Self-Protection against Gliotoxin—A Component of the Gliotoxin Biosynthetic Cluster, GliT, Completely Protects Aspergillus fumigatus Against Exogenous Gliotoxin

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Abstract

Gliotoxin, and other related molecules, are encoded by multi-gene clusters and biosynthesized by fungi using non-ribosomal biosynthetic mechanisms. Almost universally described in terms of its toxicity towards mammalian cells, gliotoxin has come to be considered as a component of the virulence arsenal of Aspergillus fumigatus. Here we show that deletion of a single gene, gliT, in the gliotoxin biosynthetic cluster of two A. fumigatus strains, rendered the organism highly sensitive to exogenous gliotoxin and completely disrupted gliotoxin secretion. Addition of glutathione to both A. fumigatus ΔgliT strains relieved gliotoxin inhibition. Moreover, expression of gliT appears to be independently regulated compared to all other cluster components and is up-regulated by exogenous gliotoxin presence, at both the transcript and protein level. Upon gliotoxin exposure, gliT is also expressed in A. fumigatus ΔgliZ; which cannot express any other genes in the gliotoxin biosynthetic cluster, indicating that gliT is primarily responsible for protecting this strain against exogenous gliotoxin. GliT exhibits a gliotoxin reductase activity up to 9 µM gliotoxin and appears to prevent irreversible depletion of intracellular glutathione stores by reduction of the oxidized form of gliotoxin. Cross-species resistance to exogenous gliotoxin is acquired by A. nidulans and Saccharomyces cerevisiae, respectively, when transformed with gliT. We hypothesise that the primary role of gliotoxin may be as an antioxidant and that in addition to GliT functionality, gliotoxin secretion may be a component of an auto-protective mechanism, deployed by A. fumigatus to protect itself against this potent biocide.

Introduction

Gliotoxin, which has a molecular mass of 326 Da and is an epipolythiodioxopiperazin (ETP), contains a disulfide bridge of unknown origin and has been shown to play a significant role in enabling the virulence of Aspergillus fumigatus [1–3]. The cytotoxic activity of gliotoxin is generally mediated by direct inactivation of essential protein thiois [4] and by inhibition of the respiratory burst in neutrophils by disrupting NADPH oxidase assembly, thereby facilitating in vivo fungal dissemination [5,6]. The enzymatic machinery responsible for gliotoxin biosynthesis, and metabolism, is encoded by a multi-gene cluster in A. fumigatus which is coordinately expressed during gliotoxin biosynthesis [7,8]. This cluster encodes gliP, a bimodular nonribosomal peptide synthetase (NRPS) which has been conclusively shown to be responsible for the biosynthesis of a Phe-Ser dipeptide, a gliotoxin precursor, by gene disruption (ΔgliP mutant) [9–12]. In fact, disruption of gliP within the gliotoxin biosynthetic cluster has resulted in the effective inhibition of all cluster gene expression in a ΔgliP mutant [9]. A putative transporter, encoded by gliA, has been shown to facilitate gliotoxin efflux, and increased tolerance to exogenous gliotoxin, when expressed in Leptosphaeria maculans [13]. sirA is a gliA ortholog in this organism and L. maculans ΔsirA was more sensitive to exogenous gliotoxin and sirodesmin than wild-type, however restoration of sirA in the mutant led to greater tolerance towards these metabolites [13]. Bok et al. [14] have demonstrated that disruption of a fungal Zn(II)̿ Cys(6) binuclear cluster domain transcription factor (gliZ) results in the complete inhibition of all gliotoxin cluster gene expression and effective diminution of gliotoxin production [14]. Although GliP has been shown to activate and condense L-Phe and L-Ser to form a precursor diketopiperazine moiety, no information relating to subsequent modification (e.g., thiolation) is available [9–12,15] and it is also unclear if A. fumigatus might need to protect itself against potential gliotoxin cytotoxicity [13].

Interestingly, addition of gliotoxin (up to 5 µg/ml) to A. fumigatus ΔgliP resulted in the up-regulation of selected gene expression (gliT,
Author Summary

The pathogenic fungus *Aspergillus fumigatus* causes disease in immunocompromised individuals such as cancer patients. The fungus makes a small molecule called gliotoxin which helps *A. fumigatus* bypass the immune system in ill people, and cause disease. Although a small molecule, gliotoxin biosynthesis is enabled by a complex series of enzymes, one of which is called GliT, in *A. fumigatus*. Amazingly, nobody has really considered that gliotoxin might be toxic to *A. fumigatus* itself. Here we show that absence of GliT makes *A. fumigatus* highly sensitive to added gliotoxin and inhibits fungal growth, both of which can be reversed by restoring GliT. Neither can the fungus make or release its own gliotoxin when GliT is missing. We also show that gliotoxin sensitivity can be totally overcome by adding glutathione, which is an important anti-oxidant within cells. We demonstrate that gliotoxin addition increases the production of GliT, and that GliT breaks the disulphide bond in gliotoxin which may be a step in the pathway for gliotoxin protection or release from *A. fumigatus*. We conclude that gliotoxin may mainly be involved in protecting *A. fumigatus* against oxidative stress and that it is an accidental toxic.

J. T and N] within the gli cluster and Cramer et al. [9] noted complete activation of the gene cluster (except gliP) following gliotoxin exposure (20 μg/ml). However, exposure of wild-type *A. fumigatus* AF293 to gliotoxin (20 μg/ml), for 24 h, did not result in any significant alteration in gliotoxin cluster expression [9]. The biological significance of these observations is unclear, apart from implying a role for gliotoxin in the regulation of the gli cluster in the absence of gliotoxin production.

It has recently been demonstrated that gliotoxin and sporidesmin, also an ETP toxin containing a disulphide bridge, are both substrates and inactivators of glutaredoxin (Grx1) [16]. These authors also confirmed that the intact disulphide form of these ETP moieties was essential for Grx1 inactivation and that prior reduction of sporidesmin, using glutathione, prevented subsequent Grx1 inactivation. Oxygen presence was also required for Grx1 inactivation by sporidesmin and mass spectrometric analysis confirmed the formation of mixed disulphides between one molecule of Grx1 and either gliotoxin or sporidesmin, respectively. Combined, these data suggest interplay between oxygen availability and selective protein inactivation in the presence of oxidised ETP-type molecules. This indirectly suggests either a protective, or neutral, involvement of the oxidised forms of gliotoxin or sporidesmin in protecting against the deleterious effects of oxygen by selective protein inactivation.

In mammalian cells it has been demonstrated that the oxidized form of gliotoxin is actively concentrated in a glutathione-dependent manner and that it then exists within the cell mostly exclusively in the reduced form [17]. As glutathione levels fall due to apoptosis, the oxidized form of gliotoxin effluxes from the cell where the cytotoxic effects of gliotoxin are perpetuated in a pseudocatalytic manner. Conversely, it has been shown that gliotoxin may substitute for 2-cys peroxiredoxin activity in HeLa cells by accepting electrons from NADPH via the thioredoxin reductase–thioredoxin redox system to reduce H₂O₂ to H₂O. In this way, nanomolar levels of gliotoxin may actually protect against intracellular oxidative stress [18].

Although the cytotoxic effects of gliotoxin on mammalian cells have been extensively investigated, and yeast have been deployed as a model system to study this interaction [19], no direct investigation of any self-protective mechanism used by *A. fumigatus* against this intriguing molecule has been undertaken. Here, we demonstrate that deletion of *gliT* results in transformants which cannot grow in the presence of even modest levels of exogenous gliotoxin and that exogenous gliotoxin up-regulates gene expression within the gliotoxin cluster, especially that of *gliT*. We propose that GliT is the key cellular defence against gliotoxin in *A. fumigatus* and that this finding yields a new selection marker system for detecting transformation.

Results

Deletion and complementation of *gliT* in *A. fumigatus*

*ΔgliT* mutants were generated by transformation of *A. fumigatus* strains ATCC46645 and ATCC26933, respectively, as described in Materials and Methods, using the bipartite marker technique and pyrithiamine selection, with modifications [20,21] (Figure S1). Deposition number: IMI CC 396691 (CABI Bioscience Centre, Egham, Surrey, UK). These two strains were chosen because ATCC26933 is a gliotoxin producer, whereas ATCC46645 lacks significant gliotoxin production using the Minimal Media described in Materials and Methods (see below). Complementation of *gliT* mutant strains was carried out as described in Materials and Methods and Figure S1). Complemented strains (*ΔgliT*) (Deposition number: IMI CC 396692) exhibited wild-type like features in all subsequent experiments, demonstrating that the occurrence of a single ectopic integration of a *gliT* fragment is insignificant in the *A. fumigatus* ATCC26933 background.

