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Original Article

Original Article

GliA in *Aspergillus fumigatus* is required for its tolerance to gliotoxin and affects the amount of extracellular and intracellular gliotoxin

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Abstract

Gliotoxin is an important virulence factor of *Aspergillus fumigatus*. Although GliA putatively belongs to the major facilitator superfamily in the gliotoxin biosynthesis cluster, its roles remain unclear. To determine the function of GliA, we disrupted *gliA* in *A. fumigatus*. *gliA* disruption increased the susceptibility of *A. fumigatus* to gliotoxin. The *gliT* and *gliA* double-disrupted mutant had even higher susceptibility to gliotoxin than each individual disruptant. The extracellular release of gliotoxin was greatly decreased in the *gliA* disruptant. Mice infected with the *gliA* disruptant of *A. fumigatus* showed higher survival rates than those infected with the parent strain. These results strongly indicate that GliA, in addition to GliT, plays a significant role in the tolerance to gliotoxin and protection from extracellular gliotoxin in *A. fumigatus* by exporting the toxin. This also allows the fungus to evade the harmful effect of its own gliotoxin production. Moreover, GliA contributes to the virulence of *A. fumigatus* through gliotoxin secretion.

Key words: *Aspergillus fumigatus*, GliA, gliotoxin, major facilitator superfamily transporter.

Introduction

Aspergillus fumigatus is not only a ubiquitous environmental fungus but also an important pathogenic fungus in clinical settings where it can cause theserious infection aspergillosis [1]. Aspergillosisis a highly prevalent fungal infection with a high mortality rate, even after antifungal treatment [2]. Several aspects of the mechanism of infection remain unclear; however, the secondary metabolites of the fungus have received attention recently. *Aspergillus fumigatus* produces several secondary metabolites, some of which are believed to play important roles during the infection

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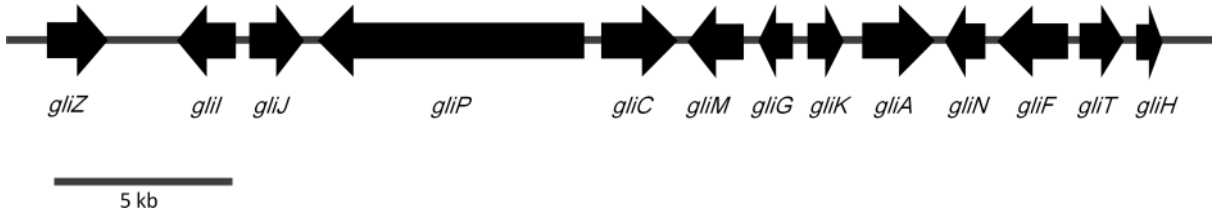

Figure 1. Gliotoxin biosynthetic gene cluster in *Aspergillus fumigatus*. The gene cluster is composed of the following: *gliZ* encoded a zinc finger–type transcription factor; *gliI*, a carbon-sulfur lyase; *gliJ*, a dipeptidase; *gliP*, a nonribosomal peptide synthase; *gliC*, a cytochrome P450 monooxygenase; *gliM*, an *O*-methyltransferase; *gliG*, a glutathione- *S*-transferase; *gliK*, a hypothetical protein participating in the production and the efflux of gliotoxin; *gliA*, a major facilitator superfamily transporter; *gliN*, a methyltransferase; *gliF*, another cytochrome P450 monooxygenase; *gliT*, an oxidoreductase; and *gliH*, a hypothetical protein.

process [3,4]. Gliotoxin, an important secondary metabolite of *A. fumigatus* [5], belongs to the class of epipolythiodioxopiperazines (ETPs) and has pleiotropic immunosuppressive properties that include induction of apoptosis in macrophages and lung epithelial cells, inhibition of nuclear factor κ-B activation, and inhibition of phagocytosis [6,7]. Several groups have recently reported that gliotoxin plays an important role in invasive aspergillosis [8,9]; however, the mechanism remains unclear.

Gliotoxin is synthesized by one of the biosynthetic gene clusters in *A. fumigatus*, which comprises 12 genes [9–11]. The gene cluster has a significant homology with the ETP biosynthetic cluster in other fungal species, such as *Leptosphaeria maculans* [11]. Recently, Schrettl et al. reported that *gliH* adjacent to the gliotoxin biosynthetic cluster was also essential for the production of gliotoxin [12] and that the cluster comprises 13 genes [12] (Fig. 1). GliZ works as atranscriptional regulator of gliotoxin biosynthesis [13], while *gliP* encodes a nonribosomal peptide synthase that catalyzes the first biosynthetic step in gliotoxin synthesis [14]. Schrettl et al. recently indicated that GliT (AFUA_6G09740) is an oxidoreductase that catalyzes the reduction and formation of the disulfide bond in gliotoxin [15]. A *gliT* disruptant was unable to produce gliotoxin and was more susceptible to exogenous gliotoxin than the parent strain, indicating that GliT has important roles not only in gliotoxin production but also in the self-protection of the fungus against gliotoxin [15]. However, the extracellular export mechanism of gliotoxin, which is deemed critical for the virulence and self-protection of the fungus from the toxin, remains unknown.

The efflux pump of the fungus, which is required to export the intracellularly produced mycotoxin, is considered to be encoded by the biosynthetic cluster. GliA, which is encoded by the gliotoxin biosynthetic cluster, exhibits a homology with the putative fungal major facilitator superfamily (MFS) transporters of *Neosartorya fischeri* (96% homology) and *Trichophyton rubrum* (52% homology). In addition, the conserved domain of the EmrB/QacA subfamily, TIGR00711, predicted to have 14 potential transmembrane-spanning regions, has been found in GliA [16] (Fig. 2), indicating that GliA is an MFS transporter, containing 14 transmembrane domains. *Leptosphaeria maculans* produces sirodesmin, another ETP molecule like gliotoxin, and Gardiner et al. investigated the role of SirA, which is an ABC transporter encoded by the biosynthetic gene cluster for the toxin. In their experiment, they expressed GliA from *A. fumigatus* in *L. maculans* and revealed that the susceptibility to exogenous gliotoxin was decreased [17]. This suggests that GliA functionsas a gliotoxin exporter, although the function of GliA was examined in *L. maculans* but not in *A. fumigatus*. Amnuaykanjanasin and Daub suggested that the roles of the fungal transporter encoded by the secondary metabolite biosynthetic gene cluster can be classified into toxin efflux, self-protection, or both [18]. Considering this classification, we hypothesized that GliA in *A. fumigatus* not only functioned as an exporter but was also deeply involved with the exclusion of the toxin for protection. In addition, disruption of the export system may lead to a reduction in the extracellular gliotoxin level and *A. fumigatus* pathogenicity. Therefore, in the present study, we investigated whether GliA had a significant role in protection against gliotoxin in *A. fumigatus*. In addition, we investigated whether GliA was involved with pathogenicity during *A. fumigatus* infections to elucidate the significance of *gliA* in the virulence of the fungus.

