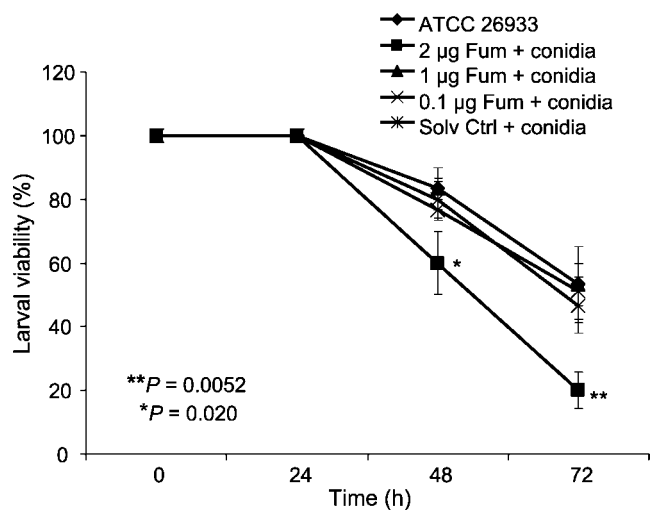

Fig. 1. Effect of fumagillin on susceptibility of *G. mellonella* larvae to *A. fumigatus* infection. Larvae were inoculated with different concentrations of fumagillin (Fum) and were subsequently injected with 1×10^7 *A. fumigatus* conidia and incubated at 37 °C. Larval viability was determined at 24, 48 and 72 h post fungal inoculation. Larvae that were administered 2 µg fumagillin ml^{-1} prior to conidial inoculation showed a significant reduction in viability compared with those which only received conidia (Solv Ctrl + conidia) ($P=0.0052$).

infection ($P \geqslant 0.7$). Interestingly, we have previously detected fumagillin concentrations of 3 μg (g larval tissue)$^{-1}$ in insects infected with *A. fumigatus* for 24 h (data not shown). As a result of this finding, subsequent work concentrated on examining the ability of 2 μg fumagillin ml^{-1} to alter haemocyte function.

Fumagillin retards the killing and phagocytic abilities of *G. mellonella* haemocytes

The insect immune response consists of cellular and humoral components, and the cellular immune response is mediated by immune cells called haemocytes, which can phagocytose and kill invading pathogens (Morton *et al.*, 1987). Haemocytes (viability 95 %) were extracted from *G. mellonella* larvae and exposed to 2 μg fumagillin ml^{-1} for 25 min. The viability of haemocytes after this incubation period was 88.8 ± 1.7 %. Toxin-treated haemocytes were mixed with opsonized cells of *C. albicans* in a ratio of 1 : 2, and the ability of the haemocytes to kill the fungal cells was assessed as described above. The results demonstrated that haemocytes exposed to 2 μg fumagillin ml^{-1} had a significantly reduced ability to kill *C. albicans* compared with solvent-treated or control haemocytes ($P=0.039$ at 60 min; Fig. 2). For example, at 60 min, *C. albicans* cell viability was 33.56 ± 4.3 % when fumagillin-treated haemocytes were employed but 11.59 ± 1.0 % when the solvent-treated haemocytes were employed in the assay ($P=0.048$).
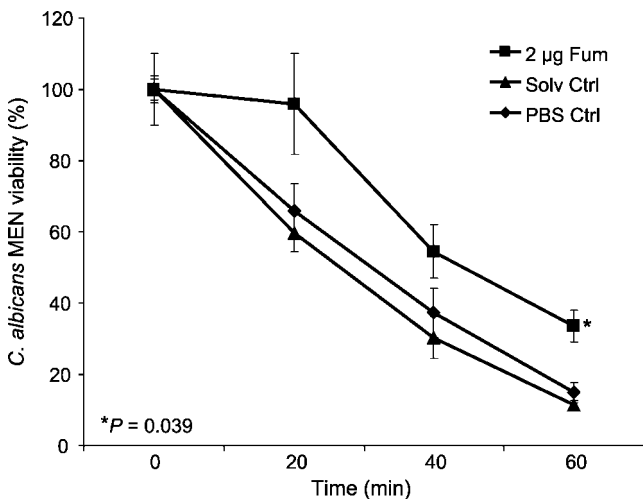
The capacity of fumagillin to alter the phagocytic ability of haemocytes was investigated and revealed that those haemocytes that had been exposed to 2 μg fumagillin ml^{-1} for 25 min prior to mixing with *A. fumigatus* conidia demonstrated reduced phagocytosis (Fig. 3). At 60 min, 43.2 ± 3.8 % of fumagillin-treated haemocytes had engulfed a conidium, while 54.9 ± 5.6 % of solvent-treated haemocytes had phagocytosed ($P=0.016$).