Gliotoxin prevents growth of *ΔgliT* strains

*ΔgliT* protoplasts grew and regenerated mycelia perfectly in the absence of gliotoxin (Figure 1A). The *ΔgliT* strain grew at identical rates to wild-type (data not shown). However, *ΔgliT* protoplasts were unable to grow in the presence of gliotoxin (10 μg/ml) (Figure 1A) whereas exogenous gliotoxin had no effect on wild-type growth. Subsequent phenotypic analysis of *A. fumigatus* ATCC46645, ATCC26933, and respective *ΔgliT* conidia (*ΔgliT*46645 and *ΔgliT*26933) demonstrated that gliotoxin (5 μg/ml) significantly inhibited *ΔgliT* growth on minimal medium and completely inhibited *ΔgliT* growth on both AMM and Sabouraud medium (gliotoxin, 10 μg/ml) (Figure 1B & C; *p*<0.0001 and Figure S2). Moreover, germination rates of *ΔgliT* strains were comparable to those of wild-type *A. fumigatus*, even in the presence of gliotoxin up to 10 μg/ml. These results clearly indicated that *ΔgliT* was highly sensitive to exogenous gliotoxin. Consequently, *ΔgliT*46645 and *ΔgliT*26933 mutant complementation was carried out by introducing *gliT* only (no antibiotic resistance gene) to complement *ΔgliT* with selection in the presence of gliotoxin (10 μg/ml). Transformants, which had recovered resistance to exogenous gliotoxin, were confirmed by Southern analysis to have an intact and functional copy of *gliT* present (Figure S1). This result confirms that *gliT* confers resistance to gliotoxin in *A. fumigatus* and that *ΔgliT* mutants have significant potential for future functional genomic studies involving *A. fumigatus* since gene deletions in this strain are selectable by *gliT* reintroduction, with selection in the presence of gliotoxin.

Remarkably, addition of reduced glutathione (GSH; 20 mM) to test plates completely abolished the cytotoxic effects of exogenous gliotoxin which indicated that *gliT* loss resulted in depletion of intracellular GSH, when exposed to gliotoxin, or that only the oxidized form of gliotoxin is imported into *A. fumigatus* (Figure 1B & C). Prior reduction of gliotoxin, using 50 mM NaBH₄, resulted in a statistically significant inhibitory effect of gliotoxin on growth of *ΔgliT*26933 (*p*<0.05) (Figure 1B & C). NaBH₄ was selected as...
reductant as it avoided complications associated with the introduction of additional thiols, or GSH, and the formation of gliotoxin conjugates, which may have resulted from GSH, DTT or β-mercaptoethanol-mediated reduction. It was also observed that GSH presence (8 mM) partially alleviated the growth inhibitory effects of gliotoxin (with or without prior reduction; p<0.01 and p<0.005, respectively) (Figure 1C). However, wild-type levels of growth were only achieved in the presence of 20 mM GSH (Figure 1B). The enhanced GSH-mediated alleviation of gliotoxin-induced cytostatic effects observed in ΔgliT suggest that depletion of intracellular glutathione may be a consequence of gliT loss. GSH-mediated relief of A. fumigatus ΔgliT growth inhibition, by exogenous NaBH4-reduced gliotoxin, indicates that intracellular GSH depletion plays a role in the inhibitory effect of gliotoxin and that GSH is merely acting to reduce exogenously added gliotoxin and prevent uptake (Figure 1B & C). Exogenous gliotoxin or reduced gliotoxin had no effect on growth of ΔgliZ (gliZ complemented strain) [14] (kind gifts from Professor Nancy Keller, University of Wisconsin-Madison) and an identical pattern was observed in the presence of GSH (data not shown). Moreover, A. fumigatus ΔgliH did not exhibit any phenotype when exposed to either H2O2 or phleomycin (data not shown). A. fumigatus gliT strains were resistant to exogenous gliotoxin (Figure 1D).

Gliotoxin induces expression of the gliotoxin gene cluster

gliZ, A and G encode the gliotoxin cluster transcription factor, transporter and a putative glutathione s-transferase (generally a detoxification enzyme), respectively, and all are conceivably involved in protection against gliotoxin toxicity [3,8,22]. Northern analysis showed that expression of these 3 genes plus gliT, from the gliotoxin gene cluster, was induced in A. fumigatus ATCC46645 within 3 h following gliotoxin (5 μg/ml) addition at 21 h.
Genes immediately adjacent to \(gltI\) in the gliotoxin gene cluster do not mediate resistance to exogenous gliotoxin

Sequence analysis of the 5' and 3' regions adjacent to the original \(gltI\) locus in \(A. fumigatus\) \(ΔgltI^{26933}\) confirmed that \(gltF\) was intact but revealed two mutations (C23R and E160G) in the open reading frame of a gene (AFUA_6G09745; identified as a conserved hypothetical protein at http://www.cadre-genomes.org.uk) but here termed \(glh_2\), located 3' with respect to the \(gltI\) locus. Although expression of \(gltF\) and \(glh\) was confirmed by RT-PCR in \(A. fumigatus\) \(ΔgltI^{26933}\) (Figure 2E), there was concern that the altered sequence of \(glh\) may have resulted in a mutant enzyme, which could possibly have also contributed to gliotoxin sensitivity in \(ΔgltI^{26933}\). However, \(A. fumigatus\) \(ΔgltF^{26933}\) grew in the presence of gliotoxin (10 \(\mu\)g/ml) (Figure 1D) which completely eliminated the possibility that this gene, located adjacent to \(gltI\) in the \(A. fumigatus\) genome, contributed to gliotoxin resistance and established, beyond question, the key role of \(gltI\) in mediating resistance to exogenous gliotoxin. \(A. fumigatus\) \(Δglh^{26933}\) (Figure S1) was also resistant to exogenous gliotoxin, as expected (Figure 1D).

Gliotoxin is not produced by \(A. fumigatus\) \(ΔgltI\)

Gliotoxin (500 ng/ml) was detectable in organic extracts from \(A. fumigatus\) ATCC26933 but not \(ΔgltI^{26933}\) cultures, grown under identical conditions, by RP-HPLC and LC-MS analysis (Figure 3). Gliotoxin production was recovered in \(A. fumigatus\) ATCC26933 \(ΔgltI^{26933}\) (Figure S4) Interestingly, \(ΔgltI^{26933}\) exhibited an identical phenotype to \(ΔgltI^{26933}\) which was generated from \(A. fumigatus\)