Materials and methods

Fungal strains and growth conditions

Aspergillus fumigatus strains used in this study (Table 1) were routinely cultured at 25°C on *Aspergillus* minimal medium [19]. Conidia were collected as described previously [20].

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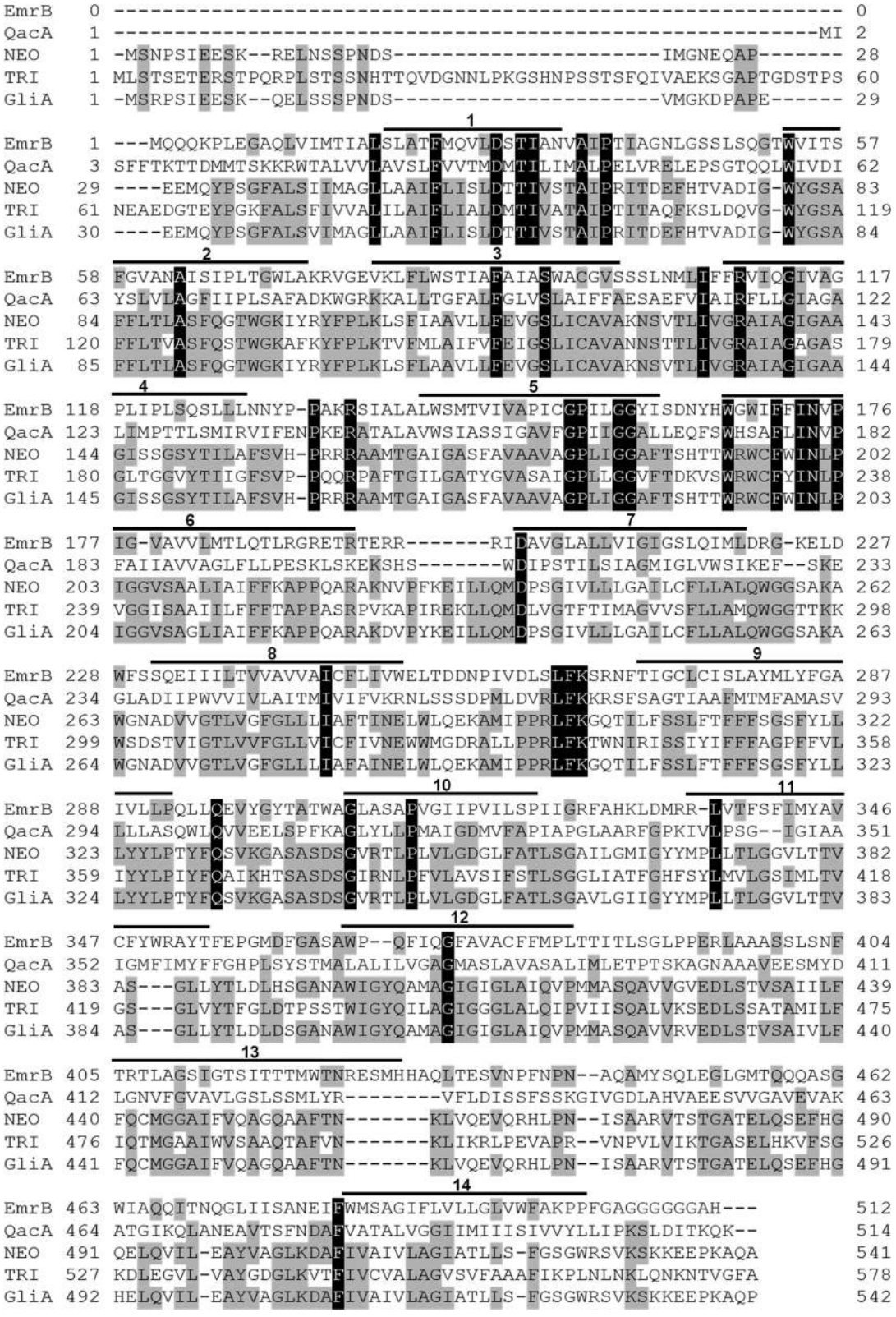

Figure 2. Amino acid sequence of *Aspergillus fumigatus* GliA (accession no. AAW03302) aligned with other major facilitator superfamily proteins with 14 transmembrane-spanning domains (TMS): *Escherichia coli* EmrB (accession no. ZP_03032909), *Staphylococcus aureus* QacA (accession no. YP_003813123), *Neosartorya fischeri* NEO (accession no. XP_001258088.1), and *Trichophyton rubrum* TRI (accession no. XP_003234604.1). The lines above the alignment correspond to the predicted positions of TMS [16].

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Table 1. *Aspergillus fumigatus* strains used in this study.

Name	Genotype	Source
Afs35	*aku::loxP*	The parent strain for gene disruption of *gliA* and/or *gliT* [a]
Δ*gliA*	*aku::loxP*, *gliA::HygB*^{R}	This study
Δ*gliT*	*aku::loxP*, *gliT::phleo*^{R}	This study
Δ*gliT*Δ*gliA*	*aku::loxP*, *gliA::HygB*^{R}, *gliT::phleo*^{R}	This study
gliAC	*aku::loxP*, *gliA*, *ptrA*^{R}, *gliA::HygB*^{R}	This study
Δ*gliTAC*	*aku::loxP*, *gliA*, *ptrA*^{R}, *gliA::HygB*^{R}, *gliT::phleo*^{R}	This study

[a]Provided by Fungal Genetics Stock Center (Kansas City, MO, USA).

Disruption of genes in *A. fumigatus*

Disruptions of target genes in *A. fumigatus* were performed using the homologous recombination technique [21]. The primers used for these disruptions are listedin Table 2. In brief, using the double-joint polymerase chain reaction (PCR) method [22], the 5′- and 3′-flanking regions of *gliA* and *gliT* were fused to the hygromycin-resistance gene [23] and the gene encoding phleomycin-binding protein [24], respectively. PrimeSTAR GXL enzyme (Takara Bio Inc., Otsu, Japan), a high-fidelity DNA polymerase, was used for PCR according to the manufacturer's instructions. The amplification consisted of 30 cycles each of 10 s at 98°C, 15 s at 60°C, and 1 min/kb at 68°C; the last step was for 8 min at 68°C. The resulting fragments were introduced into *A. fumigatus* cells by electroporation [25]. The transformed DNA fragment was replaced with a target gene by homologous recombination. The strain with both *gliA* and *gliT* disrupted (Δ*gliA*Δ*gliT*) was generated from the Δ*gliT* strain. The correct recombination of transformants was confirmed by PCR (data not shown) and Southern blotting (Supplementary Fig. 1A and 1E) [14].

To complement *gliA*, the DNA fragment, including *gliA* and the 1.4-kb upstream region, was cloned into pPTRI (Takara Bio Inc.) and the resultant plasmid was transformed into the Δ*gliA* or Δ*gliT*Δ*gliA* strain. Correct recombination was confirmed by Southern blotting (Supplementary Fig. 1A).

