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FUNGAL AND PARASITIC INFECTIONS

The Human Cathelicidin Antimicrobial Peptide LL-37 Promotes the Growth of the Pulmonary Pathogen *Aspergillus fumigatus*

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ABSTRACT The pulmonary mucus of cystic fibrosis (CF) patients displays elevated levels of the cathelicidin antimicrobial peptide LL-37, and the aim of this work was to assess the effect of LL-37 on the growth of *Aspergillus fumigatus*, a common pathogen of CF patients. Exposure of *A. fumigatus* to LL-37 and its derived fragment RK-31 (1.95 μg/ml) for 24 h had a positive effect on growth (199.94% $\pm$ 6.172% [$P < 0.05$] and 218.20% $\pm$ 4.63% [$P < 0.05$], respectively), whereas scrambled LL-37 peptide did not (85.12% $\pm$ 2.92%). Exposure of mycelium (preformed for 24 h) to 5 μg/ml intact LL-37 for 48 h increased hyphal wet weight (4.37 $\pm$ 0.23 g, $P < 0.001$) compared to the control (2.67 $\pm$ 0.05 g) and scrambled LL-37 (2.23 $\pm$ 0.09 g) treatments. Gliotoxin secretion from LL-37 exposed hyphae (169.1 $\pm$ 6.36 ng/mg hyphae, $P < 0.05$) was increased at 24 h compared to the results seen with the control treatment (102 $\pm$ 18.81 ng/mg hyphae) and the scrambled LL-37 treatment (96.09 $\pm$ 15.15 ng/mg hyphae). Shotgun proteomic analysis of 24-h LL-37-treated hyphae revealed an increase in the abundance of proteins associated with growth (eukaryotic translation initiation factor 5A [eIF-5A] [16.3-fold increased]), tissue degradation (aspartic endopeptidase [4.7-fold increased]), and allergic reactions (Asp F13 [10-fold increased]). By 48 h, there was an increase in protein levels indicative of cellular stress (glutathione peroxidase [9-fold increased]), growth (eIF-5A [6-fold increased]), and virulence (RNase mitogillin [3.7-fold increased]). These results indicate that LL-37 stimulates *A. fumigatus* growth and that this stimulation can result in increased fungal growth and secretion of toxins in the lungs of CF patients.

KEYWORDS *Aspergillus fumigatus*, antimicrobial peptide, cathelicidin, LL-37, cystic fibrosis, innate immunity

Cathelicidin antimicrobial peptides (CAMP) are expressed in all mammals as α-helical peptides ranging from 23 to 40 residues in sequence length (1). The human CAMP is located on chromosome 3p21.31 as 4 exons and is expressed as hCAP-18, a 16-kDa preprotein consisting of a 30-residue signal sequence, a highly conserved cathelin domain, and a C-terminal antimicrobial domain (2). Cleavage of the C-terminal domain by neutrophil protinease-3, keratinocyte kallikrein (KLK), seminal plasma gastricsin, and microbial proteases liberates bioactive LL-37, a cationic (+6 at physiological pH) membrane-active amphipathic peptide (3–5). The peptide is abundant in the specific granules of mature neutrophils contributing to both intracellular and extracellular killing and is present in other leukocytes, lymphocytes, wound fluid, and a variety of epithelial and mucosal sites at concentrations of 2 to 5 μg/ml and under conditions ranging from homeostasis to a concentration of 50 μg/ml during inflammation/infection (6, 7). LL-37 displays a range of functions in relation to local infection directly by eliciting microbicidal activity targeting the outer layers of microbial cells and indirectly by its alarmin, angiogenic, and reepithelialization properties (8–10). Skin-

Received 1 February 2018 **Returned for modification** 24 February 2018 **Accepted** 20 April 2018

Accepted manuscript posted online 30 April 2018

Citation Sheehan G, Bergsson G, McElvaney NG, Reeves EP, Kavanagh K. 2018. The human cathelicidin antimicrobial peptide LL-37 promotes the growth of the pulmonary pathogen *Aspergillus fumigatus*. Infect Immun 86:e00097-18. https://doi.org/10.1128/IAI.00097-18.

Editor George S. Deepe, University of Cincinnati

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derived kallikrein 5/7 cleavage of LL-37 in sweat yields shorter peptide fragments KR-20, RK-31, and KS-30 that display augmented antimicrobial activity and decreased interleukin-8 (IL-8) expression compared with that seen with LL-37; initial low concentrations of LL-37 are primarily responsible for cell recruitment and accumulation followed by peptide saturation in the infection microenvironment (11).

hCAP-18 is constitutively expressed by many cell types and modulated by inflammatory triggers and by-products of microbial growth. Vitamin D3 response elements are present in the hCAP-18 promoter and act synergistically with Dectin-1, Nod-like receptor (NLR), and Toll-like receptor (TLR) pathways in monocytes and keratinocytes for induction. An absence of saliva and plasma LL-37 is characteristic of morbus Kostmann syndrome, a severe congenital neutropenia associated with recurrent periodontal disease (12). LL-37 levels are increased in the bronchoalveolar lavage fluid (BALF) of cystic fibrosis (CF) patients, and elevated LL-37 levels were correlated with local neutrophil density in *Pseudomonas aeruginosa*-colonized individuals, indicating that LL-37 plays a central role in the pulmonary inflammatory aspect of CF (13). The presence of LL-37 also enhanced lung epithelial cell barrier function, which directly prevents *P. aeruginosa* invasion (14). Under chronic inflammatory dermatological conditions (e.g., in cases of rosacea), increased cutaneous TLR-2, LL-37, and KLK-5/7 levels with neutrophil infiltration and increased serum hydroxy vitamin D status are correlated with the symptoms of aberrant immune responses and vascular dysfunction with local neurogenic involvement (15, 16).

LL-37 displays pleotropic immunomodulatory properties, with many groups reporting classical ligand receptor interactions and others reporting LL-37-mediated transactivation of transmembrane-bound receptors (17). Nevertheless, LL-37 induces monocyte, neutrophil, and T lymphocyte chemotaxis; modulates inflammatory cytokine production; and impacts key nonimmune biological processes such as apoptosis, angiogenesis, wound healing, and maintenance of epithelial barrier integrity. LL-37 was found to activate eosinophils and bronchial epithelial cells, promoting an inflammatory milieu which may have consequences for allergic bronchopulmonary aspergillosis, a disease characterized by eosinophil infiltration (18).