Figure 2. \(gltI\) expression. (A) Northern analysis of the induction of gliotoxin gene cluster expression in \(A. fumigatus\) ATCC26934 and \(ΔgltI\). Lanes 1, 2 and 3 correspond to \(A. fumigatus\) RNA extracts from 21 h AMM, 21 h AMM shifted to gliotoxin (5 \(\mu\)g/ml) for 3 h and 24 h AMM, respectively. (B) Gliotoxin induction of \(gltI\) expression in \(A. fumigatus\) strains. Lanes 1–6 and 7–12 contain RNA from strains in the ATCC26934 and ATCC26933 backgrounds, respectively. Lanes 1 and 7: \(A. fumigatus\) \(ΔgltI\) 24 h AMM; Lanes 2 and 8: \(A. fumigatus\) \(ΔgltT\) 21 h AMM +3 h gliotoxin (5 \(\mu\)g/ml); Lane 3 and 9: \(A. fumigatus\) \(ΔgltI^{24}\) 24 h AMM; Lanes 4 and 10: \(A. fumigatus\) \(ΔgltI^{21}\) 21 h AMM+3 h gliotoxin (5 \(\mu\)g/ml); Lanes 5 and 11: \(A. fumigatus\) \(ΔgltF\) 24 h AMM; Lane 6 and 12: \(A. fumigatus\) \(ΔgltI\) 21 h AMM +3 h gliotoxin (5 \(\mu\)g/ml). (C) Expression of \(gltI\) in \(Δgliz\) following exposure to gliotoxin. Cultures of \(A. fumigatus\) ATCC26934 (lanes 1 and 2) and \(Δgliz\) (lanes 3 and 4) were grown for 24 h in AMM (Lane 1 and 3) or pulsed with gliotoxin (5 \(\mu\)g/ml) after 21 h and cultured for a further 3 h (Lane 2 and 4). Although gliotoxin induced expression of \(glh\) and \(glg\) in wild-type, neither \(glh\) or \(glg\) are expressed in \(Δgliz\). All Northern analyses were performed with 10 \(\mu\)g of total RNA isolated from strains grown in AMM for 24 h with or without gliotoxin. (D) \(A. fumigatus\) \(gltJ\) expression and identification. Quantitative 2-DPAGE analysis confirmed increased expression (threefold) of \(gltI\) following exogenous gliotoxin (GT) addition to \(A. fumigatus\) cultures (\(gltI\) appears to exist as two isoforms of different pi (5.5–5.6) and Mr). Peptides identified by MALDI-ToF mass spectrometry are highlighted in bold (33% sequence coverage) and mass spectrum is given in Figure S3. (E) Semi-quantitative RT-PCR of \(gltI\) adjacent genes in \(A. fumigatus\) wild-type (wt) (ATCC26933) and isogenic mutant strains. Expression of \(gltF\) and AFUA_6g09745 (\(glh\)) was examined. As a control \(tub\) expression was monitored. As a negative control genomic DNA (gDNA) was used as template. Lane 1: \(A. fumigatus\) \(ΔgltI^{26933}\) 24 h AMM. Lane 2: \(A. fumigatus\) \(ΔgltI^{26933}\) 21 h AMM+3 h gliotoxin (5 \(\mu\)g/ml). Lane 3: \(A. fumigatus\) \(ΔgltI^{24}\) 24 h AMM. Lane 4: \(A. fumigatus\) \(ΔgltI^{21}\) 21 h AMM+3 h gliotoxin (5 \(\mu\)g/ml). Lane 5: \(A. fumigatus\) \(ΔgltF\) 24 h AMM. Lane 6: \(A. fumigatus\) \(ΔgltI\) 21 h AMM+3 h gliotoxin (5 \(\mu\)g/ml).

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ATCC46645, yet gliotoxin production was undetectable, under the culture conditions employed, in both A. fumigatus ATCC46645 and ΔgliT strains, indicating that sensitivity to exogenous gliotoxin is not associated with a de novo gliotoxin biosynthetic capacity. A metabolite with retention time (Rt) = 11.7 min (λ220 nm) was apparent in ΔgliT extracts which was absent in wild-type extracts (Figure 3). This material was purified to assess any growth inhibitory effect, however when added to AMM cultures of ΔgliT or wild-type no alteration of growth rates was observed (data not shown). High resolution LC-ToF MS analysis of the metabolite (from Figure 3B) confirmed the presence of a molecular ion with a mass of 279.0796 m/z (M+H)+ (Figure S4). This accurate mass value (279.0796 m/z) corresponded to a predicted molecular formula of C13H15N2O3S for the ion whereby the calculated exact mass for C13H15N2O3S + H+ was 279.0798 Da using Agilent Technologies Masshunter workstation software. This result suggests that a monothiol form of gliotoxin could have been secreted from A. fumigatus ΔgliT. A molecular species of m/z 279, which yielded daughter ions of m/z 261.1, 231.0 and 203.1, upon MS2 analysis, was also detected by LC-MS analysis of the purified gliotoxin-related metabolite from A. fumigatus ΔgliT (Figure S4). Gliotoxin was not produced by A. fumigatus ΔgliH (Figure S4) which strongly supports a role for this gene in gliotoxin biosynthesis or secretion, but not protection against exogenous gliotoxin. This result was further consolidated whereby no gliotoxin production was detectable, by HPLC-DAD or LC-MS, in A. fumigatus ΔgliTΔgliH (data not shown), which was generated by restoration of the fully intact gliH in gliT-deficient A. fumigatus (Figure S1).

GliT exhibits a gliotoxin reductase activity

Recombinant GliT was expressed in, and purified by differential extraction from, E.coli with a yield of approximately 5.7 mg per gram of cells. However the protein was completely insoluble and was refractory to any attempts at refolding for activity analysis (data not shown). SDS-PAGE analysis confirmed a subunit molecular mass of 38 kDa for recombinant GliT (Figure S5), which appears to migrate as a dimer under non-reducing conditions (Figure S3), and protein identity was unambiguously confirmed by MALDI-ToF MS whereby peptides (following tryptic digestion) were identified yielding 21% sequence coverage (Figure S6). Immunoaffinity purification of GliT-specific human IgG was achieved by incubation of human sera with Sepharose-coupled recombinant GliT. The specificity of this GliT-specific human IgG was confirmed by the successful detection of native GliT in both A. fumigatus cell lysates, and partially-purified extracts of A. fumigatus (Protocol S1; Figure 4). Notably, GliT was not detectable in A. fumigatus ΔgliT (Figure 4).

Previous hypotheses have suggested that GliT may only exhibit gliotoxin oxidase activity (responsible for disulphide bridge closure during biosynthesis) (3, 8, 22). However, following gliotoxin induction of A. fumigatus ATCC46645, enhanced GliT activity was evident in cell lysates and native GliT was partially purified by ammonium sulphate precipitation and ion-exchange chromatography (Figure S7). Data presented in Figure 5A confirm that partially-purified native GliT specifically catalyses the NADPH-mediated reduction of oxidized gliotoxin, whereby NADPH oxidation is only evident in the presence of both gliotoxin (9 μM) and GliT-containing lysates. Hence, GliT appears to exhibit gliotoxin reductase activity which can catalyse disulphide bridge cleavage, at concentrations up to 9 μM gliotoxin (Figure 5B). This activity is inhibited at higher gliotoxin concentrations (>12 μM). Not unexpectedly, A. fumigatus cell extracts appear to contain basal NADPH oxidase activity which yields background, non-specific NADPH oxidation (Figure 5A). Thus, A. fumigatus ATCC46645 and ΔgliT lysates, generated without prior gliotoxin induction of GliT expression, exhibit near-identical activity. However, significantly greater gliotoxin reductase activity (2.1) was apparent in A. fumigatus ATCC46645, than ΔgliT, cell lysates following gliotoxin exposure (Figure 5C).
Immunoprecipitation of GliT from partially purified *A. fumigatus* cell lysates (Figure S7) using human IgG [anti-GliT] resulted in a 51% reduction of gliotoxin reductase (NADPH oxidase) activity (Figure 5D), in complete accordance with data in Figure 5C, further confirming enzyme specificity. Interestingly, GliT activity was not enhanced in the presence of thioredoxin from *Spirulina* sp., in activity assays, which indicates that GliT is specific for gliotoxin reduction and that it may operate independently of cellular thioredoxin reductase/thioredoxin systems.

Expression of GliT in *A. fumigatus* was further explored by fluorescence confocal microscopy. Data in Figure S8A-C confirm transformation of *A. fumigatus ΔgliT* and that *gliT-gfp* expression is enhanced by gliotoxin addition. As shown in Figure S8A, it appears that low-level GliT expression is evident throughout mycelia without gliotoxin addition. However, following mycelial exposure to gliotoxin (5 μg/ml), an enhancement of GliT expression in the cytoplasm, and in nuclei, as shown by fluorescence intensities (Figure S8B & C), is observed - which is in

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**Figure 4. Immunoaffinity purified human IgG detects native GliT in A. fumigatus.** (A) SDS-PAGE and (B/C): Western blot analysis of *A. fumigatus* cell lysates. Immuno-affinity purified human IgG[anti-GliT] was used for Western analysis followed by anti-human IgG-HRP conjugate with visualization by either (B) dianinobenzidine or (C) ECL detection. Lane M: Mr marker; Lane 1: *A. fumigatus* ATCC26933 lyse (72 h culture); Lane 2: *A. fumigatus* ATCC46645 lysate (24 h culture); Lane 3: *A. fumigatus* ATCC46645 ΔgliT lysate (24 h culture) and Lane 4: Recombinant GliT (2 μg). Immunooaffinity purified human IgG to GliT identified GliT in all except *A. fumigatus ΔgliT*, however ECL substrate was required to detect low level GliT expression in *A. fumigatus* ATCC46645 (lane C.2).