Analysis of *gliA* expression by quantitative PCR

Total RNA was prepared as follows. First, 1×10^8 conidia of each strain were inoculated into 50 ml of Roswell Park Memorial Institute (RPMI) 1640 medium with morpholinepropanesulfonic acid (MOPS) buffer adjusted to pH 7.0 (RPMI–MOPS; Sigma-Aldrich, St. Louis, MO, USA) and shaken at 180 rpm in humidified 5% CO_2 at 37°C for 24 h. Total RNA from freeze-dried mycelia was extracted using ISOGEN (Nippon Gene Co. Ltd., Tokyo, Japan), followed by treatment with Turbo DNA-free DNase I (Ambion, Austin, TX, USA). After synthesizing cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany), gene expression was quantified with Power SYBR Green PCR Master Mix using the 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Primers used for quantitative PCR analysis are listed in Table 2.

Measurement of susceptibility of *A. fumigatus* strains to gliotoxin

Gliotoxin (Sigma-Aldrich) was dissolved in filter-sterilized dimethyl sulfoxide (Wako), and the stock solution (10 mg/ml) was stored at −20°C. Next, 1×10^3 conidia of *A. fumigatus* strains were inoculated into 100 μl RPMI–MOPS with or without various concentrations (0–150 μg/ml) of gliotoxin in 96-well plates (Thermo Scientific, Roskilde, Denmark). After 24 h or 48 h culture at 35°C, the amount of viable fungal cells was determined using the 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5- [(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) assay [26]. Inhibitory concentration (IC_{50}) values and 95% confidence intervals (CIs) were calculated by nonlinear curve-fitting analysis using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

Measurement of gliotoxin susceptibility of *gliA*-expressing *Saccharomyces cerevisiae*

gliA fused with FLAG-tag was cloned into pYES2, a yeast multiple copy vector that carries the galactose-inducible promoter GAL1 [27]. The resultant plasmid and pYES2 were transformed into *S. cerevisiae* BY4742 (kindly provided by Dr A. Toh-e and Dr K. Shimizu) and were designated as *Sc-gliA* and *Sc-vec*, respectively. To repress *gliA* expression, these strains were cultured in a yeast nitrogen base (YNB) without amino acids (Difco Laboratories Inc., Detroit, MI, USA) supplemented with 0.03% (w/v) leucine, 0.02% (w/v) histidine, 0.03% (w/v) lysine, and 2% (w/v) glucose (YNB-glu). To induce GliA production in the yeast,

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Table 2. Primers used in this study.

Name	Sequence (5′ to 3′)
Primers used for gene disruptions and gene complementations	
gliT up-F[a]	TGCGGGTAGAAGCGCAGCACCATC
gliT up-R[a]	TTCGCTTACTGCCGGTGATTCGATGAAGCTCCATATTCTCCG-ATGGTTGTGGTATGCGCGAGAGTAGT
gliT down-F[b]	GCCGGCAACTGCGTGCACTTCGTGGCCGAGGAGCAGGACT-GACCAAGTTTGTTATAGCTGTACATAAA
gliT down-R[b]	TATCCCGACAACATCCAGATGATT
gliA up-F[c]	CGCCCAGTGCGCGCTACCTGGTGA
gliA up-R[c]	TAGGCATTCATTGTTGACCTCCACTAGCTCCAGCCAAGCCC-AAAAATGGTCGATGTCAGTAGAGAGCT
gliA down-F[d]	GGAAACCGACGCCCCAGCACTCGTCCGAGGGCAAAGGAAT-AGGTGTATCTGGTCGAAACATGTCTGCT
gliA down-R[d]	CCAGTCCATCTCGGACCCCTGGCC
gliA cf [15][e]	AAAGGTACCGAGAATCGAGGCATCAAAGC
gliA cr [15][e]	AAAAAGCTTGGACTTTGATCCGATCCTCA
Af-Hind[f]	AAAAAGCTTATGTCCCGTCCGTCTATCGAAGAG
Ar-FLAG-Bam[f]	AAAGGATCCTTACTTGTCATCGTCGTCCTTGTAGTCAGGCT-GCGCCTTGGGCTCCTCCTTC
Primers used for quantifications of gene expression by real-time polymerase chain reaction	
5′ *AfactinII* [14]	TCACTGCCCTTGCTCCCTCGTC
3′ *AfactinII* [14]	GCACTTGCGGTGAACGATCGAA
5′ *gliA* [14]	TTTGCGATCAACGAACTCTG
3′ *gliA* [14]	CCCTTGACGGACTGGAAGTA
5′ *gliZ* [14]	ACGACGATGAGGAATCGAAC
3′ *gliZ* [14]	TCCAGAAAAGGGAGTCGTTG
gliP- F1	GATCTCAACAGCGTGCAGAA
gliP- R1	GAACTTCCGTTCCTGCTCTG
gliK- F1	GACCTTGCAAGCCAAGTACC
gliK- R1	TAGTCGCTGTATGCCGTGAG

[a,c] *gliA* up-F and *gliA* up-R, and *gliT* up-F and *gliT* up-R were used for the amplification of 5′-flanking regions of *gliA* and *gliT*, respectively.
[b,d] *gliA* down-F and *gliA* down-R, and *gliT* down-F and *gliT* down-R were used for the amplification of 3′-flanking regions of *gliA* and *gliT*, respectively.
[e] Primers used to amplify *gliA* gene and the upstream region.
[f] Primers used for the amplification of FLAG-tagged *gliA*.

2% (w/v) galactose and 1% (w/v) raffinose were added to YNB broth (YNB-gal) instead of glucose. GliA production in *S. cerevisiae* was confirmed by Western blotting (Fig. 4A) [28]. Monoclonal anti-FLAG antibody F3165 (Sigma-Aldrich) as primary antibody and peroxidase-conjugated anti-mouse immunoglobulin-G (whole molecule) antibody A4416 (Sigma-Aldrich) as secondary antibody were used for the Western blot analysis.

To measure the susceptibility of *S. cerevisiae* strains to gliotoxin, *Sc-gliA* and *Sc-vec* were adjusted to 1×10^2 yeast cells in 100 μl of appropriate media with or without various concentrations of gliotoxin (0–8 μg/ml) and cultured at 30°C for 48 h. To estimate the growth of the yeast strains, the optical densities of each well at 630 nm were determined.