LL-37 is believed to exert its antimicrobial activity via membrane interactions according to the carpet model, where peptides coat the membrane, inducing curvature until a critical concentration is reached, resulting in breakdowns in membrane integrity, most likely as a consequence of the formation of toroidal pores as a result of oligomeric structure formation (19). Mammalian membranes are protected from the pore-forming effects of LL-37 due to the presence of cholesterol in their plasma membranes, which increases bilayer thickness and allows tight packing of acyl chain and compressibility while decreasing the translational diffusion rates of phospholipids. For example, *Helicobacter pylori* displays resistance to LL-37 that is dependent on scavenging and utilization of host cholesterol (20). Interestingly, LL-37 can induce some degree of leakage in unsaturated and cholesterol-containing membranes (21). LL-37 displays candidacidal activity by targeting the fungal membrane, resulting in complete membrane disintegration and efflux of cellular ATP and protein (22). LL-37 can bind immunomodulatory β-glucan and also alters cell wall architecture and induces wall reorganization events via interactions with Xog1p (β-1,3-exoglucanase), resulting in exposure of β-glucan and loss of cellular integrity (23). LL-37 demonstrates microbicidal activity against the clinically important microbes *Burkholderia cepacia* (79 μg/ml), *Pseudomonas aeruginosa* (16 μg/ml), *Staphylococcus aureus* (9 μg/ml), *Haemophilus influenzae* ($<$10 μg/ml), and *Stenotrophomonas maltophilia* (1.9 μg/ml) (23–25). The aim of the work presented here was to establish the response of the pulmonary pathogen *Aspergillus fumigatus*, a common pathogen in the CF lung, to LL-37.

RESULTS

Growth response of *A. fumigatus* to LL-37. An *in vitro* susceptibility assay was employed to establish the effects of intact LL-37, scrambled LL-37, and an LL-37-derived fragment (RK-31) on the growth of *A. fumigatus* at concentrations ranging from 31.25

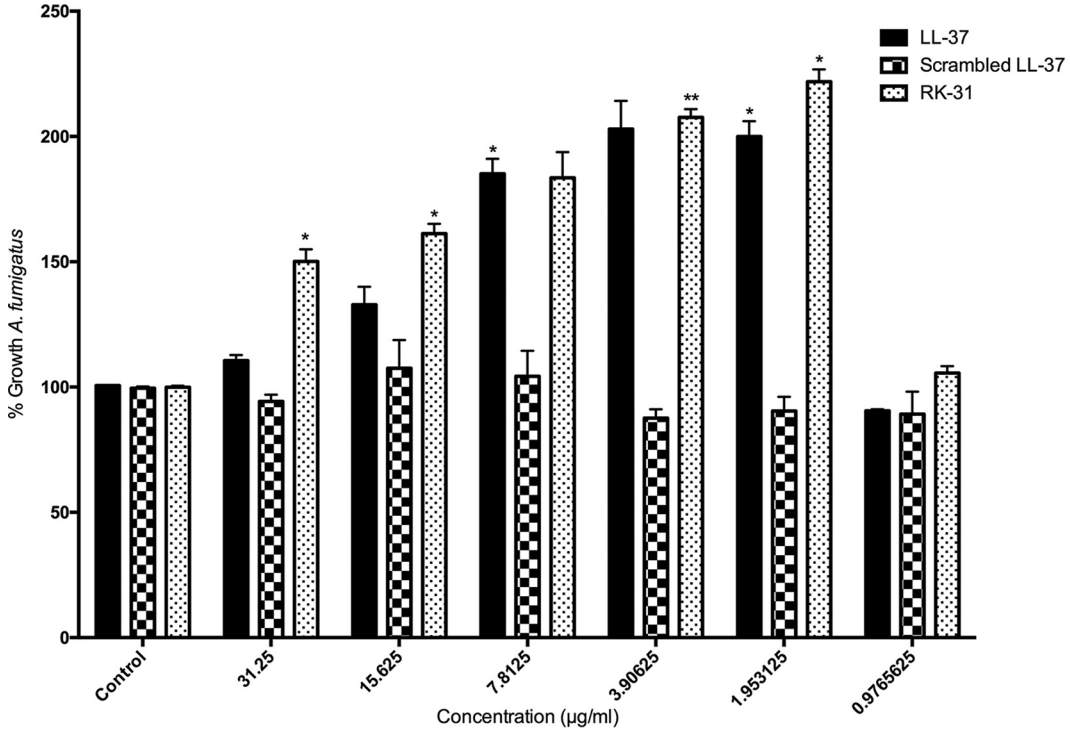

FIG 1 Susceptibility of *A. fumigatus* conidia (initial concentration, 10^4/well) to LL-37, scrambled LL-37, and RK-31 peptides at concentrations ranging from 31.25 μg/ml to 0.97 μg/ml after 24 h growth in minimal medium. LL-37 and RK-31 increased the growth of *A. fumigatus* at low concentrations of peptide, while scrambled LL-37 peptide had no significant effect on *A. fumigatus* growth (*, $P < 0.05$; **, $P < 0.01$).

μg/ml to 0.97 μg/ml in glucose minimal medium after 24 h ($n = 3$). A concentration of 31.25 μg/ml increased the growth of *A. fumigatus* to 110.57% $\pm$ 2.24% (LL-37) and 147.35% $\pm$ 5.54% (RK-31) ($P < 0.05$), while scrambled LL-37 did not show such growth (91.91% $\pm$ 2.04%). A concentration of 7.81 μg/ml LL-37 also increased the growth of *A. fumigatus* (185.09% $\pm$ 6.02%) ($P < 0.05$). Interestingly, a low concentration (1.95 μg/ml) of LL-37 and RK-31 induced maximum growth of *A. fumigatus* to 199.94% $\pm$ 6.17% ($P < 0.05$) and 218.20% $\pm$ 4.63% ($P < 0.05$), whereas the scrambled LL-37 peptide did not show such induction (85.12% $\pm$ 2.92%) (Fig. 1). High concentrations (100 μg/ml to 3.125 μg/ml) of LL-37 also significantly increased the growth of *A. fumigatus* in minimal essential medium (see Fig. S1a in the supplemental material), minimal essential medium with 5% fetal calf serum (FCS) (Fig. S1b), and yeast extract-peptone-dextrose (YEPD) (Fig. S1c). Other cellular stress agents targeting the cell wall (calcofluor white [Fig. S1d]), cell membrane (saponin [Fig. S1e]), and cellular oxidative capacity (hydrogen peroxide [H_2O_2] [Fig. S1f]) were employed to determine if sublethal levels of cellular stress agents could stimulate increased growth by *A. fumigatus*. Exposure of *A. fumigatus* to calcofluor white (31.25 μg/ml; 195.37% $\pm$ 35.54%) ($P < 0.001$) and saponin (100 μg/ml; 111.90% $\pm$ 3.29%) ($P < 0.05$) resulted in increased cell growth; however, exposure to H_2O_2 decreased growth at higher concentrations and did not increase growth at low concentrations compared to the relative control results.