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This activity is inhibited at higher gliotoxin concentrations (ATCC46645 with gliotoxin addition during culture. Wild-type lysates exhibit enhanced gliotoxin reductase activity (47%) consequent to *A. fumigatus* elevated GliT expression. (D) Immunodepletion of GliT from semi-purified *S. cerevisiae* IgG [anti-GliT], results in a 51% decrease in gliotoxin reductase activity.

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Figure 5. GliT exhibits a gliotoxin reductase activity. (A) No GliT activity (gliotoxin reductase) is detectable in the absence of gliotoxin or GliT (1 & 2). Background NADPH oxidase activity is detectable in semi-purified *A. fumigatus* cell extracts (3) (Figure S5), however, GliT-mediated gliotoxin reductase activity is detectable upon addition of gliotoxin (4). (B) In vitro, optimal GliT gliotoxin reductase activity is observed up to 9 μM gliotoxin. This activity is inhibited at higher gliotoxin concentrations (>12 μM). (C) Relative gliotoxin reductase activity in cell lysates from ΔgliT compared to *A. fumigatus* ATCC46645 with gliotoxin addition during culture. Wild-type lysates exhibit enhanced gliotoxin reductase activity (47%) consequent to elevated GliT expression. (D) Immunodepletion of GliT from semi-purified *A. fumigatus* cell extracts (Figure S5), using immunoaffinity purified human IgG [anti-GliT], results in a 51% decrease in gliotoxin reductase activity.

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complete agreement with proteomic, molecular and enzyme activity observations. Expression of GliT-GFP fusion protein completely restored gliotoxin resistance (10 μg/ml), although colonies appeared white (Figure S9). The concordance of these data lead us to conclude that a GliT-mediated gliotoxin reductase activity is induced by exposure of *A. fumigatus* to gliotoxin.

**GliT is not required for *A. fumigatus* virulence in *Galleria mellonella***

A prerequisite for testing *A. fumigatus* ΔgliT virulence was to evaluate the utility of our *G. mellonella* infection model. To this end, assessment of the relative virulence of *A. fumigatus* ΔgliT and corresponding wild-type in *G. mellonella*, in either the presence or absence of added gliotoxin, was assessed (Figure S10). Here, all *Galleria* exposed to *A. fumigatus* ΔgliT were alive at 24 h and the wild-type strain exhibited greater virulence than ΔgliT (60% (12/20) versus 20% (4/20) mortality, respectively), at 48 h post-inoculation, thereby confirming the utility of the model system for detection of alteration in virulence associated with gliotoxin production. To assess now the relative contribution of gliT to virulence of *A. fumigatus* we compared the survival of larvae of the greater wax moth *G. mellonella* following infection with 10⁶ conidia/larvae of *A. fumigatus* ATCC26933 and gliT* to that of larvae (n = 20) infected with the same dose of ΔgliT (Figure S10). For all groups of infected larvae, 100% mortality was recorded after 72 h and the degree of melanisation was not distinguishable between these groups. Also, pretreatment of larvae with gliotoxin (0.5 μg/larva in 20 μl) did not lead to an attenuation of virulence of ΔgliT (Figure S10). Notably, similar results were obtained using ATCC46645 and ΔgliT-ATCC46645 strains (data not shown). These results clearly show that, gliT has a minimal, if any, role to play in the virulence of *A. fumigatus* employing a Galleria model.

**GliT confers protection against exogenous gliotoxin in *Aspergillus nidulans* and *Saccharomyces cerevisiae***

Reintroduction of gliT into *A. fumigatus* ΔgliT was selected for in the presence of gliotoxin and no additional selection marker was required (Figure S1 and Figure 1D). To further test the ability of gliT to confer resistance to gliotoxin, and its future potential as a selection marker gene, we introduced gliT into *A. nidulans* which does not produce gliotoxin and neither does it contain any genes involved in gliotoxin biosynthesis [22,23]. The absence of gliT, and cognate gene expression, in *A. nidulans* was confirmed by Southern and Northern analysis (Figure 6A & B). Subsequent transformation of *A. nidulans* with *A. fumigatus*-derived gliT resulted in the generation of three transformants (Δ*gliT* 1, 2 and 3) (Deposition number: IMI CC 396693), which were shown by Northern analysis to express gliT to different extents (Figure 6B). This led to acquisition of resistance to high levels of exogenous gliotoxin (50 μg/ml) (Figure 6C) thereby confirming the key role of gliT in protection against gliotoxin toxicity in gliotoxin-naïve fungi. The gliT coding sequence was also transformed into the genetically distant yeast, *S. cerevisiae* BY4741, under control of the constitutive SSA2 promoter [24] in plasmid pC210. As can be seen in Figure 6D, yeast transformed with plasmid-encoded gliT were capable of growth in the presence of gliotoxin (16 and 64 μg/ml, respectively) depending on whether minimal or rich media was used to support growth, while those transformed with empty vector were unable to grow, irrespective of what media conditions were used. These observations further confirm the pivotal role of...
gliT in mediating resistance to gliotoxin, even in fungal species which do not normally contain the gene or biosynthesise gliotoxin.

Discussion

Studies into the biosynthesis and pathogenicity of gliotoxin have attracted significant recent attention, stimulated in part by the plethora of fungal genome data now emerging [3,22]. Here, we demonstrate for the first time that disruption of gliT, which do not normally contain the gene or biosynthesise gliotoxin, even in fungal species which do not normally contain the gene or biosynthesise gliotoxin.

Exposure of A. fumigatus ΔgliT to gliotoxin appears to result in depletion of intracellular GSH since the inhibitory phenotype can be completely relieved by GSH supplementation. Furthermore, we demonstrate the enzymic functionality of GliT as a gliotoxin reductase and that GliT reactivity is evident in human sera. We demonstrate that gliT confers resistance to exogenous gliotoxin, independently of the extent of gliT expression, following transformation in naive hosts, A. nidulans and S. cerevisiae. Finally, identification of gliT complementation in A. fumigatus ΔgliT ΔgliA46645 and Δ"nss", respectively, was selected for in the presence of gliotoxin which supports a selection marker role for gliT in A. fumigatus transformation experimentation.

To date, the potential requirements for self-protection against gliotoxin, in A. fumigatus, have not been studied. The ETP toxin, sirodesmin, is produced by the fungus Leptosphaeria maculans with biosynthesis encoded by a multigene cluster similar to that responsible for gliotoxin production in A. fumigatus [13]. Deletion of the sirodesmin transporter gene, sirA, in L. maculans led to increased sensitivity to exogenous sirodesmin and gliotoxin, however the A. fumigatus gliotoxin transporter, GliA, was shown to confer resistance to exogenous gliotoxin (10 μM), but not sirodesmin, in L. maculans ΔsirA. Interestingly, production and secretion of sirodesmin was actually increased by 39% in L. maculans ΔsirA compared to wild-type and resulted in speculation as to the presence of alternative toxin efflux mechanisms [13]. Based on our observations, we hypothesise that in addition to the likely role of gliA in gliotoxin efflux in A. fumigatus, GliT may play an essential role in the auto-protective strategy against the deleterious effects of the ETP toxin. Moreover, we predict that gliT orthologs in other fungi [22] may play similar, if not identical roles.

Our results indicate that absence of GliT may lead to accumulation of intracellular gliotoxin which is reduced, non-enzymatically, by GSH, analogous to the situation in animal cells as demonstrated by Bernardo et al. [17]. The concomitant depletion of intracellular GSH levels, allied to the cytotoxicity of reduced gliotoxin, results in strong growth inhibition, possibly mediated by disruption of the cellular redox status and significant protein modification by gliotoxin. This conclusion is strongly supported by the observation that addition of GSH, during exposure of A. fumigatus ΔgliT to gliotoxin, effectively completely reverses the cytostatic effects of gliotoxin. While we cannot exclude the possibility that added GSH is merely reducing exogenously added gliotoxin and preventing import of the reduced form, it is...
clear from Figure 1 that addition of NaBH₄-reduced gliotoxin results in significant growth inhibition of *A. fumigatus* ΔgliT (p<0.05). The observed alleviation of this inhibition (by NaBH₄-reduced gliotoxin), in the presence of added GSH, supports the proposal that intracellular GSH depletion is a consequence of gliT disruption, when growth occurs in the presence of exogenous gliotoxin.