Detection of gliotoxin from *A. fumigatus*

In total, 7.5×10^6 conidia were inoculated into 150 ml of RPMI 1640 medium supplemented with L-glutamine and

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$NaHCO_3$ (Sigma-Aldrich) and incubated in 5% CO_2 in a rotary shaker at 37°C under gentle agitation at 140 rpm for 24 h. The culture supernatant was filtered through 0.2-μm pore-size filters (Thermo Scientific). Next, 1-ml aliquots of the culture filtrate (CF) of each strain were used for the viability assays with mammalian cells, as described below. Secondary metabolites, including gliotoxin, were extracted two times with chloroform. Extraction from harvested hyphae was performed as described previously [29]. The extracts from CF and hyphae were dried in a rotary evaporator (Rotavapor, Buchi, Switzerland) at 40°C. The evaporated extracts were dissolved in 200 μl of methanol and stored at −20°C until further analysis. Gliotoxin was detected using a reversed-phase high-performance liquid chromatography (RP-HPLC) L-7000 system (Hitachi High-Technologies Co., Tokyo, Japan) equipped with a diode array detector and an Agilent 6530 time-of-flight liquid chromatography coupled with mass spectrometry (LC/MS/MS) system (Agilent Technologies, Santa Clara, CA, USA). For HPLC analysis, 20 μl of the samples were injected into a reversed-phase C18 XTerra column (150 × 4.6 mm, 5 μm) (Waters Co., Milford, MA, USA) at 40°C. The mobile phases used for the analysis were gradient solvent A, H_2O, 0.1% (v/v) trifluoroacetic acid (Wako), and solvent B, acetonitrile (Wako). The ratios were varied as follows: 20%–65% B for 20 min, followed by 65% B for 8 min and 100% B for 10 min. The flow rate was 1 ml/min. The effluent absorbance was monitored at 280 nm. Average values obtained from three independent experiments were compared with a standard curve (50–10000 ng gliotoxin) of peak areas obtained with gliotoxin (Sigma-Aldrich).

For LC/MS/MS analysis, 3 μl of the sample was separated using the Zorbax Eclipse Plus C18 column (100 × 2.1 mm, 1.8 μm) (Agilent Technologies) at 40°C. The mobile phases comprised gradient solvent A, 0.1% (v/v) HCOOH + 10 mM $HCOONH_4$, and solvent B, acetonitrile; the ratios were varied as follows: 10% B for 30 min, followed by 100% B at a flow rate of 0.2 ml/min. MS spectra were collected in the positive ion mode, with scanning between 100 and 1000 *m/z*.

Assay of viability of mammalian cells

Viability of mammalian cells was measured as follows [30]. In total, 4×10^3 A549 cells, a human lung adenocarcinoma epithelial cell line (American Type Culture Collection CLL 185), were cultured in 100 μl of Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (Gibco BRL, Grand Island, NY, USA, or Hyclone, Logan, UT, USA) in 5% CO_2 at 37°C for 48 h. The 50% or 20% (v/v) CF of each strain was added to A549 cells, followed by incubation for an additional 24 h. The number of viable cells was estimated using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Cells incubated without CF were used as controls.

Infection with *A. fumigatus* strains in immunosuppressed mice

All animal studies were approved by the institutional animal care and use committee of Chiba University (DOU24-294 and DOU24-309). *Aspergillus fumigatus* strains Afs35, Δ*gliA*, and *gliAC* were used to infect immunosuppressed mice (10 mice per group). In this experiment, 10- to 11-week-old male BALB/c mice weighing 24–29 gr were supplied by Charles River Laboratories Japan Inc. (Yokohama, Japan). The mice were immunosuppressed by subcutaneous injection of 2 mg of hydrocortisone acetate (Sigma-Aldrich) in 100 μl phosphate-buffered saline (PBS)–0.1% Tween 20 on days–4, –2, 0, 2, and 4 [6]. The mice were housed in sterile cages with sterile bedding andprovided with sterile feed and drinking water containing 300 mg/l tetracycline hydrochloride to prevent bacterial infection. The mice were intratracheally inoculated with 5×10^6 conidia in 20 μl PBS–0.01% Tween 20 on day 0. The mice were then monitored for up to 21 days. The mice were euthanized and lungs were dissected 72 h after infection to determine fungal burdens by colony count (10 mice per fungal strain) and to facilitate histopathological examination (three mice per fungal strain). To count viable fungal cells, the lungs were weighed and homogenized using a Polytron homogenizer (Kinematica AG, Luzern, Switzerland). After 24 h incubation on potato dextrose agar containing 0.05 mg/ml chloramphenicol at 35°C, the colonies were counted. For histopathological examination, the excised lungs were fixed with 10% (v/v) formalin and stained with hematoxylin and eosin (H&E) or using Grocott's modification of Gomori's methenamine-silver nitrate procedure (GMS). Images were obtained using an Axio imager A1 microscope equipped with AxioCam MRc (Carl Zeiss, Jena, Germany). The numbers and areas of the site containing fungal hyphae with inflammatory cell infiltration in the three lungs were determined using an Axiovision Imaging System (Carl Zeiss) [31]. In brief, eight lesions were outlined and average areas for each mouse were calculated. The means and standard errors of the areas from the three mice were calculated and compared among groups infected with each strain.

Statistical analysis

All experiments conducted in this study were repeated at least three times, and the results were expressed as mean ± standard deviations (SDs). One-way analysis of variance with Tukey multiple comparison test was used for

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experiments comparing multiple groups, and the Student *t* test (two-tailed) was used for experiments comparing two groups. Survival curves were plotted using the Kaplan–Meier method, and comparisons among groups were performed using the log-rank test. A *P* value threshold of 0.05 was used to determine significant differences.

Results

GliA is important for tolerance to exogenous gliotoxin

To investigate the roles of *gliA* in *A. fumigatus*, we constructed a Δ*gliA* strain that was confirmed using Southern blotting (Supplementary Fig. 1). By macroscopic assessment, the growth and conidiation of the Δ*gliA* strain at 25°C (data not shown), 35°C (Supplementary Fig. 2), and 42°C (data not shown) on the *Aspergillus* minimal medium plate were indistinguishable from that of the parent strain. To examine the role of *gliA* in the tolerance to gliotoxin, the Δ*gliA* strain and other strains were treated with exogenous gliotoxin (Fig. 3). At 24 h after treatment (Fig. 3A), the IC_{50} value of gliotoxin for the Δ*gliA* strain was 1.160 μg/ml (95% CI = 1.006–1.339 μg/ml), and at 48 h after treatment, it was 6.870 μg/ml (95% CI = 6.312–7.477 μg/ml).

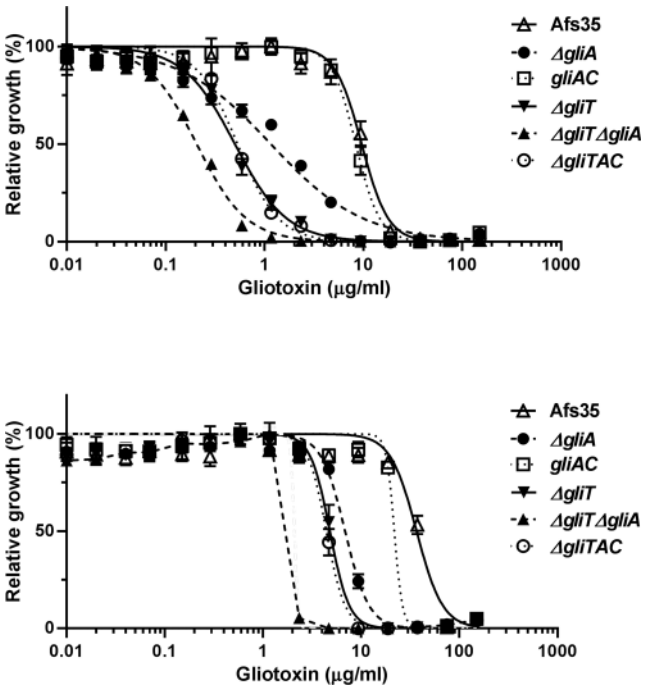

Figure 3. Susceptibility of *Aspergillus fumigatus* to gliotoxin. The strains indicated in the graph legend were treated with gliotoxin for 24 h (A) or48 h (B). The number of viable fungal cells is shown relative to the values for each gliotoxin-free well. The curves presented in the graph were estimated using GraphPad Prism 6 software. The results are representative of three independent experiments performed in triplicate and are expressed as the mean ± standard deviationof three replicates.