Cultures of *A. fumigatus* (24 h) were supplemented with intact or scrambled LL-37 to give a final concentration of 5 μg/ml and were incubated for a further 24 or 48 h ($n = 3$). Addition of LL-37 to cultures for 24 h induced a small increase in growth (2.4 $\pm$ 0.23 g versus 2.18 $\pm$ 0.10 g [50 ml culture]$^{-1}$); however, by 48 h, there was a significant increase in growth ($P < 0.001$) (4.37 $\pm$ 0.23 g versus 2.67 $\pm$ 0.05 g [50 ml culture]$^{-1}$) (Fig. 2). This increase in growth was not observed following the addition of scrambled LL-37 (5 μg/ml) after 24 h (1.85 $\pm$ 0.15 g [50 ml culture]$^{-1}$) or 48 h (2.23 $\pm$ 0.09 g). As a positive control, cultures were supplemented with DMSO (dimethyl sulfoxide) (final

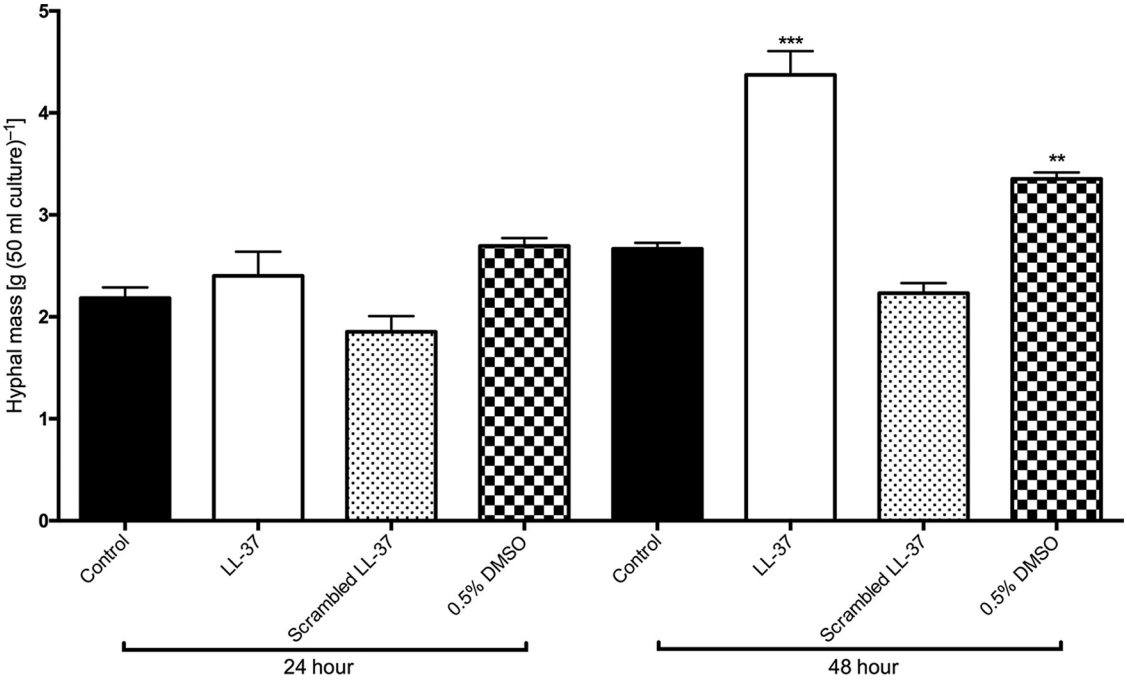

FIG 2 The effect of LL-37 on growth of *A. fumigatus*. A 24-h culture of *A. fumigatus* (10^5/ml) in Sabouraud dextrose broth was supplemented with LL-37, scrambled LL-37, DMSO (0.5% [vol/vol]), or ddH_2O (control) to give a final peptide concentration of 5 μg/ml. After 24 and 48 h, mycelium were weighed. LL-37 increased hyphal mass significantly after 48 h exposure (**, $P < 0.01$; ***, $P < 0.001$).

concentration, 0.5% [vol/vol]), which is known to alter the permeability of fungal cells (26). The results demonstrated increases in hyphal mass at 24 h (2.7 $\pm$ 0.077 g) and 48 h (3.35 $\pm$ 0.06 g) ($P < 0.05$) (Fig. 2). Microscopic examination of LL-37, scrambled LL-37, and RK-31 peptide-treated and control *A. fumigatus* hyphae revealed no changes in cell morphology (Fig. 3). We also examined the effect of LL-37, scrambled LL-37, and RK-31 on the growth of pulmonary pathogen *Aspergillus flavus*. LL-37 increased the growth of *A. flavus* at 1.93 μg/ml (137.64% $\pm$ 5.90%) ($P < 0.05$); however, RK-31 and scrambled LL-37 had no significant effect on growth (Fig. S2). Moreover, these peptides had no effect on *A. flavus* cell morphology (Fig. S3). Low concentrations of cellular-stress-inducing agents were used to determine if cell wall, membrane, or oxidative stress can cause an increase in wet weight such as was observed with LL-37, which acts at the membrane-wall interface. Interestingly, a low concentration (0.46875 mM) of H_2O_2 resulted in a significant decrease in mycelium wet weight at 24 and 48 h ($P < 0.05$) (Fig. S4A). However, exposure of *A. fumigatus* to 50 μg/ml of the cell wall stressor calcofluor white resulted in increased mycelium wet weight at 48 h (3.11 $\pm$ 0.15 g, $P < 0.05$) compared to the relative control.

Effect of LL-37 on gliotoxin secretion by *A. fumigatus*. Culture filtrates of intact LL-37-, scrambled LL-37-, or DMSO-treated *A. fumigatus* mycelium were collected at 24 and 48 h and assessed for gliotoxin leakage by reversed-phase high-performance liquid chromatography (RP-HPLC) in order to determine the effects of LL-37 (intact and scrambled) and DMSO on membrane integrity ($n = 3$). The biological solvent DMSO is known to increase cell permeability (26) and at low concentrations induced gliotoxin leakage from *A. fumigatus*. Mycelium cultured in the presence of intact LL-37 (5 μg/ml) for 24 h released significantly more extracellular gliotoxin (169.15 $\pm$ 6.36 ng/mg hyphae, $P < 0.05$) than control mycelium (102.72 $\pm$ 18.81 ng/mg hyphae). The addition of scrambled LL-37 (5 μg/ml) had no significant effect on the extracellular gliotoxin concentration after 24 h (96.09 $\pm$ 15.15 ng/mg hyphae) or 48 h (99.88 $\pm$ 4.08 ng/mg hyphae) compared to their respective controls. An increase in extracellular gliotoxin was also noted for DMSO-treated *A. fumigatus* cultures (211.30 $\pm$ 15.42 ng/mg hyphae, $P < 0.01$). By 48 h, cultures supplemented with LL-37 showed gliotoxin