Addition of gliotoxin (up to 20 µg/ml) for 24 h resulted in the complete up-regulation of the gene cluster (except gliP) in *A. fumigatus* ΔgliP, but not in *A. fumigatus* wild-type [9]. We demonstrate that exposure to exogenous gliotoxin for 3 h does induce GliT expression in *A. fumigatus* wild-type at the transcript and protein level, in fact these data also represent the first confirmed identification of a protein encoded by the gliotoxin biosynthetic cluster. The discrepancy, possibly due to 3 versus 24 h experimental windows, nonetheless, indicates differential GliT expression relative to other gli genes. Disruption of *gliZ*, the transcriptional regulator of the gliotoxin biosynthetic cluster, has been shown to result in abolition of gliotoxin production and loss of gliotoxin cluster gene expression [14]. Our data demonstrate that although growth of *A. fumigatus* ΔgliZ and ΔgliG is unaffected by exogenous gliotoxin, *gliZ* expression is up-regulated in response to exogenous gliotoxin exposure in *A. fumigatus* ATCC62645, but to a lesser extent than that of gliT (Figure 2). In addition, we have shown that gliT expression is induced by gliotoxin addition to liquid cultures of *A. fumigatus* ΔgliZ thereby confirming the independence regulation of gliT expression to other cluster components (e.g., gliA and gliG). In combination, these observations further confirm the minimal role played by any other component of the gli gene cluster in protection against gliotoxin presence since *gliZ* absence results in complete cluster attenuation [14], except for gliT.

A thioredoxin system in *A. nidulans* has recently been described whereby a thioredoxin mutant exhibited decreased growth, impaired reproductive function and altered catalase activity [25]. These authors also identified a thioredoxin reductase (termed AnTrxR) which functions to regenerate reduced thioredoxin in *A. nidulans*. Our BLAST analysis indicates minimal identity between GliT and AnTrxR as well as between GliT and a second putative thioredoxin reductase in *A. fumigatus* (Genbank accession number: EAL83952; 30% identity). This strongly indicates distinct functionality of gliT and confirms that alternative thioredoxin reductase activities cannot compensate for loss of gliT in *A. fumigatus*. It further appears unlikely that thioredoxin is involved in mediating GliT activity since no thioredoxin reductase present in *A. fumigatus* cell lysates appears capable of compensating for GliT absence. Consequent to its bioinformatic classification as a thioredoxin reductase, GliT has been predicted by many authors to encode disulfide bond formation in gliotoxin and to play a role in gliotoxin biosynthesis [3,8,22]. While this ‘gliotoxin oxidase’ activity cannot be ruled out completely, our demonstration that GliT exhibits gliotoxin reductase activity (Figure 5) suggests that direct gliotoxin reduction is a pre-requisite for secretion from *A. fumigatus* via a GliT-mediated pathway or as a component of the auto-protective mechanism deployed against exogenous gliotoxin secreted by adjacent fungi in the environment (Figure 7). This hypothesis is firmly supported by the absence of gliotoxin secretion in *A. fumigatus* ΔgliT([25]). Given the potential of reduced gliotoxin to thiolate cellular proteins, we speculate that reduced gliotoxin may be sequestered into intracellular vesicles where it is converted to the oxidized form, by an unidentified activity, prior to release from the cell by an exocytic mechanism complementary to GliA-mediated efflux (Figure 7). It remains possible that GliT-mediated gliotoxin oxidase activity may be associated with disulfide bridge closure during gliotoxin biosynthesis when intracellular levels of gliotoxin can be regulated more precisely by the organism. Thus, GliT could be necessary to maintain a balance between reduced and oxidised gliotoxin in *A.

![Figure 7. A proposed model for GliT functionality in *A. fumigatus* based on experimental observations.](image-url)

Exogenous gliotoxin enters *A. fumigatus* (1) and is converted to the reduced form intracellularly by GliT (gliotoxin reductase activity (2)). GliT may also be necessary to oxidize reduced gliotoxin during biosynthesis in *A. fumigatus*. Given the toxicity of the intracellular form of reduced gliotoxin, we predict that it may be imported into intracellular vesicles, possibly with concomitant oxidation for storage (3). GliA function to facilitate gliotoxin efflux (4) is extrapolated from the observation in *L. maculans* that this protein confers resistance to exogenous gliotoxin [13]. In the absence of GliT, gliotoxin may be alternately reduced by intracellular GSH (5) leading to a depletion in GSH and cell death/growth arrest and also modification of other cellular proteins leading to inactivation or activity modification (6). In this model, absence of GliT would lead to the build up of gliotoxin within the cell and also the inability to reduce exogenously added gliotoxin. Reduced gliotoxin may not enter but converts to the oxidized form in a time-dependent manner (7, 7A).

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Aspergillus spp. was co-cultured, at both 30 and 37°C. A. regio, gliotoxin production has been detected when A. fumigatus was observed against methicillin-resistant A. fumigatus. A. nidulans, where the latter does not produce gliotoxin is somewhat at variance with the A. fumigatus ΔgliZ findings. We suggest that alterations in the levels of additional metabolites in A. fumigatus ΔgliZ as noted in [14], or a possible cytotoxic role in G. mellonella for the putative monothiol form of gliotoxin secreted by A. fumigatus ΔgliT may account for this dichotomy. Our demonstration that gliT is expressed independently of other cluster components implies that previous virulence model experimentation, involving gliP- and gliZ- deficient mutants [9–12], may require interpretation in light of the possibility of independently regulated gliT expression, or GliT functionality. Indeed, if it is ever demonstrated that gliT expression occurs in the absence of gli cluster expression/gliotoxin biosynthesis (as has been demonstrated herein for A. fumigatus ΔgliZ), or is regulated by factors other than exposure to exogenous gliotoxin, then consideration may need to be given to this phenomenon in future studies. This consideration is based on the fact that independent regulation of gliT may have enabled acquisition of functionality beyond a role in gliotoxin biosynthesis or auto-protection.

Genetic modification of filamentous fungi for the improved production of food additives, industrial enzymes or pharmaceuticals is an ongoing requirement of the biotechnological industry [26,27]. Antibiotic-producing fungi are continually subjected to strain improvement, with a concomitant requirement for new selection markers, to increase product yield and decrease the level of unwanted side-products [28]. Our observation that gliT complementation in A. fumigatus can be selected for in the presence of gliotoxin, without the use of conventional selection markers, and that transformation of A. nidulans and S. cerevisiae with gliT confers enhanced resistance to gliotoxin offers the possibility of using the gliT/gliotoxin combination to select for fungal transformation. Moreover, acquired gliotoxin resistance in A. nidulans and S. cerevisiae resulting from gliT presence, underpins the important role played by this gene in mediating resistance to exogenous gliotoxin. Gliotoxin isolated from cultures of a marine fungus from the genus Pseudallescheria has been shown to possess both anti-bacterial and free-radical scavenging capability whereby an MIC50 of 1 µg/ml was observed against methicillin-resistant Staphylococcus aureus [29]. Gliotoxin may also provide a competitive advantage for A. fumigatus when grown in the presence of other fungi [30]. In this regard, gliotoxin production has been detected when A. fumigatus was co-cultured, at both 30 and 37°C, with a range of other Aspergillus spp., leading the authors to speculate that co-expression of resistance genes may allow toxin producers to resist the effects of their own biological arsenal in competitive co-culture situations [30]. The parallel between this supposition, and our observation of GliT-mediated resistance to exogenous gliotoxin, is vivid.