These values were significantly lower than those for the parent strain Afs35 (IC_{50} of 9.608 μg/ml [95% CI = 0.9430–1.022] after 24 h treatment and 36.73 μg/ml [95% CI = 32.49–41.53] after 48 h treatment) but were comparable to those in Δ*gliT* (IC_{50} of 0.5018 μg/ml [95% CI = 0.4607–0.5465] after 24 h treatment and 4.809 μg/ml [95% CI = 4.480–5.163] after 48 h treatment; Fig. 3). The Δ*gliT*Δ*gliA* strain exhibited the highest sensitivity in this study (Fig. 3). The IC_{50} value was 2.090 μg/ml at 48 h after treatment. The *gliA*-complemented strains *gliAC* and Δ*gliTAC* regained tolerance to exogenous gliotoxin (Fig. 3). These results suggest that GliA, in addition to GliT, has an important role in the protection of fungal cells against gliotoxin.

To further investigate the function of *gliA*, *gliA* with FLAG-tag was introduced into *S. cerevisiae* BY4742 and expressed under the control of the inducible *GAL1* promoter (Fig. 4A) [27]. As presented in Figure 4B, the *gliA*-overexpressing strain increased the tolerance to gliotoxin. However, no differences were observed between the two transformants in gliotoxin sensitivity under conditions that suppressed *gliA* expression (Fig. 4C). These results suggest that GliA also has an important role in the tolerance of gliotoxin by yeast.

Decrease in gliotoxin in CF of the *gliA* disruptant

To examine gliotoxin production by the *A. fumigatus* strains, we measured the cytotoxicity of fungal CF using A549 cells. After treatment with 50% or 20% (v/v) CF of the Afs35 or *gliAC* strain, the viable cells were reduced to <20% of the control cells (Fig. 5). In contrast, the number of viable A549 cells after incubation with CF of the Δ*gliA* and Δ*gliT* strains was significantly higher than that after incubation with CF of the Afs35 strain ($P < 0.01$; Fig. 5), while no significant differences were observed between the viability of A549 cells treated with 20% (v/v) CFs of the Δ*gliA* strain and those treated with 20% (v/v) CFs of the *gliT* disruptants (Fig. 5B). We detected and measured gliotoxin production by the Afs35, Δ*gliA*, and *gliAC* strains using RP-HPLC and LC/MS/MS. RP-HPLC analysis of CFs revealed that the gliotoxin concentration in CF of the Δ*gliA* strain was 144.2 ± 12.9 ng/ml, which was significantly lower than that in CFs of the Afs35 strain (569.5 ± 76.5 ng/ml) and the *gliAC* strain (625.9 ± 34.4 ng/ml; $P < 0.01$; Fig. 5C). By LC/MS/MS analysis (the fragment ion pattern of gliotoxin is shown in Supplementary Fig. 3), the amount of gliotoxin present in the hyphae of the Δ*gliA* strain was 0.1± 0.1 ng/mg hyphae, which was significantly lower than that in hyphae of the Afs35 strain (3.8 ± 1.5 ng/mg hyphae; $P < 0.05$), whereas the amount of gliotoxin in hyphae of the *gliAC* strain (2.5 ± 2.4 ng/mg hyphae) was similar to that of the parental strain (Fig. 5D).

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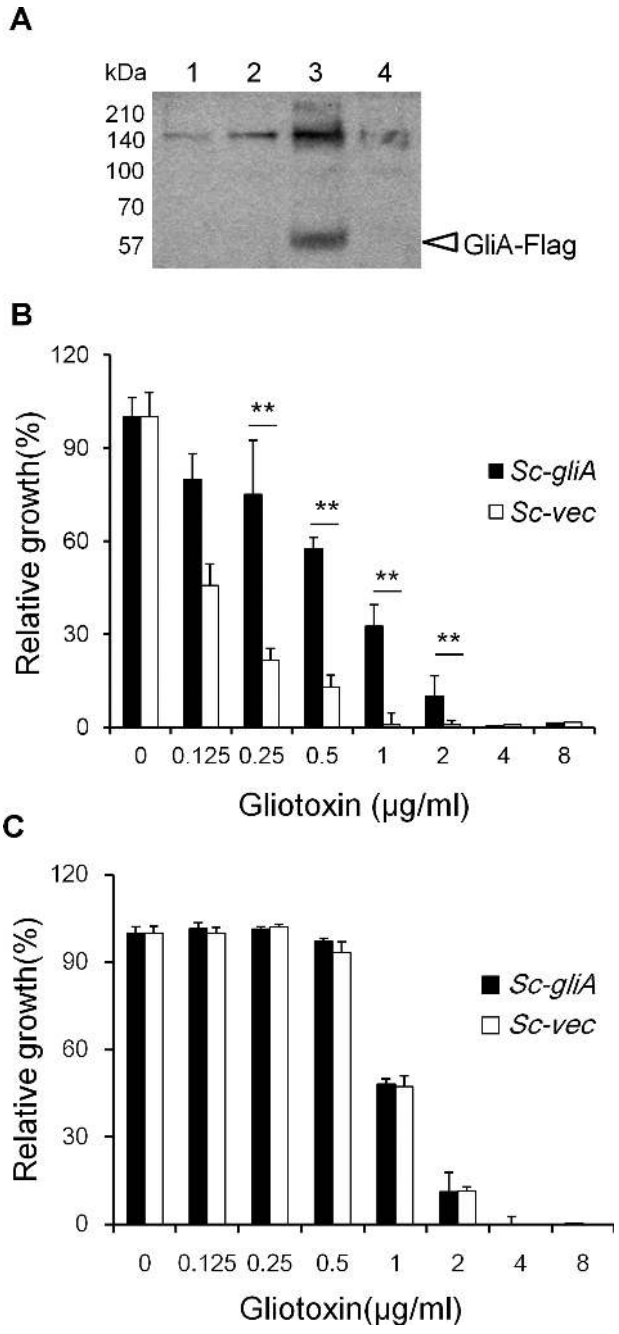

Figure 4. The role of GliA in tolerance to gliotoxin in *Saccharomyces cerevisiae*. (A) GliA expression was confirmed by Western blotting. Lane 1, *Sc-gliA* incubated in yeast nitrogen base (YNB)-glucose (YNB-glu); lane 2, *Sc-vec* incubated in YNB-glu; lane 3, *Sc-gliA* incubated in YNB-galactose (YNB-gal); lane 4, *Sc-vec* incubated in YNB-gal. A 57-kDa product of FLAG-fused GliA protein was detected in lane 3. The susceptibility under conditions ofinduction (B) or suppression (C) of the GAL1 promoter. Each experiment was repeated three times with triplicate samples. **, $P < 0.01$.

