Disruption of a Nonribosomal Peptide Synthetase in *Aspergillus fumigatus* Eliminates Gliotoxin Production

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The fungal secondary metabolite gliotoxin produced by *Aspergillus fumigatus* has been hypothesized to be important in the development of invasive aspergillosis. In this study, we addressed this hypothesis by disrupting a nonribosomal peptide synthetase (NRPS) (encoded by *gliP*) predicted to be involved in gliotoxin production. Mutants with a disrupted *gliP* locus failed to produce gliotoxin, which confirmed the role of the NRPS encoded by *gliP* in gliotoxin biosynthesis. We found no morphological, developmental, or physiological defects in Δ*gliP* mutant strains. In addition, disruption of *gliP* resulted in down regulation of gene expression in the gliotoxin biosynthesis gene cluster, which was restored with addition of exogenous gliotoxin. This interesting result suggests a role for gliotoxin in regulating its own production. Culture filtrates from the Δ*gliP* mutant were unable to inhibit ionomycin-dependent degranulation of mast cells, suggesting a role for gliotoxin in suppressing mast cell degranulation and possibly in disease development. However, the Δ*gliP* mutant did not have an impact on survival or tissue burden in a murine inhalational model of invasive aspergillosis. This result suggests that gliotoxin is not required for virulence in an immunosuppressed host with an invasive pulmonary infection.

New medical therapies for life-threatening diseases such as solid-organ transplantation, aggressive cancer therapies, and other immunomodulating therapies have resulted in an increase in invasive fungal infections. In particular, the mortality due to invasive aspergillosis (IA) has increased 357% over the last 25 years (19). IA has become one of the leading causes of death in immunocompromised patients, with mortality rates ranging from 60 to 90% (18, 19). Although IA can be caused by several members of the genus *Aspergillus*, *Aspergillus fumigatus* remains the most prevalent causal organism (13, 26).

A. fumigatus is a saprophytic, asexually reproducing fungus that is found in soil and compost piles and primarily functions to recycle carbon and nitrogen throughout the environment (35). Yet the high frequency of *A. fumigatus* infections strongly suggests that this saprophytic fungus has attributes unique among *Aspergillus* species that make it an effective opportunistic pathogen. Several physiological and morphological characteristics of *A. fumigatus* have been hypothesized or demonstrated to be involved in fungal pathogenesis. These include thermotolerant growth, fast growth rates, conidial size and

morphology, and the production of cell wall-degrading enzymes and proteases (3, 6, 13, 16).

Like other *Aspergillus* species, *A. fumigatus* is known to produce immunosuppressant secondary metabolites, including the epipolythiodioxopiperazine (ETP) toxin gliotoxin (11). Secondary metabolites are compounds produced by many filamentous fungi that are not required for growth but often have important biological activities. For instance, fungal secondary metabolites often have antimicrobial activity, and it is hypothesized that their production is an adaptation that allows fungi to compete with other microorganisms in their ecological niches. In several plant-fungal interactions, secondary metabolites are primary virulence factors that determine outcomes of host-pathogen interactions (25). While several indirect lines of evidence have suggested a role for gliotoxin and other secondary metabolites in invasive fungal infections, no direct evidence has definitively shown their involvement in human-fungal pathogenesis.

With the recent completion of the *A. fumigatus* genome project, the ability to address the role of secondary metabolites in IA has greatly increased (23). Recently, an ETP toxin and the nonribosomal peptide synthetase (NRPS) involved in its production were identified in the plant pathogen *Leptosphaeria maculans* (9). With this sequence information, a gene cluster containing an NRPS (*gliP*) was identified in the genome sequence of *A. fumigatus* and predicted to be involved in the

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TABLE 1. Primers used in this study