The vast majority of literature surrounding the role of gliotoxin in A. fumigatus focuses on its function as a cytotoxic molecule which has deleterious effects on cells within infected individuals and exhibits anti-microbial activity [5,6,9–12,29,30]. However, based on our observations and significant other literature [16,18,31], a credible alternative hypothesis is that gliotoxin may actually be part of the intracellular antioxidant defense system within A. fumigatus, and is a molecule, analogous to thioredoxin or 2-ctys peroxiredoxin, which may undergo rapid changes in redox status to buffer against specific exogenous or endogenous oxidants. In other words, the cytotoxic effects of gliotoxin in infected host cells may actually be an indirect consequence of its role within A. fumigatus. This alternative hypothesis is not without support. Firstly, Watanabe et al. [31] have shown that the cytotoxicity of A. fumigatus culture filtrates was significantly attenuated, or absent, when cultures were grown under reduced aerobic or anaerobic conditions. Interestingly, gliotoxin production was detectable by GC-MS analysis from aerobic but not in reduced aerobic culture supernatants. Although Watanabe et al. concluded that their results indicated that gliotoxin production is increased to facilitate fungal pathogenicity (mimicking the aerobic lung environment), an alternative conclusion, which is in accordance with our thinking, is that gliotoxin production is actually elevated to cope with increased oxygen levels and that secretion of gliotoxin forms part of the gliotoxin homeostasis control mechanism within A. fumigatus to prevent the side-effect of intracellular oxidative stress. As noted earlier, in animal cells it has been shown that gliotoxin may substitute for 2-ctys peroxiredoxin activity in HeLa cells by accepting electrons from NADPH via the thioredoxin reductase–thioredoxin redox system to reduce H2O2 to H2O. In this way, nanomolar levels of gliotoxin may actually protect against intracellular oxidative stress [18]. Additionally, as demonstrated by Srinivasan et al. [16], oxidized gliotoxin facilitates selective protein inactivation in the presence of molecular oxygen which, we hypothesise, could prevent global intracellular damage due to resultant reactive oxygen species. Moreover, a protective role for gliotoxin against environmental stress in A. fumigatus has been considered [2,13]. Our observations and consequent hypothesis now provide a vehicle to explore this proposal.

In summary, we have demonstrated that GliT plays a major auto-protective role against gliotoxin toxicity in A. fumigatus which points to alternative gliotoxin functionality in A. fumigatus. From a utilitarian viewpoint, gliT/gliotoxin sensitivity represents a potential new selection marker strategy for fungal transformation. The transfungal implications of our observations remain to be explored.

Materials and Methods

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. Ethical permission was obtained from The Ethics Committee of NUI Maynooth for the use of human serum specimens. Anonymous serum specimens were obtained with the signed agreement of the Irish Blood Transfusion Service.

Strains, growth conditions, and general DNA manipulation

In general, A. fumigatus strains (Table 1) were grown at 37°C in Aspergillus minimal media (AMM). AMM contained 1% (w/v)
genomic DNA was purified using a ZR Fungal/Bacterial DNA Kit used for general plasmid DNA propagation and

A. fumigatus

10^4 conidia of the respective strains were point inoculated on Erlenmeyer flasks inoculated with 10^8 conidia. For growth assays, Liquid cultures were performed with 200 ml AMM in 500 ml A. fumigatus

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glucose as carbon-source, 5 mM ammonium tartrate as nitrogen-source, and trace elements according to Pontecorvo et al. [32]. Liquid cultures were performed with 200 ml AMM in 500 ml Erlenmeyer flasks inoculated with 10^8 conidia. For growth assays, 10^8 conidia of the respective strains were point inoculated on AMM plates, containing the relevant supplements and incubated for 48 h at 37°C.

TOPO TA cloning system (Invitrogen) and TOP10 E. coli cells (F-merA Δmer-hisRMS-merBC) ϕ80lacZAM15 ΔlacX74 recA1 araD139 galU galK Δ (ara-leu-7697) rpsL (Str^R) endA1 nupG) were used for general plasmid DNA propagation and A. fumigatus genomic DNA was purified using a ZR Fungal/Bacterial DNA Kit (Zymo Research).

glT26933 and ATCC26933 were co-transformed with two DNA constructs, technique was used [20]. Briefly, for flanking region B (1.2 kb). Subsequent to gel-purification, the amplified from ATCC46645 genomic DNA using primer ogliT1 of PCR generated each fragment. First, each flanking region was the intact resistance gene at the site of recombination. Two rounds gliHgfp homologous integration of each fragment into the genome flanking recombination site during transformation. During transformation, 557 bp overlap within the ptrA cassette, which served as a potential cassette, which served as a potential selection marker was released from plasmid pSK275 (a kind gift of SpeI described above. For generation of gliT, two overlapping fragments were amplified from the ligation products using primers ogliT-5 and optrA-2 for fragment C (2.6 kb) and primers ogliT-6 and optrA-1 for fragment D (2.2 kb). Subsequently ATCC46645 and ATCC26933 were transformed simultaneously with the overlapping fragments C and D. In the generated mutant allele of ΔgliT- ptrA the deleted region comprises amino acids 1–325 of gliT.

For reconstitution of the ΔgliT strain with a functional gliT copy, a 3.2 kb PCR fragment, amplified using primers ogliT-5 and ogliT-6, was subcloned into pCR2.1-TOPO (Invitrogen). The resulting 7.1 kb pgliT was linearised with AatI and used to transform A. fumigatus ΔgliT protoplasts. Taking advantage of the decreased resistance of the ΔgliT mutant to exogenously added gliotoxin ΔgliT protoplasts were transformed with pgliT and screened for wild-type resistance to gliotoxin for genetic complementation. Positive deletion- and reconstituted-strains were screened by Southern analysis (Figure S1) and DIG-hybridisation probes were generated using primers ogliT-5 and ogliT-4.

To obtain knock-out constructs for the deletion of gliH a 5′ flanking region with oligos ogliH1 and ogliH4 was amplified. For the 3′ flanking region a PCR with oligos ogliH2 and ogliH3 was performed. Amplicons were digested with SpeI and HindIII, respectively. Resulting fragments were ligated to a ptrA cassette, released from pSK275 via SpeI and HindIII digest. Final PCR products were obtained using oligos ogliH5/optrA2 and ogliH6/optrA1 and used for transformation.

To complement ΔgliH and ΔgliT^T325 with a functional copy of gliH, oligos ogliI7 and M13 were used to amplify a PCR fragment using pgliT as template. This fragment digested with EcoRI and SacI was cloned into pBS-KS (Stratagene), resulting in pgliH. Together with pAN7-1 [33], pgliH was used to complement A. fumigatus ΔgliH and ΔgliT^T325.

GliT was C-terminally fused in frame to gfp (green fluorescent protein) to determine its subcellular localisation. To this end, a fragment containing gliT was amplified using oligos ogliT-5-SphI and ogliT-16. The resulting 2.2 kb fragment was sub-cloned into pCR2.1-TOPO (Invitrogen) and sequenced. Via SpeI digest a fragment containing the gliT promoter region and the coding sequence was released and cloned into the corresponding SphI site of pgfp, resulting in pgliTgfp. To obtain pgfp, a gfp containing fragment was released from pUCG-H [34] via SmaI and SacI, and subcloned into the corresponding Eco RV and SacI sites of pGEM5zf+ (Promega). The plasmid pgliTgfp was used to transform ΔgliT protoplasts via co-transformation using a phleomycin resistance gene. Phleomycin resistant transformants carrying an in-frame gliTgfp fusion were used to localize GliT using fluorescence microscopy. Positive, GliT-GFP harbouring strains were screened by Southern analysis and hybridization probes were generated using oligos ogliT-7 and ogliT-8. A. fumigatus transformation was carried out according to Tilburn et al. [35]. In order to obtain homokaryotic transformants, colonies from single homokaryotic spores were picked and single genomic integration was confirmed by PCR (data not shown) and Southern blot analysis.