Gliotoxin could not be detected in CFs of the Δ*gliT* and Δ*gliT*Δ*gliA* strains (data not shown). These data indicate that GliA is not essential for gliotoxin synthesis and that the extracellular and intracellular amounts of gliotoxin were significantly decreased in the *gliA* disruptant.

Quantification of the expression levels of *gliA*, *gliP*, *gliK*, and *gliZ* in wild-type, *gliA*-disruptant, and *gliA*-complemented strains

To examine the effect of the absence of GliA on other genes in the gliotoxin biosynthetic cluster, we quantified the expression of *gliP*, *gliK*, and *gliZ*, as well as *gliA* (Fig. 6; Supplementary Fig. 4). As shown in Supplementary Figure 4, the expression of *gliA* in Δ*gliA* could not be determined and *gliA* expression in *gliAC* was significantly higher than the parental strain Afs35. The expressions of *gliP* (Fig. 6A) and *gliK* (Fig. 6B) in Δ*gliA* were not significantly changed compared with the parental strain Afs35. The expressions of *gliP* and *gliK* in the *gliAC* strain were <1.5 times higher than those in Afs35. On the other hand, the expression of *gliZ* in Δ*gliA* was about 2 times higher than that in Afs35 or *gliAC* (Fig. 6C).

Disruption of *gliA* leads to increased survival of the *gliA* disruptant-infected mice

To determine the effects of *gliA* disruption on virulence, the *A. fumigatus* Afs35, Δ*gliA*, and *gliAC* strains were intratracheally administrated to BALB/c mice immunosuppressed with hydrocortisone acetate. As shown in Figure 7A, the survival rate of the *gliA* disruptant-infected mice increased significantly compared with that of the parental strain Afs35-infected mice ($P < 0.05$).

The pulmonary fungal burdens of the Δ*gliA* strain-infected mice 72 h after infection were not different from those of the Afs35 strain- and *gliAC* strain-infected mice (Fig. 7B). The results of H&E and GMS staining 72 h after infection revealed that the hyphae were widely scattered throughout the lungs and were surrounded by extensive inflammatory infiltrates of neutrophils in the pulmonary lesions of the mice infected with the Afs35 strain (Fig. 7C and 7F; Supplementary Fig. 5A and D) and the *gliAC* strain (Fig. 7E and 7H; Supplementary Fig. 5C and F). Nuclear fragmentation was frequently detected in the pulmonary lesions of the Afs35 strain- and *gliAC* strain-infected mice (Fig. 7F and H), suggesting that gliotoxin produced by the Afs35 or *gliAC* strain induced apoptosis in neutrophils around the infection foci. In contrast, neutrophils in the pulmonary sections of the Δ*gliA* strain-infected mice were mostly intact (Fig. 7G), suggesting that their apoptosis was relatively reduced. Fungal growth and the size and number of lesions per lung caused by the Δ*gliA* strain (Fig. 7D and G; Supplementary Fig. 5B and 5E) were not statistically different from those caused by the Afs35 or *gliAC* strain (Fig. 7I and 7J), whereas the lesions of the Δ*gliA* strain-infected mice were slightly smaller than those of the parental/complemented strain-infected mice.

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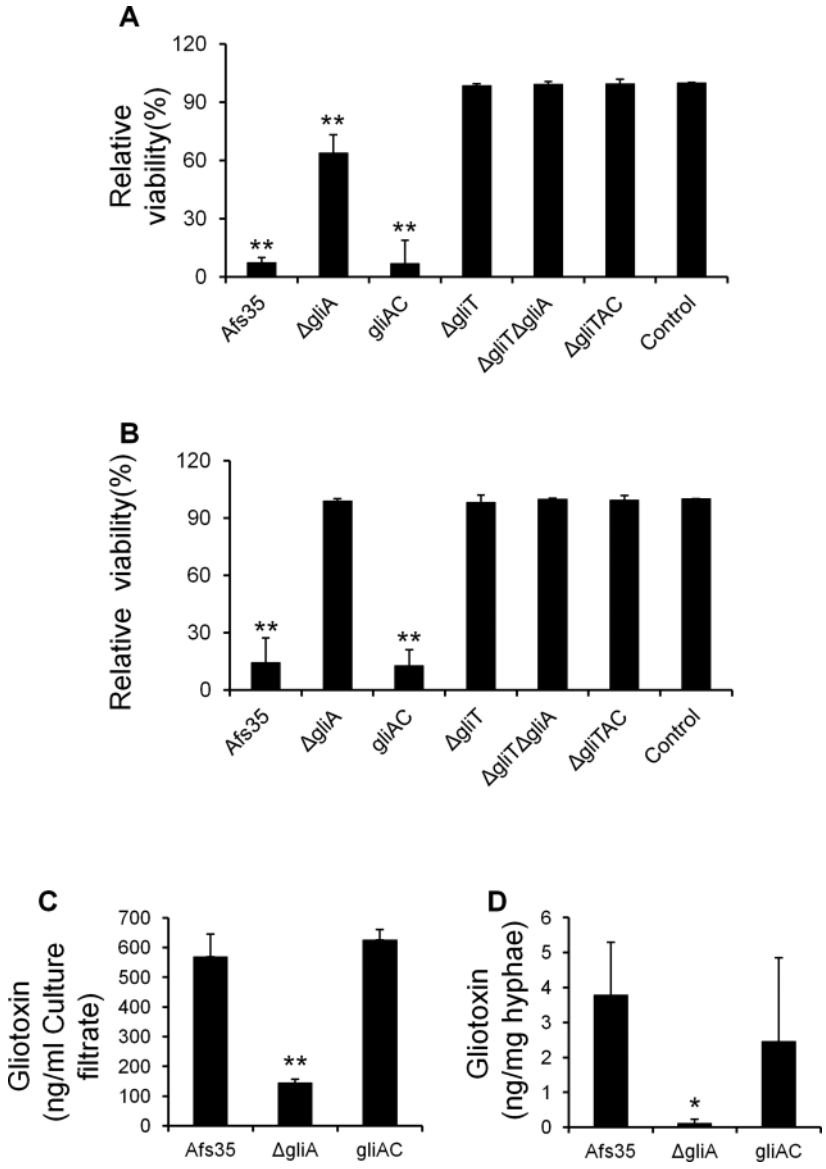

Figure 5. Toxicity of *Aspergillus fumigatus* culture filtrates (CFs) to A549 cells. The amount of viable cells was determined 24 h after treatment with (A) 50% (v/v) and (B) 20% (v/v) CFsof *A. fumigatus* Afs35 strain, Δ*gliA* strain, *gliAC* strain, Δ*gliT* strain, Δ*gliT*Δ*gliA* strain, and Δ*gliTAC* strain. Each experiment was repeated three times with triplicate samples. **, $P < 0.01$, compared with untreated A549 cells (control). (C) The amount of gliotoxin in CFs of *A. fumigatus* strains was confirmed by reversed-phase high-performance liquid chromatography (RP-HPLC) analysis. Each experiment was repeated three times with triplicate samples. **, $P < 0.01$, compared with the Afs35 strain. (D) The amount of gliotoxin in *A. fumigatus* hyphae was confirmed by RP-HPLC analysis. Each experiment was repeated three times with triplicate samples. *, $P < 0.05$, compared with the Afs35 strain.