Fumagillin reduces the oxygen uptake of haemocytes and retards the cytosol–membrane translocation of p47 protein

Once phagocytosis has been accomplished by a haemocyte, the engulfed pathogen can be killed by the generation of ROS and the release of proteolytic enzymes in the process of degranulation. Oxygen uptake is essential for ROS production and is mediated by an NADPH oxidase complex which shows similarity to that in neutrophils (Bergin *et al.*, 2005). The oxygen consumption ability of fumagillin-treated haemocytes was reduced significantly (Fig. 4). After 10 min, the difference in oxygen consumption between fumagillin-treated haemocytes and solvent-treated haemocytes was significant at $P=0.003$.

ROS production is mediated by an NADPH oxidase complex that consists of membrane (gp91^{phox}) and cytosolic (p40^{phox}, p47^{phox} and p67^{phox}) proteins in neutrophils and of homologous proteins in haemocytes
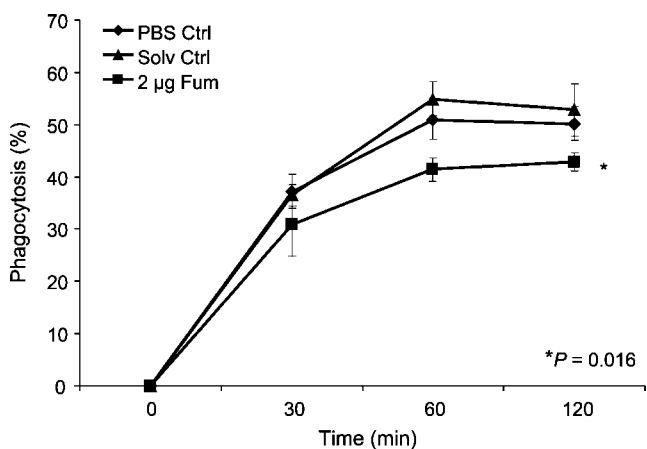

Fig. 2. Fumagillin-treated haemocytes are less effective in killing *C. albicans* cells. Haemocytes (5×10^6) were incubated in the presence of 2 μg fumagillin ml^{-1} (Fum) prior to incubation with opsonized *C. albicans* (2.5×10^6). Aliquots were removed at regular intervals and diluted in ice-cold MEM to quench phagocytosis, and a 100 μl volume was spread on a YEPD-erythromycin agar plate. Significant reductions in cell-mediated killing by toxin-treated haemocytes were observed compared with the PBS control (PBS Ctrl) and solvent control (Solv Ctrl) ($P=0.039$).

Fig. 3. Phagocytosis ability of fumagillin-treated haemocytes. Haemocytes (5×10^6) were incubated in the presence of 2 μg fumagillin ml^{-1} (Fum) and mixed with opsonized conidia. Aliquots were removed at 0, 30, 60 and 120 min and examined by light microscopy to ascertain the level of phagocytosis. Cells pre-treated with fumagillin demonstrated a significant ($P=0.016$) reduction in phagocytosis compared with cells which were pre-incubated in the PBS control (PBS Ctrl) or the solvent control (Solv Ctrl) at 120 min.
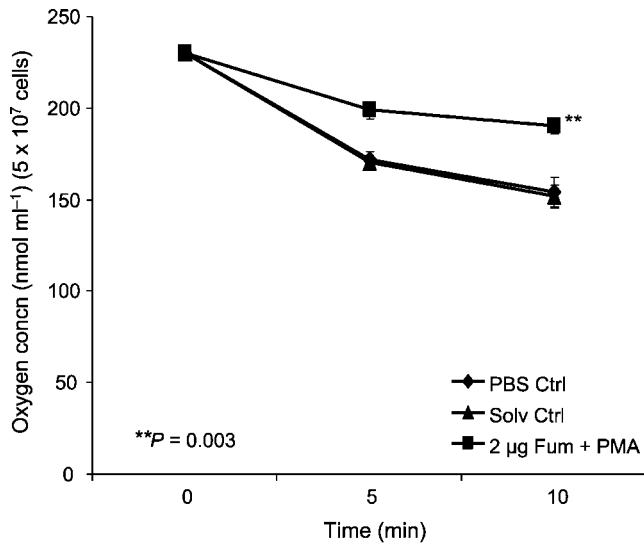
Fig. 4. Oxygen consumption of fumagillin-treated haemocytes. Haemocytes (5×10^7) were incubated in the presence of 2 μg fumagillin ml^{-1} (Fum) and stimulated with 1 μg PMA ml^{-1}. A significant reduction in the consumption of oxygen was observed compared with control cells (Solv Ctrl) ($P=0.003$).