Primer[a]	Sequence 5′ to 3′	Function
5′GliZ	ACG ACG ATG AGG AAT CGA AC	Real-time PCR of gliotoxin cluster
3′GliZ	TCC AGA AAA GGG AGT CGT TG	Real-time PCR of gliotoxin cluster
5′GliI	AGG CCA TCC TCG TGT GTA AC	Real-time PCR of gliotoxin cluster
3′GliI	GCC GAG GTC TTT GCT GAT AC	Real-time PCR of gliotoxin cluster
5′GliJ	CTC TGA TCG ACG GCC ATA AT	Real-time PCR of gliotoxin cluster
3′GliJ	TCG AGC TGT TGG AGT GTC TG	Real-time PCR of gliotoxin cluster
5′NRPS10	CAT CGA GGG GCT GGA GGT GAA A	Real-time PCR of gliotoxin cluster
3′NRPS10	TCG GAT GCT GTC CTC GAT GGT G	Real-time PCR of gliotoxin cluster
5′GliC	ATT GAC CGG GAT GAC ACA TT	Real-time PCR of gliotoxin cluster
3′GliC	ACC GTC GAG GAT TGT ATT GC	Real-time PCR of gliotoxin cluster
5′GliM	CGA TCT GTA CCC CAA CGA GT	Real-time PCR of gliotoxin cluster
3′GliM	TTC TGG AAC TTT GCC AGC TT	Real-time PCR of gliotoxin cluster
5′GliG	GAA ACT GCG CAG CAA CAT TA	Real-time PCR of gliotoxin cluster
3′GliG	TTG GCC ATT TCT CAA ACT CC	Real-time PCR of gliotoxin cluster
5′GliK	CTC ACG GCA TAC AGC GAC TA	Real-time PCR of gliotoxin cluster
3′GliK	ATA ATC CAA CCG AGC CAC TG	Real-time PCR of gliotoxin cluster
5′GliA	TTT GCG ATC AAC GAA CTC TG	Real-time PCR of gliotoxin cluster
3′GliA	CCC TTG ACG GAC TGG AAG TA	Real-time PCR of gliotoxin cluster
5′GliN	GCA AGA GGT GCA AGA GAA GG	Real-time PCR of gliotoxin cluster
3′GliN	GGA TCG GAT CAA AGT CCT CA	Real-time PCR of gliotoxin cluster
5′GliF	GGG GGC CGA TAA TAT CAA CT	Real-time PCR of gliotoxin cluster
3′GliF	AAG ATG GCC AAT CCA CCA TA	Real-time PCR of gliotoxin cluster
5′GliT	ACT CCA CCA TCC AGT TCC AG	Real-time PCR of gliotoxin cluster
3′GliT	TCC GAG TAT CCC TCG ATG TC	Real-time PCR of gliotoxin cluster
5′AfactinII	TCA CTG CCC TTG CTC CCT CGT C	Real-time PCR of gliotoxin cluster
3′AfactinII	GCA CTT GCG GTG GAC GAT CGA A	Real-time PCR of gliotoxin cluster
5′SalIOL1GliP	TCG TCG ACG ATC GAG GAA CAG ACC CAT C	GliP disruption construct
3′EcoRIOL2GliP	GCG AAT CCG GCA AGC TCT GTG AAG ATG A	GliP disruption construct
5′XbaIOL3GliP	TCT CTA GAC GAT TGA TCA TTC CGT CTC	GliP disruption construct
3′SacIOL4GliP	GTG AGC TCC TCT GCA TCA TGC CAT CAG T	GliP disruption construct
5′GlioPReplace	GGA TGC GAG TCG GTG TAA CT	Amplifies 0.7-kb internal region of *gliP*
3′GlioPReplace	ATC TCT TGG GCG ACA TTG AC	Amplified 0.7-kb internal region of *gliP*
5′GliPKOLF	TGG CTC GCT GCA CGC GTA GAC	Check homologous recombination left (5′)
3′PyrGKOLF	TAG GGT ACC TGT CCG CGC GGG G	Check homologous recombination left (5′)
5′PyrGKORT	TGG CGA CCA CAC CCG TCC TGT G	Check homologous recombination right (3′)
3′GliPKORT	GCC ATA TGA AGC ATC GAC TTA CC	Check homologous recombination right (3′)

[a] Gli RT primers are from reference 10. Primers 5′NRPS10 and 3′NRPS10 are for *gliP*.

synthesis of gliotoxin (10). Yet this prediction has not been functionally confirmed.

In this study, we definitively demonstrate that the NRPS *gliP* is involved in gliotoxin biosynthesis as disruption of the *gliP* locus eliminated gliotoxin production in *A. fumigatus*. This mutant was used to address the hypothesis that gliotoxin plays a critical role in the development of IA. Effects of gliotoxin on the expression of the biosynthetic gene cluster involved in its production and on the morphology and physiology of *A. fumigatus* are also presented.