Discussion

Gliotoxin is known to be one of the most important virulence factors of *A. fumigatus*, and its metabolism in the fungus has been a focus of interest, particularly the toxin export system. GliA, which is encoded by the gliotoxin biosynthetic gene cluster, is homologous to the MFS EmrB/QacA subfamily with 14 transmembrane domains (Fig. 2); therefore, it was suspectedto be agliotoxin efflux pump of *A. fumigatus* [10]. Some reports support this hypothesis [9,32], but the function of *gliA* has not been analyzed in *A. fumigatus*.

Our data suggest that, through its capacity to export gliotoxin extracellularly, GliA functions to protect the fungus from the harmful effects of extracellular gliotoxin, which strongly suggests that GliA also contributes to protection from its own produced gliotoxin by constantly exporting the toxin. *gliA* disruption caused the fungus to be highly susceptible to extracellular gliotoxin, and when the gene was introduced into gliotoxin-sensitive *S. cerevisiae*, the fungus acquired a resistance to gliotoxin. Amnuaykanjanasin and Daub analyzed the roles of the fungal transporter encoded by the secondary metabolite biosynthetic

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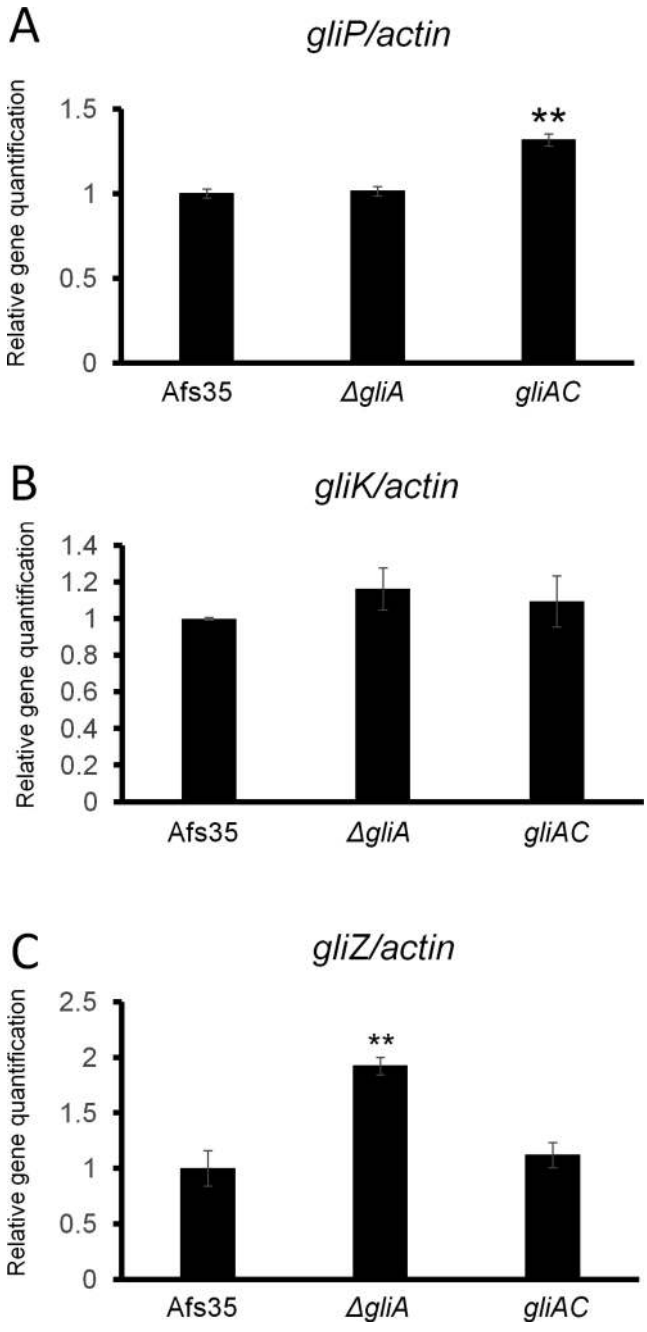

Figure 6. Expression of the *gliP* (A), *gliK* (B), and *gliZ* (C) in *Aspergillus fumigatus* Afs35, Δ*gliA*, and *gliAC*, where the values were normalized relative to the expression ratios of the Afs35 strain. The relative expression levels of these genes were calculated using the comparative threshold cycle (C_T) method, with the *A. fumigatus* actin gene used as the normalization control [14]. Each experiment was repeated three timeswith triplicate samples. **, $P < 0.01$, compared with the Afs35 strain.

gene cluster and classified the roles into three categories: toxin efflux, self-protection, or both [18]. On the basis of their classification, GliA has the third role, that is, extracellular efflux and self-protection. Gardiner et al. found a similar phenomenon in *L. maculans*, reporting that SirA, an ABC transporter but not an MFS transporter, plays a role in the self-protection of the fungus from its own toxin, sirodesmin [32]. In fact, gliotoxin could be a double-edged sword for *A. fumigatus* [3] because it is toxic not only to the host cells but also to the fungus. Thus, *A. fumigatus* has two distinct mechanisms that mediate tolerance to gliotoxin: extracellular export by GliA and intracellular detoxification of the toxin by GliT [15]. We revealed that the extracellular gliotoxin concentration in the *gliA* disruptant was significantly lower than that in the Afs35 parental strain and the *gliA*-complemented strain (Fig. 5D), whereas *gliT* expression was upregulated in the *gliA* disruptant (data not shown), suggesting that GliT overexpression decreased the intracellular gliotoxin concentration in the *gliA* disruptant and prevented the toxic effect. Although further investigation is required, it has become evident that GliA, in addition to GliT, plays a significant role in protection from the toxic effects of gliotoxin.

An interesting finding of our study was that the amount of gliotoxin was reduced not only in extracellular but also in intracellular spaces, which suggests that gliotoxin production was greatly reduced by the *gliA* disruption. Bradshaw et al. revealed that deletion of the MFS transporter gene in the dothistromin biosynthetic gene cluster, *dotC*, decreased the production of dothistromin, but its mechanism was not determined [33]. On the other hand, the deletion of the MFS transporter gene, *aflT*, in the aflatoxin biosynthetic gene cluster has no effect on aflatoxin production [34]. In contrast to aflatoxin, the intracellular accumulation of gliotoxin would directly lead to fungal cell death, and this may be related, in part, to the difference in the phenotypes.