(Bergin *et al.*, 2005). The cytosolic proteins translocate to the membrane upon cellular activation and lead to the formation of a functional NADPH oxidase (Renwick *et al.*, 2007). The ability of fumagillin to retard the translocation of p47 was assessed by exposing cells to 2 μg fumagillin ml^{-1} and subsequently isolating cytosolic and membrane fractions. These were resolved by 1D SDS-PAGE, blotted and probed with an antibody that reacts with an insect p47 protein previously shown to be homologous to the p47^{phox} protein in the NADPH oxidase complex of neutrophils (Bergin *et al.*, 2005; Renwick *et al.*, 2007). The results revealed that cells exposed to fumagillin showed lower levels of p47 in the membrane fraction than those in the PMA-activated cells ($P=0.05$) (Fig. 5). Immunoblot analysis of haemocyte fractionation showed the presence of p47 protein in the cytosolic fraction of unstimulated cells, with a translocation of the protein to the membrane fraction upon PMA stimulation ($P=0.014$). Haemocytes which had been pre-exposed to fumagillin and subsequently PMA-stimulated showed retention of $50.2\pm2.3\%$ of p47 in the cytosolic fraction compared with $37.4\pm1.09\%$ of the protein in the cytosolic fraction of PMA-stimulated haemocytes ($P=0.01$).

Fumagillin-treated haemocytes demonstrate reduced levels of degranulation

During phagocytosis and the generation of ROS, neutrophils undergo the process of degranulation, and one of the most abundant proteins released during degranulation is MPO, which accounts for up to 5 % of the protein content of the cell (Klebanoff, 1999). Using an anti-MPO antibody, the rate of haemocyte degranulation was quantified. Cells were induced to degranulate by exposure to PMA in a stirred thermostatically controlled reaction chamber, and the presence of protein in the secretome to which the anti-MPO antibody reacted was quantified by Western blotting (Fig. 6). The results revealed that the level of protein to which the anti-MPO antibody reacted in fumagillin-treated haemocytes was significantly lower than that in PMA-activated or in solvent-treated PMA-activated haemocytes ($P=0$ and 0.049, respectively). The anti-MPO antibody was observed to react with two proteins, and these were approximately 59.5 kDa in size. Both proteins to which the anti-MPO antibody reacted were excised and analysed by LC-MS as described above. The results revealed the larger protein to be a prophenoloxidase (PPO), an enzyme known to be present in insect haemocytes (Mandato *et al.*, 1997) and released during degranulation (the excised protein Mascot score was 501, 24 % coverage, with 10 distinct peptide matches to *G. mellonella* PPO, accession no. AF336289.1). The second protein was also identified as PPO (Mascot score 309, 13 % coverage with five distinct peptide matches).

DISCUSSION

The insect immune response demonstrates a number of similarities to the innate immune response of mammals and, as a consequence, insects have become an attractive alternative to the use of mammals for assessing the virulence of microbial pathogens (Brennan *et al.*, 2002; Fuchs & Mylonakis, 2006; Johny *et al.*, 2007) or for assessing the efficacy of novel antimicrobial drugs (Rowan *et al.*, 2009; Hamamoto *et al.*, 2009; Lionakis *et al.*, 2005).

Larvae of *G. mellonella* offer many advantages for this type of work in that they are inexpensive to purchase and inoculate, and have no ethical or legal impediments to their use (Kavanagh & Reeves, 2004; Fuchs & Mylonakis, 2006; Mylonakis, 2008). *G. mellonella* larvae have been used to assess the virulence of *A. fumigatus* (Renwick *et al.*, 2006; Jackson *et al.*, 2009), but little attention has been given to understanding the interaction of this fungus with the insect immune response. We previously demonstrated a correlation between gliotoxin production and virulence of *A. fumigatus* in *G. mellonella* larvae (Reeves *et al.*, 2004), and also demonstrated the ability of gliotoxin to block p47 and p67 translocation and thus prevent the formation of a functional NADPH oxidase complex in insect haemocytes (Renwick *et al.*, 2007).