MATERIALS AND METHODS

Fungal strains, cell lines, media, and growth conditions. *A. fumigatus* strain AF293.1 was used to create *gliP* knockout strain ARC2 (Δ*gliP::A. parasiticus pyrG pyrG1*) and ectopic transformant strain ARC5 (*pyrG1 A. parasiticus pyrG::gliP*). *A. fumigatus* strain AF293.1 is a uracil-auxotrophic (*pyrG1*) mutant of *A. fumigatus* strain AF293 (36). *A. fumigatus* strains were cultivated at 37°C in Czapek Dox broth (Difco Laboratories, Sparks, MD) to measure gliotoxin production and in glucose minimal medium for all other assays as previously described (4, 28). For growth on solid medium, glucose minimal medium with appropriate supplements was used as previously described (28). Rat basophilic leukemia (RBL-2H3) cells were cultured in minimum essential medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum. Cells were trypsinized and replated in 96-well plates prior to experiments.

Construction of gliotoxin-deficient *A. fumigatus* strain ARC2. Disruption of the NRPS gene *gliP* in *A. fumigatus* strain AF293.1 was accomplished by replac-

ing an ~3.7-kb internal fragment of the *gliP* coding region (~6.4 kb; GenBank accession no. DQ457015) with *A. parasiticus pyrG*. The disruption construct was created by cloning a sequence homologous to the *gliP* locus into plasmid pJW24 (donated by Nancy Keller, University of Wisconsin—Madison). 5′ and 3′ *gliP* homologous sequences, each 1 kb in length, were cloned to flank *A. parasiticus pyrG* in pJW24. The resulting plasmid, pGLIPKO, was used as a template to amplify the 5.1-kb disruption construct for use in fungal transformation. The primers utilized in vector construction and all of the other primers utilized in this study are presented in Table 1.

Creation of fungal protoplasts and polyethylene glycol-mediated transformation of *A. fumigatus* were performed as previously described (5). Briefly, 2 μg of the *gliPKO* PCR-generated disruption construct was incubated on ice for 50 min with 1×10^7 fungal protoplasts in a total volume of 100 μl. Transformants were initially screened by PCR to identify potential homologous recombination events at the *gliP* locus. PCR was performed with primers designed to amplify a 700-bp internal region of the *gliP* locus (5′GliPReplace and 3′GliPReplace), which should be absent in *gliP* disruptant strains. In addition, PCR was performed with primers designed to amplify only the disrupted *gliP* locus (5′GlipKOLF and 3′PyrGKOLF; 5′PyrGKORT and 3′GliPKORT). Homologous recombination was confirmed by Southern analysis with the digoxigenin labeling system (Roche Molecular Biochemicals, Mannheim, Germany) as previously described (7). To eliminate the chance of heterokaryons, each transformant was streaked with sterile toothpicks a minimum of two times to obtain colonies from single spores.

Determination and quantification of gliotoxin production. Czapek Dox broth was used to determine the production of gliotoxin in liquid broth cultures. Freshly harvested *A. fumigatus* conidia from wild-type strain AF293, Δ*gliP* mutant strain ARC2, or ectopic transformant strain ARC5 were inoculated into 50 ml of Czapek Dox broth and incubated for defined periods of time at 37°C while shaking at 200 rotations/min. Following growth, mycelia were separated from

culture filtrates (CFs) by vacuum filtration. Mycelial mats were lyophilized overnight and weighed to determine the dry weight of fungal tissue. CFs were extracted 2× with equal volumes of high-performance liquid chromatography (HPLC) grade chloroform, and the organic layers were combined and dried to completeness on a roto-evaporator (Brinkmann-Eppendorf, Hamburg, Germany). Dried extracts were resuspended in 1 ml of HPLC grade methanol. Gliotoxin production was measured by reverse-phase HPLC on a Waters HPLC system with a UV light detector (Waters Corporation, Milford, MA). A Zorbax SB-C$_{18}$ reverse-phase column (5 µm, 4.6 by 250 mm) was used (Agilent Technologies, Palo Alto, CA). Twenty microliters of the methanol-resuspended culture extracts was injected onto the column and eluted isocratically with 0.1% (vol/vol) trifluoroacetic acid–65% (vol/vol) HPLC grade water–34.9% (vol/vol) HPLC grade acetonitrile as previously described (27). The pressure in the column ranged from 1,400 to 1,800 lb/in^2, and the flow rate was 1.00 ml min^{-1}. Gliotoxin was detected at a wavelength of 264 nm. A standard curve of peak area versus gliotoxin concentration was constructed with gliotoxin (Sigma Aldrich, St. Louis, MO) standards ranging from 25 µg/ml to 1 mg/ml. Quantification of gliotoxin production by *A. fumigatus* strains was done by measuring CFs from cultures grown at 37°C for 72 h in Czapek Dox broth. The amount of gliotoxin produced by each *Aspergillus* strain is presented as milligrams of toxin produced per gram of dried fungal tissue.