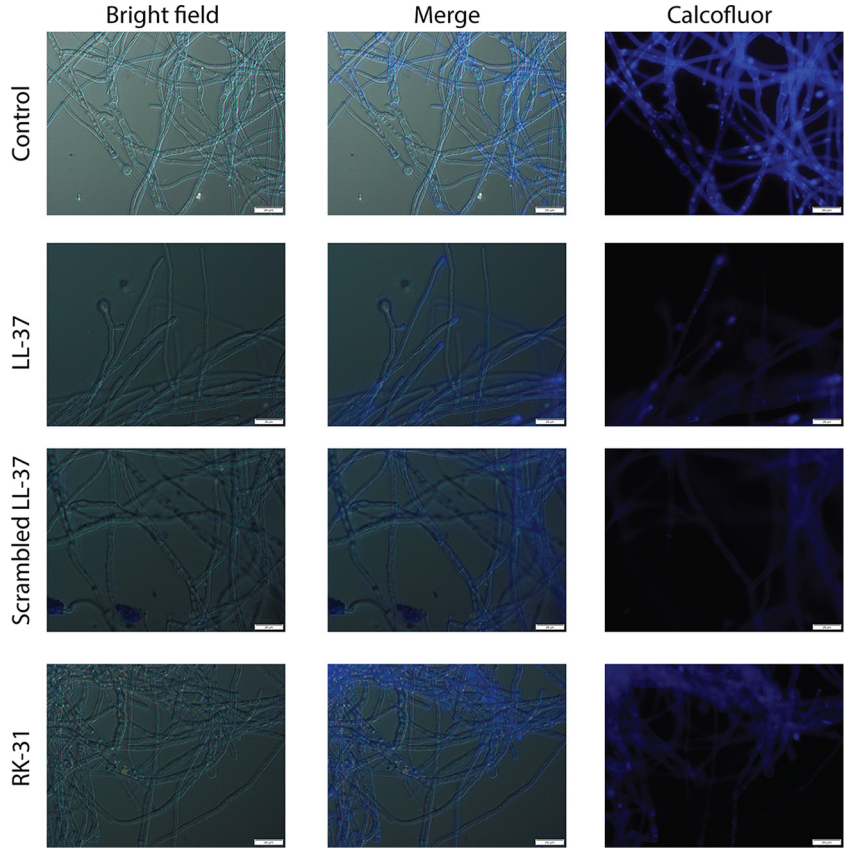

FIG 3 Photomicrographs of hyphae from cultures exposed to LL-37, scrambled LL-37, and RK-31. *A. fumigatus* mycelium exposed to 31.25 μg/ml LL-37, scrambled LL-37, and RK-31 for 24 h were stained with calcofluor white and visualized with an Olympus BX51 fluorescence microscope. LL-37, RK-31, and scrambled LL-37 had no effect on hyphal morphology.

levels of 137.82 $\pm$ 8.72 ng/mg hyphae compared to the control results (96.32 $\pm$ 8.43 ng/mg hyphae). Mycelium treated with DMSO for 48 h displayed significantly increased levels of gliotoxin (168.15 $\pm$ 15.64 ng/mg hyphae, $P < 0.01$) compared to the control (Fig. 4). Furthermore, the addition of cellular-stress-inducing agents also resulted in an increase in the levels of extracellular gliotoxin (calcofluor white, 186.15 $\pm$ 12.05 ng/mg hyphae; saponin, 171.52 $\pm$ 19.28 ng/mg hyphae) at 48 h postexposure compared to the relative controls (Fig. S4B).

Analysis of proteomic response of *Aspergillus fumigatus* to LL-37. Label-free quantitative (LFQ) proteomic analysis was conducted on *A. fumigatus* mycelium exposed to LL-37 (5 μg/ml) for 24 and 48 h. In total, 1,524 and 6,004 peptides were identified, representing 232 and 626 proteins with two or more peptides. A total of 32 proteins at 24 h and 87 proteins at 48 h were determined to be differentially abundant (analysis of variance [ANOVA], $P < 0.05$), with a fold change value of >1.5. A total of 3 (24 h) and 43 (48 h) proteins were deemed exclusive (i.e., with LFQ intensities present in all three replicates of one treatment and absent in all three replicates of the other two treatments). These proteins were also used in statistical analysis of the total levels corresponding to the differentially expressed group members following imputation of the zero values as described above. After data imputation, these proteins were included in the subsequent statistical analysis. A principal-component analysis (PCA) performed on all filtered proteins distinguished the control and LL-37 (24 and 48 h)-treated samples, indicating a clear difference between the proteomes (Fig. S5).

Proteins that increased in relative abundance in LL-37 (24 h of exposure)-treated *A. fumigatus* compared to the control were eukaryotic translation initiation factor 5A (eIF-5A) (16-fold), Asp F13 (10-fold), translation initiation factor 4B (9.5-fold), translation

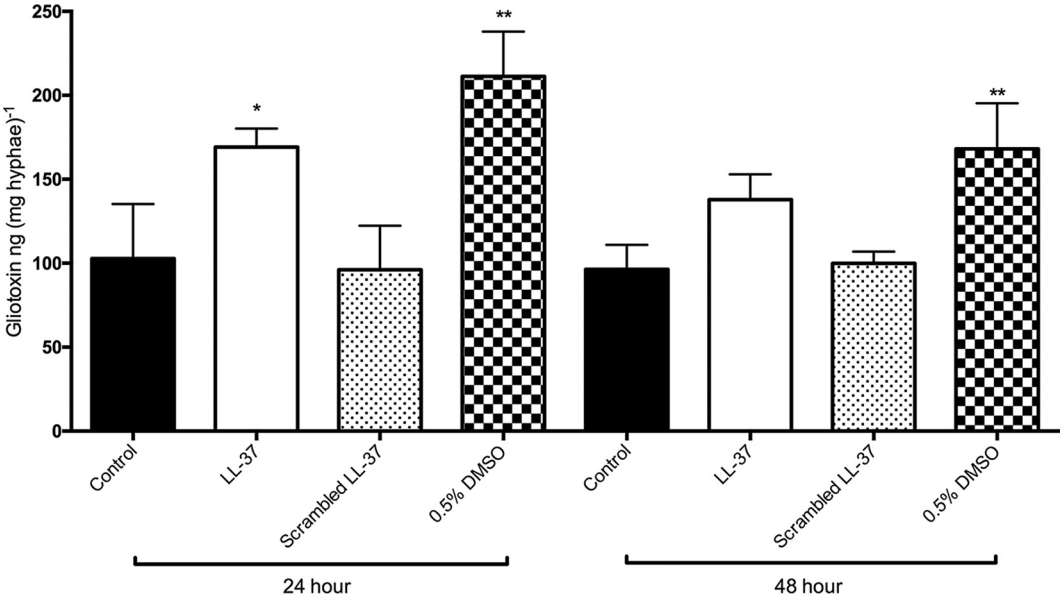

FIG 4 Gliotoxin quantification in *A. fumigatus* supernatants exposed to LL-37 (5 μg/ml), scrambled LL-37 (5 μg/ml), or DMSO (0.5% [vol/vol]) for 24 or 48 h was determined by RP-HPLC. Exposure to LL-37 increased gliotoxin levels at 24 and 48 h (*, $P < 0.05$; **, $P < 0.01$).