We found a significant improvement in the survival rate of a nonneutropenic mouse model when animals were infected with the Δ*gliA* strain. The fungal burden of Δ*gliA* strain-infected mice was slightly lower than that of the Afs35 strain-infected mice. However, the difference was not significant, revealing that there was a discrepancy between survival and the fungal burden. Spikes et al. reported similar results, where the survival rate of mice infected with a gliotoxin-deficient Δ*gliP* strain was significantly higher than that of the parent strain-infected mice; however, no difference in the pulmonary burdens was observed between these two groups [31]. Gliotoxin destroys neutrophils by inducing apoptosis, and the release of gliotoxin from the fungus in the infected foci causes neutrophil destruction and results in the decrease of the survival rate of mice. A possible explanation for this discrepancy is that, after infection with the wild-type strain, neutrophil destruction was enhanced by gliotoxin, followed by the release of tissue-damaging substances from the neutrophils that caused more extensive inflammatory reactions

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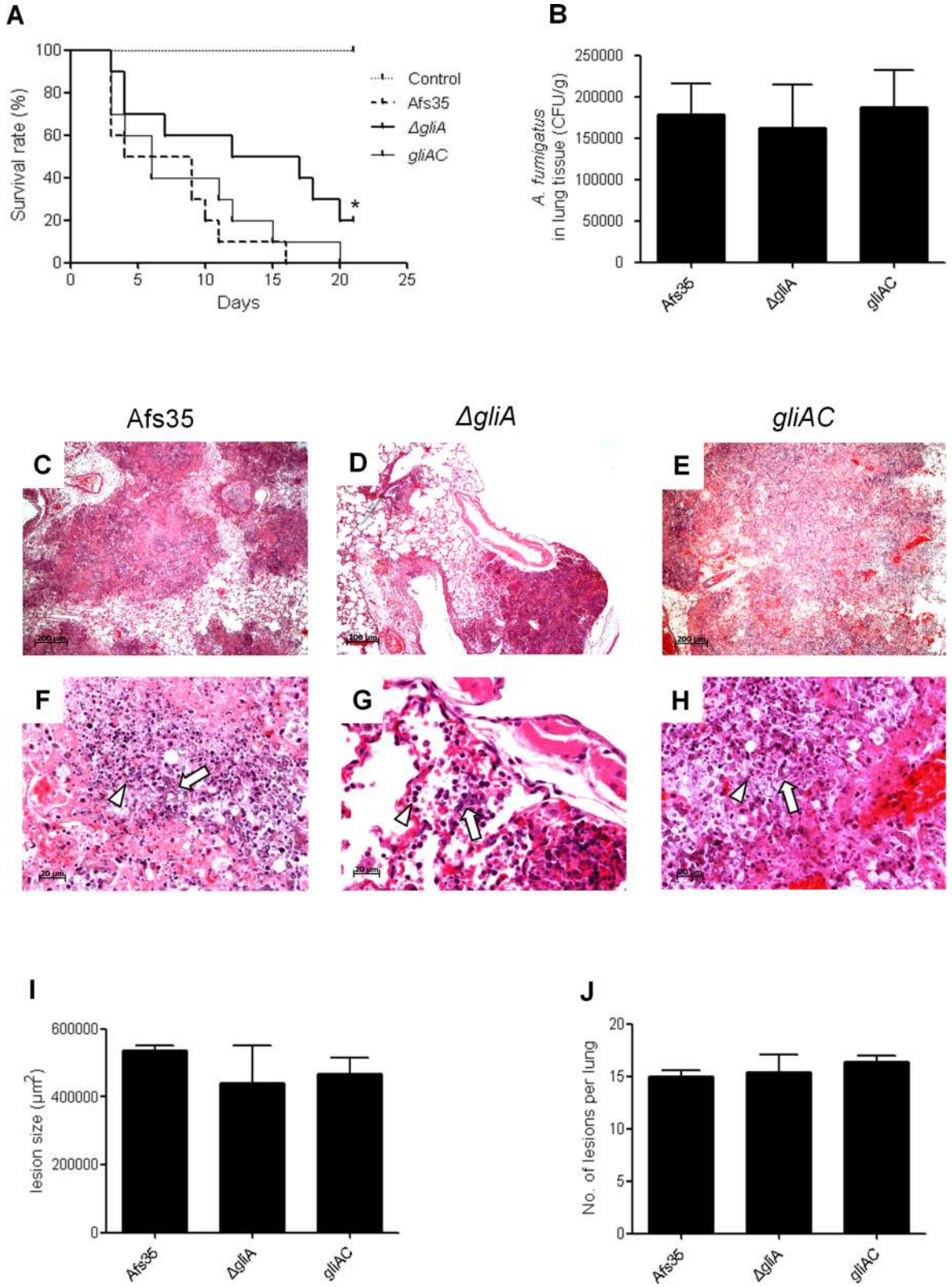

Figure 7. The survival rate, fungal burden, and histopathological analyses of mice infected with the Afs35, Δ*gliA*, or *gliAC* strain. (A) The survival rates of mice infected with Afs35, Δ*gliA*, or *gliAC* strain. The Kaplan–Meier survival plot and log-rank test were used to compare the survival rates among the groups. ∗, $P < 0.05$, compared with Afs35. (B) The fungal burden in lung tissues of mice sacrificed 72 h after infection (10 mice per fungal strain; CFU, colony-forming units). (C–H) Lung tissue sections stained with hematoxylin and eosin from mice (three mice per fungal strain) infected with Afs35 (C and F), Δ*gliA* (D and G), and *gliAC* (E and H). Arrowheads indicate neutrophils. White arrows indicate hyphae. (I) Areas containing hyphae and inflammatory cell infiltration. (J) Number of inflammatory lesions per lung.

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and resulted in more serious tissue damage [35]. In fact, in our Δ*gliA* strain-infected mice, most of the neutrophils that migrated into the infected lesions were apparently intact, whereas in the Afs35 strain- or *gliAC* strain-infected mice, neutrophils exhibited nuclear fragmentation much more frequently, which is compatible with gliotoxin-induced apoptotic changes. The differences in each of the fungal burdens and the areas of the inflamed regions were not significant, but survival improved significantly because of a combination of these factors.

In conclusion, this is the first study of disrupted *gliA* in *A. fumigatus*. We analyzed the function of this gene and provided new insights into gliotoxin metabolism in *A. fumigatus*. In *A. fumigatus*, GliA has important functions in the export of gliotoxin and the self-protection of the fungus from gliotoxin, thereby playing a critical role in virulence. Control of the expression of *gliA* and/or its related products may facilitate the development of a new strategy for the supportive management of aspergillosis. Further investigation is underway to understand the mechanism of action of GliA and its application.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

Supplementary material

Supplementary material is available at *Medical Mycology* online (http://www.mmy.oxfordjournals.org/).

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