Fumagillin is produced during the growth of *A. fumigatus* (Mitchell *et al.*, 1997) and may play a role in suppressing the immune response of the host. The aim of this work was to investigate the interaction of fumagillin with haemocytes, which are the main component of the cellular immune response of insects (Morton *et al.*, 1987). Since larvae of *G. mellonella* are a convenient and effective host
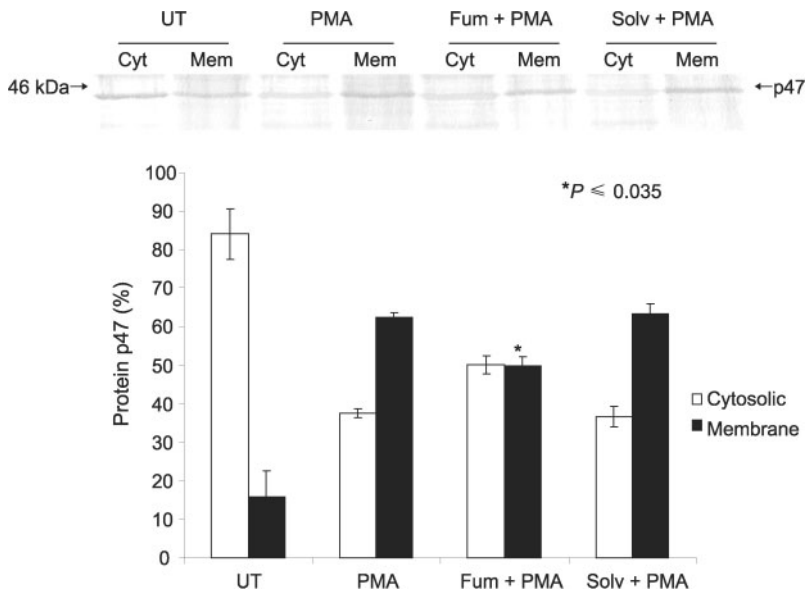
Fig. 5. Presence of p47 protein in cytosolic and membrane fractions of *G. mellonella* haemocytes. Haemocytes (1×10^8) were incubated in the presence of 2 µg fumagillin ml^{-1} (Fum) and fractionated as described in Methods. Following electrophoresis and immunoblotting, densitometric analysis was used to quantify the relative abundance of p47 in the cytosol and membrane fractions, and these values are expressed in terms of the percentage distribution of p47 in the fractions. Haemocytes which were pre-exposed to fumagillin showed a significant reduction in p47 translocation compared with controls [i.e. PMA- and solvent and PMA-stimulated (Solv+PMA) haemocytes] ($P \leqslant 0.035$). UT, untreated.

for studying fungal virulence, understanding the interaction of *A. fumigatus* with the insect immune response might give insights into how this fungus grows and develops in insects and, by extrapolation, in mammals.

Inoculation of larvae with fumagillin had no adverse effect on larval viability; however, administration of this toxin to *G. mellonella* larvae rendered them more susceptible to infection with conidia of *A. fumigatus*. In particular, those larvae that received a dose of 2 µg fumagillin ml^{-1} showed a mortality rate of 80 ± 5.7 % at 72 h compared with 53.33 ± 12.0 % in larvae that were not pre-injected ($P=0.0052$). Fumagillin reduced the ability of haemocytes to phagocytose opsonized *A. fumigatus* conidia and kill opsonized *C. albicans* cells. The percentage inhibition in phagocytosis was 14.71 %, while the reduction in killing

(relative to the control) was 44.36 %, which indicated that as well as retarding phagocytosis fumagillin was also affecting the killing mechanism of haemocytes.