elongation factor EF-Tu (7-fold), aspartic endopeptidase Pep1 (5-fold), 40S ribosomal protein S10a (4.5-fold), 1,3-β-glucanosyltransferase Gel1 (2-fold), and aegerolysin family protein (2-fold). Proteins decreased in relative abundance in LL-37 (24-h exposure)-treated *A. fumigatus* were triosephosphate isomerase (9-fold), secreted dipeptidyl peptidase (7-fold), inorganic diphosphatase (5-fold), saccharopine dehydrogenase (4-fold), and fructose-bisphosphate aldolase (4-fold) (Fig. 5A).

Proteins increased in relative abundance in LL-37 (48-h exposure)-treated *A. fumigatus* were malate dehydrogenase (27-fold), glutathione peroxidase (9-fold), ribosomal protein L15 (8.9-fold), translationally controlled tumor protein homolog (8.4-fold), Hsp70 chaperone (7.9-fold), eukaryotic translation initiation factor eIF-5A (6-fold), 60S acidic ribosomal protein P0 (6-fold), an alcohol dehydrogenase (5.2-fold), dual-functional monooxygenase/methyltransferase psoF (4.1-fold), RNase mitogillin (3.7-fold), FK506-binding protein 2 (3.5-fold), eIF4A (2.7-fold), allergen Asp F15 (2.5-fold), and glutathione oxidoreductase (1.7-fold). Proteins that had decreased in relative abundance in LL-37-treated *A. fumigatus* at that time were associated with the proteasome (proteasome subunit beta type [7-fold], proteasome endopeptidase complex [2.5-fold], Pup1 [2.1-fold], and proteasome subunit alpha type activity [2-fold]), cell wall activity (1,3-β-glucanosyltransferase Gel1 [3-fold]), and carboxypeptidase activity (carboxypeptidase [2.4-fold]) (Fig. 5B).

DISCUSSION

A. fumigatus is a significant pulmonary pathogen in individuals who are immunocompromised (e.g., myelosuppression) and in those who display defects in immune equilibrium, commonly observed in the CF lung, and presents itself in the form of allergic bronchopulmonary aspergillosis (ABPA) (27). In the latter, *A. fumigatus* airborne conidia penetrate the lower airways, germinate, and grow within the thick tenacious CF mucus. CF patients colonized by *A. fumigatus* can become sensitized to secreted *A. fumigatus* allergens and eventually develop a type 2 hypersensitivity reaction to *A. fumigatus* allergens, toxins, and proteases, which are readily replenished into the mucus and can directly cause cell damage and peeling at the bronchiole-epithelium interface (28). Therefore, we hypothesized that components of the CF mucus could affect the growth of *A. fumigatus*. LL-37 is an important antimicrobial peptide produced by a variety of pulmonary cells (including type II pneumocytes and neutrophils) and aug-