Table 1. A. fumigatus and A. nidulans strains used in this study.

| Strain            | Genotype          | Reference          |
|-------------------|-------------------|--------------------|
| ATCC46645         | Wild-type         | Hearn et al. [43]  |
| ATCC26933         | Wild-type         | Taylor et al. [44] |
| gliIZ             | gliIZ::pyrG       | Bok et al. [14]    |
| gliT^PDR          | gliIZ::gliZ::pyrG  | Bok et al. [14]    |
| ΔgliT^PDR         | ATCC46645::gliT::ptrA | This study |
| ΔgliT^T325        | ATCC26933::gliT::ptrA | This study |
| ΔgliT^T325pDR     | ATCC26933::gliT::ptrA | This study |
| ΔgliT^T325        | ΔgliT::gliT::gliT | This study |
| ΔgliT^T325        | ΔgliT::gliT::gliT | This study |
| ΔgliH             | ATCC26933::gliH::ptrA | This study |
| ΔgliT^T325        | ΔgliT::gliT::gliT | This study |
| ΔgliT^T325        | ΔgliT::gliT::gliT | This study |

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P. J. Sabouraud media followed by gliotoxin addition for 3 h (final

Northern analysis

RNA was isolated using TRI-Reagent (Sigma-Aldrich). Equal concentrations of total RNA (10 μg) were size-separated on 1.2% agarose-2.2 M formaldehyde gels and blotted onto Hybond N+ membranes (Amersham Biosciences). The hybridisation probes used in this study were generated by PCR using primers ogliA1 and ogliA2 for AFUA_6G09710, ogliG8 and ogliG16 for AFUA_6G09690, ogliT7 and ogliT8 for AFUA_6G09740, and ogliZ1 and ogliZ2 for AFUA_6G09630. All primers used in this study are listed in Table 2.

Proteomic analysis of GliT expression

A. fumigatus ATCC26933 was cultured (n = 3) for 21 h in Sabouraud media followed by gliotoxin addition for 3 h (final
Table 2. Primers used in this study.

| Primer   | Sequence (5'-3') |
|----------|-----------------|
| ogliA-1  | TGG ATC GTT GAT CTG CGC |
| ogliA-2  | ATG GCC TGG TAT CCG ATC |
| ogliG-7  | GAC CCT CCG ATC TTG TAG |
| ogliG-8  | TTC TCG CCA TGG TCA AAC |
| ogliT-1  | AGC GTA GAC AGG TTG TAG |
| ogliT-2  | GCA GAC GTC TAG CAT GGA CTG G |
| ogliT-3  | GCT AGT CTG CCG GAG TTT CGT CTC |
| ogliT-4  | GCA GTA ATG GCG GAG ATG AGT GG |
| ogliT-5  | TCT GCG CAT CTT GAT CGG |
| ogliT-6  | ACG GTC CTG GGA ATC ATC |
| ogliT-7  | GTG GAC CTC ATC ATC |
| ogliT-8  | GCC GGC CAT AAC CAC GAC |
| ogliT-S-SphI | CGG CAT GCT CGG TTC TTG ATC |
| ogliT-16 | AAA GCA TSG TAG TCTCG TAC GAG ACG |
| ogliI-1  | GCT ATG CAT GGT CAG TCG |
| ogliI-2  | CGG CCA TGC TAA TAC TGC |
| optrA1   | GAG GAC CTG GAC AAG TAC |
| optrA2   | CAT GCT GAC CAG TAC |
| ogliIgG  | CCA GAT CTA TGT CGA TGG GAC AAG TAC |
| ogliI-NTd | ATA GCC GCC GCC TAT AGC TCC TGA TCG AGA |
| ogliH1   | CAT GCA CAA CTG CCT CGG ATG |
| ogliH2   | GCT CCT GGG GAT TCT GAG CGC |
| ogliH3   | AAC AAG CTT AGA AGG GCC AGT TCG GAC |
| ogliH4   | GCT ACT AGA GAT CTG TCT GCC ATC |
| ogliH5   | TCC ACC ATC CAG TCC CAG |
| ogliH6   | GCG GTG CAG TGA ACT AAC |
| M13      | GTAAAACGACGGCCAGT |
| M13rev   | AAGCAGCTATGACAGCTAG |
| Sc-gliT-F | CCCCCGGCATATGGCTAGCCGCAAACTACTCTECAAC |
| Sc-gliT-R | CCCCCGGCATCGCATAGCTAGCCTGATGAGACAGAAC |

Added restriction enzyme sites are underlined.

Concentration: 14 μg/ml. Control cultures (n = 3), where gliotoxin was not added, were also performed. Mycelia were harvested, lysed and subject to MALDI-ToF mass spectrometric analysis as previously described [36] and Imagemaster analysis (GE Healthcare).

Analysis of gliotoxin production
To analyze gliotoxin, or related metabolite production, A. fumigatus wild-type and mutant strains were grown up at 37°C for 72 h in Czapeks-Dox. Supernatants were chloroform extracted overnight and fractions were lyophilized to complete dryness. Samples were resuspended in MeOH and analysed using a reversed phase HPLC as described in [37] and LC-MS (Agilent 6340 ETD LC-MS system). Samples (1 μl) were loaded onto a Zorbax 300SB C-18 Nano-HPLC Chip (150 mm ×75 μm, Agilent) with 0.1% (v/v) formic acid (0.6 μl/min), and compounds eluted at an increasing 0.1% (v/v) formic acid, acetonitrile gradient (90% (v/v) final). Eluted compounds were directly ionised and analysed by ion trap mass spectrometer (Agilent). For each round of MS the two most abundant compounds were automatically selected for MSn analysis. Gliotoxin was identified by its whole mass of 326.4 m/z and its characteristic MS2 fragmentation pattern (263, 245 and 227 m/z). LC-ToF analysis was performed using an Agilent HPLC 1200 series using electrospray ionisation inputted into a ToF (Aglient). LC separation was via an XDB C18 column (4.6.0×150 mm) using a water/acetonitrile (both containing 0.1% (v/v) formic acid) gradient at a flow rate of 0.5 ml/min. The gradient was started at 50% (v/v) acetonitrile, which was increased to 100% acetonitrile in 10 min; 100% acetonitrile was maintained for 5 min before the gradient was returned to starting conditions. Spectra were collected at 0.99 spectra per second.

Cloning and expression of gliT
The gliT sequence was amplified from cDNA using primers incorporating terminal Xhol and HindIII sites to facilitate downstream cloning. PCR products were cloned into the pCR2.1 cloning vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. gliT was subsequently cloned into the pProEX-HtB expression vector (Invitrogen). Ligations were performed using Quicklith ligase (Bioline, London, UK) according to the manufacturer’s instructions. pPXAgET, the resultant expression vector was transformed into E. coli strain BL21 by standard protocols. Expression of GliT was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG; to 0.6 mM) and monitored by SDS-PAGE and Western blot analysis. Recombinant GliT purification was undertaken by differential extraction. Protein concentrations were determined using the Bradford method [38] with bovine serum albumin as a standard.

Purification of native GliT from A. fumigatus by ion-exchange chromatography
A. fumigatus ATCC46645 mycelia were ground in liquid nitrogen and lysed in ice-cold lysis buffer as described [36] following incubation with gliotoxin (10 μg/ml) for 3 h. Following centrifugation (12,000 g; 30 min), the lysate supernatant (176 ml) was ammonium sulphate precipitated (10, 20, 50 and 70% ammonium sulphate). The 50% pellet was resuspended in 20 mM Bis-Tris propane pH 7.6 and dialysed three times against 50 volumes of the same buffer at 4°C. The dialysate was centrifuged (12,000 g; 20 min) and filtered (0.45 μm) to remove particulates. The dialysate was loaded onto an equilibrated Q-Sepharose ion-exchange (IEX) column (4 ml) at a flow rate of 1 ml/min. The column was washed with 20 mM Bis-Tris propane pH 7.6 before bound protein was eluted using an NaCl gradient (0.5 M final). Absorbance detection was at 280 nm and 454 nm. Collected fractions were subjected to SDS-PAGE, Western blot and activity analysis for GliT.

Immunoaffinity purification of human IgG [anti-GliT]
Serum specimens (provided by the Irish Blood Transfusion Service, Dublin, Ireland according to institutional guidelines) containing high titer IgG [anti-GliT] were pooled, diluted 1 in 4 in PBS, and applied to a GliT-Sepharose affinity column (0.5 ml), prepared as per manufacturer’s instructions. After removal of unbound proteins by PBS washing, immobiilised IgG [anti-GliT] was eluted using 100 mM Trizma base pH 8.3. Resultant immunoaffinity purified (IAP) IgG [anti-GliT] was used to detect native GliT by Western analysis.