Neutrophils are similar in structure and function to haemocytes (Bergin *et al.*, 2005), which mediate their killing by the production of ROS and by releasing degradative enzymes in the process of degranulation (Spitznagel, 1990). During the process of degranulation a wide range of enzymes are released into the phagosome and around the periphery of the cell (Standish & Weiser, 2009). The generation of ROS is a critical part of the haemocyte's ability to kill an engulfed cell (Bergin *et al.*, 2005), and the suppression of this would adversely affect its killing ability. Exposure of haemocytes to fumagillin resulted in a reduction in oxygen uptake without adversely affecting
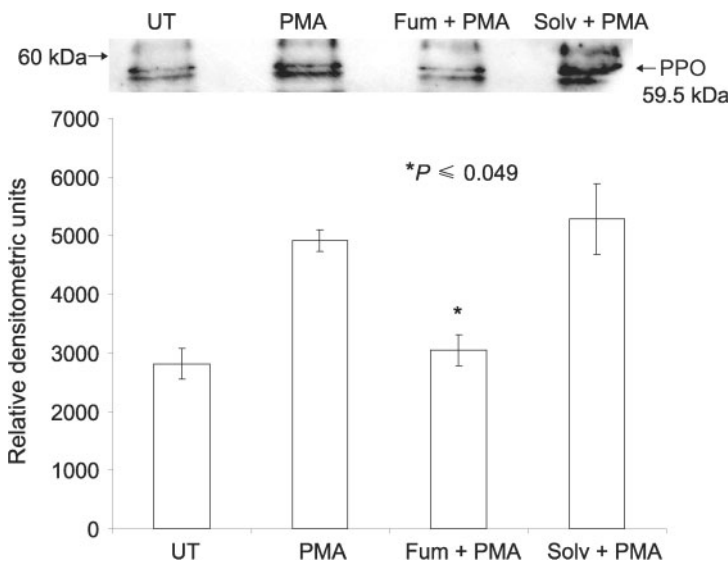

Fig. 6. Visualization of anti-MPO reactivity in the secretome of *G. mellonella* haemocytes. Haemocytes were incubated in the presence of 2 µg fumagillin ml^{-1} (Fum) and placed in a stirred chamber maintained at 37 °C. Six minutes post PMA stimulation, the contents of the chamber were centrifuged at 800 **g** for 5 min to pellet cells and cellular debris. The supernatant was subjected to electrophoresis and immunoblotting with a mammalian anti-MPO antibody. There was a significant reduction in MPO-like reactivity compared with the PMA only and solvent and PMA (Solv+PMA) controls ($P \leqslant 0.049$). UT, untreated.

haemocyte viability. Reduced oxygen uptake indicates a reduction in the generation of ROS, since this is dependent upon a sufficient supply of oxygen and the presence of a functional NADPH oxidase complex. Fumagillin-treated cells demonstrated reduced release of PPO upon stimulation with PMA.

Recent work has demonstrated that 2 μg fumagillin ml^{-1} retards the ability of human neutrophils to phagocytose and kill microbial cells, and this is also due to a reduction in the formation of a functional NADPH oxidase complex (Fallon *et al.*, 2010). We demonstrated that fumagillin adversely influences many of the structural rearrangements (phagocytosis, formation of functional NADPH oxidase, degranulation) essential for the formation of an activated cell (Fallon *et al.*, 2010). The current work demonstrates that fumagillin induces similar effects in insect haemocytes, thus demonstrating further similarities between the two cell types. At the cellular level there is a high degree of homology between the insect immune response and the innate immune response of mammals (Salzet, 2001). Both insect haemocytes and human neutrophils phagocytose and kill pathogens by the generation of ROS, and this is mediated by a similar NADPH oxidase complex in both cell types (Bergin *et al.*, 2005). In addition, both neutrophils and haemocytes respond to gliotoxin (Tsunawaki *et al.*, 2004; Renwick *et al.*, 2007) and fumagillin (Fallon *et al.*, 2010; this work) in a similar manner. These results extend the number of similarities between the two immune systems and highlight the suitability of using insects as an alternative screening system to mammals.

The use of insects for studying microbial pathogens and for assessing the efficacy of novel antimicrobial drugs is increasing (Peleg *et al.*, 2009). This work has demonstrated that fumagillin has the ability to suppress the immune response of *G. mellonella* larvae by interfering with the function of haemocytes. During growth of *A. fumigatus* in the larvae, fumagillin, together with other toxins and enzymes (Rementeria *et al.*, 2005), may play a role in suppressing the immune response, thus facilitating hyphal growth. The utility of the insect system is widely recognized, and this work, together with others, has highlighted a further similarity (Salzet, 2001) between the insect immune response and the innate immune response of mammals.

ACKNOWLEDGEMENTS

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Edited by: K. Haynes