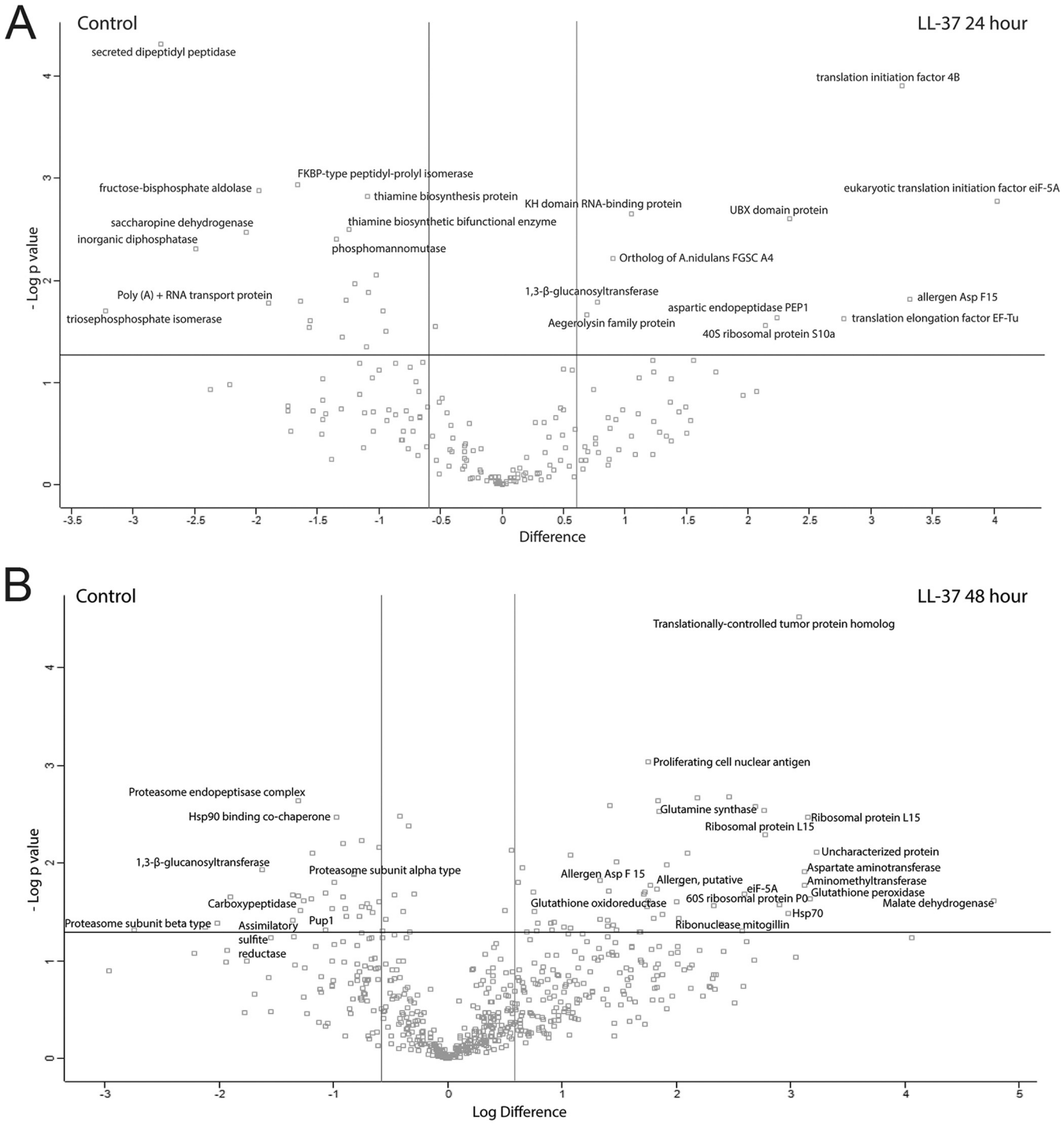

FIG 5 Label-free proteomics of *A. fumigatus* exposed to 5 μg/ml LL-37 for 24 h (A) or 48 h (B). Volcano plots showing the distribution of quantified proteins according to *P* value (−log10 *P* value) and fold change (log2 mean LFQ intensity difference). Proteins above the horizontal line are considered to represent statistically significant data (*P* value < 0.05), and those to the right and left of the vertical lines are considered to indicate relative $\pm$1.5-fold changes. LL-37 induced increased abundance of proteins associated with growth, virulence, and allergic reactions.

mentable to high concentrations transiently during inflammation and infection. LL-37 has direct and indirect antimicrobial activity against important pathogens of the CF lung and has pleotropic growth effects on eukaryotic membranes; this, together with its augmented abundance in the CF lung, which is frequently dominated by *A. fumigatus*, makes it an interesting target for the study of host-pathogen interactions.

LL-37 increased the growth of *A. fumigatus*. Doses ranging from 31.25 μg/ml, which

would be indicative of local inflammation, to 5 μg/ml, which is characteristic of CF BALF, supported the growth of *A. fumigatus in vitro* (6, 7). The addition of scrambled LL-37 peptide had no effect on *A. fumigatus* growth, while the addition of LL-37-derived fragment RK-31 also produced an increase in growth in high- and low-nutrient media. This indicates that the primary structure of LL-37 is essential to its bioactivity and that the presence of RK-31, a shorter and purportedly more microbicidal peptide of LL-37, also enhanced *A. fumigatus* growth. The presence of LL-37 at a low concentration also increased the growth of *A. flavus*, a less common CF lung pathogen (29, 30). Furthermore, microscope analyses of *A. fumigatus* and *A. flavus* revealed no changes in cellular morphology and no hyperbranching or aggregation in response to LL-37 or RK-31. The presence of LL-37 increased mycelium wet weight after 24 h (2.4 $\pm$ 0.23 g versus 2.18 $\pm$ 0.10 g [50 ml culture]$^{-1}$) and 48 h (4.37 $\pm$ 0.23 g versus 2.67 $\pm$ 0.05 g) ($P < 0.001$). Addition of scrambled LL-37 to *A. fumigatus* cultures for 24 h (1.85 $\pm$ 0.15 g [50 ml culture]$^{-1}$) and 48 h (2.23 $\pm$ 0.09 g) had no significant effect on mycelium mass. This indicates that specific characteristics with respect to peptide size, sequence, charge, helicity, hydrophobicity, and amphipathicity are essential for the growth-promoting effects induced by LL-37. The use of a low concentration of the biological solvent DMSO, which is known to alter cell permeability (26), also increased mycelium wet weight at 24 and 48 h. These findings suggest that the presence of LL-37 may increase the growth of *A. fumigatus* by acting on the fungal membrane by a mechanism similar to that observed in mammals (31, 32).

Employing low concentrations of cellular stress agents resulted in enhanced growth comparable to that observed with LL-37; the most notable results were seen with calcofluor white and saponin, which interact with the fungal cell wall and membrane, respectively (33, 34). Calcofluor white increased the growth of *A. fumigatus* to 195.37% $\pm$ 35.54% ($P < 0.001$) and also increased mycelium biomass (3.11 $\pm$ 0.15 g, $P < 0.05$). It is hypothesized that a low level of stress may activate compensatory growth pathways in *A. fumigatus*.

Previous work has demonstrated that LL-37 induced leakage of biological and fungal membranes and that the antifungal drug amphotericin B also alters *A. fumigatus* membrane permeability and induces the release of gliotoxin (19, 26, 35–37). The secretion of gliotoxin was increased in response to LL-37 at 24 h (169.15 $\pm$ 6.36 ng/mg hyphae, $P < 0.05$) and 48 h (137.82 $\pm$ 8.72 ng/mg hyphae). Gliotoxin has pleotropic effects on its host such as polymorphonuclear leukocyte apoptosis, inhibition of neutrophil phagocytosis and NADPH-dependent oxidative burst, inhibition of T lymphocyte epithelial damage, and slowed ciliary beating (38–41). Gliotoxin induces vitamin D receptor downregulation in pulmonary macrophages and epithelial cells, resulting in increased levels of IL-5 and IL-13 which skew T cell plasticity toward an allergic T_H2-dominated response (42). Small-molecule leakage may facilitate rapid nutrient uptake into the cell, possibly accounting for the observed growth increase. The use of calcofluor and saponin resulted in a significant increase in extracellular levels of gliotoxin at 48 h postexposure. Gliotoxin may play an important role in maintenance of cell redox balance and is significantly increased in level in response to oxidative stress (43). This suggests that cell stress may result in an alteration in fungal cell redox status and lead to increased gliotoxin production to restore cell redox homeostasis, which may have adverse consequences in the CF lung.

Shotgun proteomics was employed to determine the effect of LL-37 on *A. fumigatus* after 24 and 48 h of exposure. *A. fumigatus* responds to LL-37 by increasing the abundance of growth-associated proteins such as eukaryotic translation initiation factor eIF-5A (16-fold increased), translation initiation factor 4B (9.5-fold increased), translation elongation factor EF-Tu (7-fold increased), and 40S ribosomal protein S10a (4.5-fold increased). eIF-5A in eukaryotic cells is strongly associated with hyperproliferative growth and maintenance of cell wall integrity in fungal cells and is important in *A. fumigatus* during cell stress in response to H_2O_2 (44–46).

After 24 h of LL-37 exposure, *A. fumigatus* also increased the abundance of proteins which may have a role in virulence, such as Asp F13 (10-fold increased), aspartic

endopeptidase Pep1 (5-fold increased), and Aegerolysin family protein (2-fold increased). Asp F13 is a secreted alkaline protease and allergen associated with *A. fumigatus* immune subversion and virulence via cleavage of complement cascade proteins and immunoglobulin class G isotypes and binding of IgE (47–49). Pep1 is a hyphal tip secreted aspartic proteinase known to hydrolyze proteins of the basement membrane (elastin, collagen, and laminin) and is found during *A. fumigatus* invasion of the neutropenic mouse lung (50). *A. fumigatus* also produces a 14-kDa aegerolysin family protein (Asp-hemolysin) found to exhibit putative hemolytic activity on erythrocytes as well as cytotoxic effects on murine macrophages and endothelial cells (51). Asp-hemolysin can be detected *in vivo* during infection; however, experimental evidence is lacking for its role in virulence (52). Proteins associated with thiamine metabolism were decreased in abundance 24 h post-LL-37 exposure.