GliT activity assay and removal of native GliT from A. fumigatus by IAP pulldown
A. fumigatus ATCC46645 mycelia were ground in liquid nitrogen and lysed in ice-cold lysis buffer and bead-beating as described...
elsewhere [36]. Following centrifugation (12,000 g; 30 min), the lysate supernatants were used to determine gliotoxin reductase activity (ΔA330 nm) in the presence of gliotoxin (9 μM) and NADPH (200 μM) at pH 7.2 (a modified version of Hill et al. [39]). A. fumigatus cell lysates were also subjected to ion-exchange chromatography and a pooled IEX fractions (250 μl) incubated with iAP human IgG (anti-GliT) (100 μl) followed by Protein A-Sepharose addition and centrifugation (10,000 g; 10 min). Supernatant activity analysis as described above.

GliT-GFP confocal microscopy

A. fumigatus gliT^P and ATCC46645 mycelia were grown in cell culture six well plates (Corning Inc.) for 21 h before induction with (or without) gliotoxin (5 μg/ml). Mycelia were removed from the wells and centrifuged (12,000 g; 5 min). Supernatants were stored while pellets were resuspended in DAPI staining solution and incubated (5 min) at room temperature. The stained mycelia were centrifuged and washed with deionised H2O before resuspension in the original supernatant. Aliquots of these preparations were analysed for GliT-GFP presence and DAPI fluorescence on an Olympus Fluoview 1000 confocal microscope.

Virulence model

G. mellonella larvae (n = 10) were inoculated into the hind pro-leg with 10^7 A. fumigatus conidia in 20 μl (per larva) [37]. In addition, one cohort of larvae was pre-treated with gliotoxin (0.5 μg/larva in 20 μl). Control treatments were included to ensure that neither the injection procedure, or the incubation period, were responsible for any mortality observed. These controls involved G. mellonella larvae injected with 20 μl of sterile PBS or gliotoxin alone. G. mellonella larvae were placed in Petri-dishes and incubated in the dark at 30°C. Mortality rates were recorded for 72 h post-injection. Mortality was assessed based on lack of movement in response to stimulation and discolouration (melanisation) of the injection.

Generation of gliT-encoding Aspergillus nidulans and Saccharomyces cerevisiae

To introduce gliT in A. nidulans TRAN, a plasmid containing gliT coding sequence under the control of a constitutive sotf [40] promoter was used. Therefore, a 1.1 kb fragment containing gliT was amplified using oligo-BgII and oligo-NdeI and subcloned into pCR2.1-TOPO (Invitrogen). A 0.9 kb fragment containing an mellerella larvae were placed in Petri-dishes and incubated in the cuticle. response to stimulation and discolouration (melanisation) of the injection. Mortality was assessed based on lack of movement in the original supernatant. Aliquots of these preparations were analysed for GliT-GFP presence and DAPI fluorescence on an Olympus Fluoview 1000 confocal microscope.

Supporting Information

Protocol S1 Supplementary data. Found at: doi:10.1371/journal.ppat.1000952.s001 (0.03 MB DOC)

Figure S1 (A) Deletion of gliT and gliH in A. fumigatus ATCC46645 and 26933, respectively. Southern analysis of ΔgliT mutant versus wild-type DNA for A. fumigatus ATCC46645 and ATCC26933, respectively. Here, a DIG-labelled probe was used to detect the predicted presence of 3.3 and 6.4 kb fragments in XbaI restricted ΔgliT and wild-type DNA, respectively. (B) Southern Blot analysis of ΔgliT complemented strains (gliT^C). Genomic DNA of wild-type and complemented strains was digested with NdeI (ATCC46645) and ApaI (ATCC26933), respectively and probed using a DIG-labelled probe amplified using oligos gliT-4 and gliT-5. (C) Southern Blot analysis of ΔgliH, ΔgliH-complemented strains (gliH^C) and ΔgliT-complemented with gliH (ΔgliT^gliH^C). Genomic DNA of wild-type and respective mutant strains was digested with NdeI (ATCC26933) and probed using a DIG-labelled probe amplified using oligos gliH-4 and gliH-5. (1) A. fumigatus ATCC26933, (2) ΔgliH, (3) gliH^C, (4) ΔgliT, (5) ΔgliT^gliH^C. Found at: doi:10.1371/journal.ppat.1000952.s002 (1.30 MB DOC)

Figure S2 Phenotypic analysis of A. fumigatus ATCC46645 (wild-type) and ΔgliT strains in the presence of gliotoxin (GT). Compared to wild-type, gliotoxin (5 μg/ml) significantly inhibits ΔgliT growth in minimal medium (MM) and completely inhibits ΔgliT growth in both MM and Sabouraud medium (10 μg/ml). Found at: doi:10.1371/journal.ppat.1000952.s003 (0.04 MB DOC)

Figure S3 Peptide mass spectrum of GliT from A. fumigatus ATCC26933, a component of the gliotoxin biosynthetic cluster (33% sequence coverage). This MALDI-ToF identification represents the first proteomic confirmation of the expression of a component of the gliotoxin biosynthetic cluster. Found at: doi:10.1371/journal.ppat.1000952.s004 (0.11 MB DOC)

Figure S4 Analysis of gliotoxin, and related metabolite, production in A. fumigatus mutant strains. (A) Gliotoxin was detectable by RP-HPLC (data not shown) and LC-MS in A. fumigatus ATCC26933 gliT^P with identical molecular mass and fragmentation pattern to commercially available gliotoxin and as reported in [10]. (B) LC-ToF analysis of RP-HPLC purified gliotoxin-related metabolite (Figure 3B) from Aspergillus fumigatus ΔgliT^PMS. MS spectrum shows the presence of a high abundance molecular ion (Retention time = 9.153 min) with m/z 279.0796 (M+H)^+ (557.1497 (2M+H)^+), which corresponds precisely to a predicted molecular formula of C13 H14 N2 O3 S - a putative
monothiol form of gliotoxin. (C) LC-MS analysis of RP-HPLC purified gliotoxin-related metabolite (Figure 3B) from *Aspergillus fumigatus* ΔgliT*ΔgliZ*. Using a manual approach, LC-MS software identified five molecular species with m/z 279.0. The most intense peaks (1 and 5) were subjected to MS2 analysis and both yielded identical fragments ions of m/z 261.1, 231.0 and 203.1. Notably, peak 1 eluted from LC-MS and LC-ToF with an identical retention time (9.1 min) (D) Gliotoxin production was undetectable in *A. fumigatus* and identical retention time (9.1 min) (D) Gliotoxin production was undetectable in *A. fumigatus* ΔgliT*ΔgliZ* by RP-HPLC and LC-MS (data not shown), thereby indicating a role for this gene in either gliotoxin biosynthesis or secretion. Found at: doi:10.1371/journal.ppat.1000952.s006 (2.29 MB DOC)

**Figure S5** Recombinant GliT expression. (A) SDS-PAGE and (B) Western blot analysis of recombinant GliT expression and solubility. Lane 1 contains non-transformed BL21 (DE3) cells. Lane 2 contains non-induced cell extract and lanes 3–5 contain induced cell extracts taken 1–3 h post-induction with 0.6 mM IPTG. Lane 6 and 7 contain soluble and insoluble cell extracts respectively. Lane 8 contains His-tag positive control and lane 9 contains non-reducing cell extract monomeric (m) and dimeric (d) forms of GliT are evident. Lane M contains molecular mass marker. Found at: doi:10.1371/journal.ppat.1000952.s006 (2.29 MB DOC)

**Figure S6** Confirmation of recombinant GliT identity by MALDI-ToF mass spectrometry (21% sequence coverage). Found at: doi:10.1371/journal.ppat.1000952.s007 (0.03 MB DOC)

**Figure S7** (A) Partial purification and immunological identification of GliT. Absorbance (A280 nm and A454 nm) versus elution volume (ml) for a Q-Sepharose ion-exchange fractionation of GliT dialysate (post-ammonium sulphate precipitation). (B) SDS-PAGE analysis of Q-Sepharose ion-exchange chromatography (IEC) fractions. (C) Western blot analysis of Q-Sepharose IEX fractions using human IgG[anti-GliT] and anti-human IgG-HRP conjugate with ECL detection. These fractions were pooled and used for activity and immunological analysis as shown in Figures 4 and 5. Found at: doi:10.1371/journal.ppat.1000952.s008 (0.29 MB DOC)

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