At 48 h post-LL-37 exposure, the mycelium proteome displayed enrichment for proteins associated with growth (malate dehydrogenase [27-fold increased], ribosomal protein L15 [8.9-fold increased], 60S acidic ribosomal protein P0 [6-fold increased], eIF-5A [6-fold increased], FK506-binding protein 2 [3.5-fold increased], and eIF4A [2.7-fold increased]) as well as for a variety of detoxification and stress response proteins (glutathione peroxidase, Hsp70 chaperone, alcohol dehydrogenase, and glutathione oxidoreductase). As with 24-h-LL-37-treated mycelium, 48-h-exposed mycelium showed increased abundance of proteins associated with virulence. RNase mitogillin (3.7-fold increased) is an 18-kDa purine-specific RNase which cleaves the 28S RNA of eukaryotic ribosomes, resulting in protein synthesis inhibition (53). Mitogillin is considered a major allergen of *A. fumigatus* which interacts with IgE from ABPA patients (53). Allergen (AFUA_5G01440; 3.6-fold increased) is a putative secreted allergen of *A. fumigatus* which possesses domains associated with oxidoreductase activity (redoxin, thioredoxin-like fold, thioredoxin domain-containing protein) and may play a role in cell redox homeostasis. Dually functional monooxygenase/methyltransferase psoF and methyltransferase psoC are both essential in pseurotin and fumagillin (psoF) biosynthesis. Pseurotin has been noted to inhibit chitin synthesis and inhibit IgE production from mouse B cells. The toxin fumagillin can retard the ciliary beat frequency of pulmonary epithelial cells and inhibit neutrophil function and decreases host survival of invasive aspergillosis *in vivo* (54, 55). Exposure of *A. fumigatus* to LL-37 for 48 h results in decreased abundance of proteins associated with protein degradation. The reduction in the levels of these proteins may explain the increased abundance of proteins associated with regulatory processes (mRNA and protein synthesis, etc.) in response to an external stimulus (LL-37). Interestingly, Gel1 is increased at 24 h but is decreased at 48 h. Gel1 plays a role in elongation of 1,3-β-glucan chains and is induced under hypoxic conditions, and its change in abundance may alter the β-glucan concentration in the cell wall (56, 57).

The results presented here demonstrate that LL-37 stimulates the growth of *A. fumigatus* at physiologically relevant concentrations such as those found in the CF lung. This stimulation of growth may be due to the altered membrane permeability or interactions with the fungal cell wall resulting in the activation of growth-associated processes. The enhanced growth of the fungus, the elevated secretion of gliotoxin, and the increased abundance of allergic and tissue-degrading enzymes may have adverse effects on the pulmonary tissue of CF patients and contribute to the development of disease.

MATERIALS AND METHODS

Chemicals and reagents. Synthetic LL-37 peptide (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES), scrambled LL-37 (GLKLRFEFSKIKGEFLKTPEVRFRDIKLKDNRISVQR) (molecular weight, 4,493.33), and RK-31 (RKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) (molecular weight, 3,800.51) were purchased from Innovagen (Sweden) (>95% purity) and dissolved in double-distilled water (ddH_2O). Reagents used in this study were purchased from Sigma-Aldrich, unless indicated otherwise.

***A. fumigatus* culture conditions.** *A. fumigatus* ATCC 26933 was obtained from the American Type Culture Collection, and *A. flavus* was a kind gift from Ricardo Araujo, Faculty of Medicine of the University of Porto, Porto, Portugal. *A. fumigatus* was grown in Sabouraud dextrose broth (SAB), YEPD broth (2% [wt/vol] glucose, 1% [wt/vol] yeast extract, 2% peptone), minimal essential medium (Sigma), and minimal

medium broth (2% [wt/vol] glucose, 0.5% [wt/vol] yeast nitrogen base [without amino acids or ammonium sulfate], 0.5% [wt/vol] ammonium sulfate) at 37°C and 200 rpm. Stocks were maintained on malt extract agar (MEA) (Oxoid).

Assessment of susceptibility of *Aspergillus* conidia to peptide and chemical agents. MEA plates containing sporulating *A. fumigatus* (or *A. flavus*) colonies were washed with 0.1% (vol/vol) Tween 80 (Merck)–phosphate-buffered saline (PBS; Sigma-Aldrich) (pH 7.2) to isolate conidia. Conidia were washed twice in sterile PBS, centrifuged (1,500 × *g*, 5 min) in a Beckman GS-6 centrifuge, and enumerated using a hemocytometer. LL-37, scrambled LL-37, or LL-37 fragment (RK-31) was serially diluted in growth medium (minimal medium; 31.25 μg/ml to 0.97 μg/ml) on a 96-well plate (Corning Costar), and *Aspergillus* conidia (1×10^4/well) were added to each well. Cellular-stress-inducing agents (calcofluor white [31.25 μg/ml to 0.2441 μg/ml], saponin [100 μg/ml to 0.390625 μg/ml]) and hydrogen peroxide (15 mM to 0.05859375 mM) were also serially diluted in growth media to determine the effect of low concentrations on *A. fumigatus* growth and toxin production. Plates were incubated at 37°C, and growth was quantified by measuring optical density at 570 nm (OD_{570}) (using Bio-Tek Synergy HT readings) at 24 h.

Effect of LL-37 on growth of *A. fumigatus* hyphae. Flasks containing SAB (50 ml) were inoculated with 5×10^6 *A. fumigatus* conidia to give a density of 1×10^5/ml and incubated at 37°C and 200 rpm for 24 h. Cultures were supplemented with 1 ml of a 250 μg/ml LL-37–ddH_2O solution, 1 ml of a 250 μg/ml scrambled LL-37–ddH_2O solution (final concentration, 5 μg/ml), 1 ml DMSO (final concentration, 0.5% [vol/vol]), 1 ml of a stress-inducing agent (calcofluor white [final concentration, 50 μg/ml] or saponin [final concentration, 100 μg/ml]), hydrogen peroxide (final concentration, 0.46875 mM), or 1 ml ddH_2O (control) for a further 24 or 48 h, at which time hyphal wet weights were determined.

Visualization of LL-37-treated *A. fumigatus* hyphae. *A. fumigatus* mycelium exposed to 31.25 μg/ml LL-37, scrambled LL-37, and RK-31 for 24 h was washed in PBS and stained with calcofluor white (Sigma) for 15 min at 16°C on a glass slide. The cells were washed twice (PBS), and a cover slide was placed on top. The cover slides were then fixed *in situ* by applying a clear sealing solution around the perimeter of the slide which also prevented the drying of the sample. Cells were viewed with an Olympus BX51 fluorescence microscope.

Extraction of gliotoxin from *A. fumigatus* culture filtrate. Flasks containing SAB (50 ml) were inoculated with 5×10^6 *A. fumigatus* conidia to give a density of 1×10^5/ml and incubated at 37°C and 200 rpm for 24 h. Cultures were supplemented with LL-37 (5 μg/ml), scrambled LL-37 (5 μg/m), DMSO (0.5% [vol/vol]), 1 ml of a stress-inducing agent (calcofluor white [final concentration, 50 μg/ml] or saponin [final concentration, 100 μg/ml]), hydrogen peroxide (final concentration; 0.46875 mM), or ddH_2O (control) and incubated for a further 24 or 48 h. Each *A. fumigatus* culture was filtered using Miracloth and 0.22-μm-pore-size cellulose filters (Sarstedt), mixed with an equal volume (20 ml) of chloroform (Hyper Solv; BDH), and mixed for 2 h. The chloroform fraction was collected and evaporated to dryness in a Büchi rotor evaporator (Brinkmann Instruments, Westbury, NY). Dried extracts were dissolved in 500 μl methanol (Hyper Solv; BDH) and stored at −70°C.

Quantification of gliotoxin by RP-HPLC. Gliotoxin was detected by reversed-phase HPLC (Spectra-Physics). The mobile phase was 34.9% (vol/vol) acetonitrile (Hyper Solv; BDH), 0.1% (vol/vol) trifluoroacetic acid (Sigma-Aldrich), and 65% (vol/vol) ddH_2O. Gliotoxin extract (20 μl) was injected onto a C_{18} Hewlett Packard column. A standard curve of peak area versus gliotoxin concentration was constructed using gliotoxin standards (0.1, 0.2, 0.5, and 1.0 μg per 10 μl) dissolved in methanol (Sigma-Aldrich).

Whole-cell protein extraction from *A. fumigatus*. Mycelium (1 g) was ground to a fine powder in a pestle and mortar with liquid nitrogen followed by the addition of 6 ml of protein extraction buffer (0.4 M NaCl, 10 mM Tris HCl, 2 mM EDTA, 10% [vol/vol] glycerol) supplemented with aprotinin, pepstatin A, TLCK (*N*α-p-tosyl-L-lysine chloromethyl ketone), and leupeptin (1 μg/ml). The suspension was disrupted using a sonication probe (Bandelin Sonopuls; Bandelin electronic, Berlin, Germany) at 20% power (six cycles for 10 s per cycle). This was repeated twice, with the sample being cooled on ice between sonications. Protein supernatants were obtained by centrifugation (10,000 × *g* at 4°C for 20 min).

Label-free quantitative proteomics of *A. fumigatus* protein lysate. Label-free shotgun quantitative proteomics was conducted on protein extracted from control and LL-37 (5 μg/ml)-treated *A. fumigatus* hyphae for 24 and 48 h. Protein (75 μg) was reduced with dithiothreitol (DTT) (Sigma-Aldrich) (200 mM), alkylated with iodoacetamide (IAA) (Sigma-Aldrich) (1 M), and digested with sequencing-grade trypsin (Promega, Ireland) at a trypsin/protein ratio of 1:40 overnight at 37°C. Tryptic peptides were purified for mass spectrometry (MS) using C_{18} spin filters (Medical Supply Company, Ireland), and 1 μg of peptide mix was eluted onto a Q-Exactive (ThermoFisher Scientific, USA) high-resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 chromatography system (RSLCnano). Peptides were separated by the use of an increasing acetonitrile gradient on a Biobasic C_{18} Picofrit column (100-mm length, 75-mm inner diameter [ID]), using a 120-min reverse-phase gradient at a flow rate of 250 nl/min. All data were acquired with the mass spectrometer operating in automatic data-dependent switching mode. A high-resolution (300-to-2,000-Da) MS scan was performed using an Orbitrap analyzer to select the 15 most intense ions prior to tandem MS (MS/MS).

Protein identification from the MS/MS data was performed using the Andromeda search engine in MaxQuant (version 1.6.0.16; http://maxquant.org/) to correlate the data against an annotated database derived from the *A. fumigatus* Af293 reference proteome downloaded from Uniprot (58, 59). The following search parameters were used: first-search peptide tolerance of 20 ppm and second-search peptide tolerance of 4.5 ppm. Carbamidomethylation of cysteines was set as a fixed modification, oxidation of methionines and acetylation of N-terminal sequences were set as variable modifications, and the maximum number of missed cleavage sites allowed was 2. False-discovery rates (FDR) were set to 1%

for both peptides and proteins, and the FDR was estimated following searches against a target-decoy database. Peptides with a minimum length of seven amino acids were considered for identification, and proteins were considered identified only when two or more unique peptides were observed for each protein.

Processing of results, statistical analyses, and generation of graphics were conducted using Perseus v. 1.5.5.3. LFQ intensities were subjected to $\log_2$ transformation, ANOVA of significance and *t* tests for comparisons between the LL-37-treated and control *A. fumigatus* hyphal proteomes were performed using a *P* value of 0.05, and significance was determined using FDR (Benjamini-Hochberg) correction. Proteins that had nonexistent values (indicative of their absence or very low abundance in a sample) were included in the study only when they were absent from all of the replicates in one group and present in at least two of the three replicates in the second group (referred to as qualitatively differentially abundant proteins).

Statistical analysis. All experiments were performed on at least three independent occasions, and the results are expressed as means ± standard errors (SE). Statistical analysis was performed using one-way ANOVA with *post hoc* Tukey tests for the susceptibility and flask assays (wet weight and gliotoxin), with *P* values of <0.05 considered statistically significant compared to the results determined with the relevant controls. All statistical analyses listed were performed using GraphPad Prism.

Data availability. The MS proteomics data and MaxQuant search output files have been deposited in the ProteomeXchange Consortium (60) via the PRIDE partner repository with data set identifier PXD008143.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00097-18.

SUPPLEMENTARY FILE 1, PDF file, 0.6 MB.

ACKNOWLEDGMENTS

G. Sheehan is the recipient of a Maynooth University Doctoral Studentship. E. P. Reeves acknowledges funding from the Medical Research Charities Group/Health Research Board Ireland (MRCG 2014-1). The Q-Exactive mass spectrometer was funded under the SFI Research Infrastructure Call 2012, grant 12/RI/2346 (3).

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