Determination of spore morphology, colony growth, and sporulation. Scanning electron microscopy was used to assess the surface morphology of conidia from *A. fumigatus* strains AF293, ARC2, and ARC5. Sporulation and colony growth were quantified as previously described (19). Briefly, 10-µl aliquots containing 1 × 10^7 conidia from freshly harvested glucose minimal medium plates were placed in the center of glucose minimal medium and Sabouraud's agar plates. Diameters of three colonies per *A. fumigatus* strain were measured once daily over a period of 5 days. The average change in colony diameter per 24 h of growth was calculated for three independent cultures. Sporulation was quantified by harvesting conidia from 5-day-old glucose minimal medium plates inoculated as for colony growth determination. Conidia were harvested with 20 ml of sterile 0.05% Tween 80, filtered through two layers of miracloth (EMD Biosciences, La Jolla, CA), and quantified with a hemacytometer. Conidia were harvested from three independent cultures, and the mean and standard deviation of the total number of conidia collected from the plate are reported.

Mast cell ionomycin chase β-hexosaminidase release assay. Hindrance of degranulation of RBL cells was determined by measuring β-hexosaminidase release after pretreatment with fungal CF and stimulation with a calcium ionophore. Fungal CFs were prepared in Czapek Dox broth as described for gliotoxin production and sterilized by filtering through a 0.22-µm Millipore Express Plus membrane (Millipore Corporation, Billerica, MA). Briefly, monolayers of RBL cells (5 × 10^4) were exposed to 0.1 ml of fungal CF in Czapek Dox broth for 120 min. Filtrates were removed before stimulation with 0.1 ml of 1 µM ionomycin in Tyrode's buffer for 60 min. The cells were lysed with 0.1% Triton in Tyrode's buffer. Supernatants and total cell lysates were incubated with 3.4 mg/ml p-nitrophenyl-N-acetyl-β-D-galactosaminide in 0.1 M sodium citrate buffer (pH 4.5) at 37°C for 60 min. Addition of 0.1 M carbonate buffer (pH 10.0) terminated the reaction. Release of the product p-nitrophenol was evaluated by measuring optical absorbance at 405 nm. Results are expressed as the percentage of β-hexosaminidase released into the medium versus the total. The experiments were performed in quadruplicate wells for replicates 1 and 2 and triplicate wells for replicate 3. The variables are presented as the mean ± the standard error of the mean, and comparisons between groups were made by analysis of variance, followed by a multiple-comparison Tukey test. The analyses were done with the statistical package S-PLUS 7.0 for Windows (Insightful Corp., Seattle, WA), and a significance level of $P < 0.05$ was accepted.

Gene expression assays. Expression of genes in the predicted gliotoxin biosynthesis cluster was assessed by real-time reverse transcriptase PCR (RT-PCR). Expression of genes in the gliotoxin biosynthesis cluster (GenBank accession no. AY83877) was examined in Czapek Dox broth cultures after 48 h of growth at 37°C. In addition, to confirm the regulation of the gliotoxin cluster by the presence or absence of gliotoxin, gliotoxin at final concentrations of 500 ng/ml, 5 µg/ml, and 20 µg/ml was added after 24 h to the ARC2 culture, which was incubated for an additional 24 h. Total RNA was extracted from the Czapek Dox broth cultures with the Ambion RNAqueous total RNA isolation kit (Ambion, Austin, TX). Total RNA was treated with Ambion Turbo DNA-free DNase I (Ambion, Austin, TX) to remove contaminating genomic DNA. A total of 500 ng of DNase-treated total RNA from each *A. fumigatus* strain was reverse transcribed with Superscript III RT (Invitrogen, Carlsbad, CA). Real-time RT-PCR was conducted with 20-µl reaction volumes with the iQ SYBR green supermix (Bio-Rad Laboratories, Hercules, CA), 2 µl of a 1:5 dilution of first-strand cDNA, and 0.4 µl of each 10 µM primer stock. No-RT controls were used to

confirm elimination of contaminating genomic DNA. Real-time PCR was performed with an iQ Cycler real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). PCRs for each gene were done in triplicate, and data were analyzed with the gene expression software included with the iQ Cycler system. Melt curve analysis was performed after the PCR was complete to confirm the absence of nonspecific amplification products. The fold change in gliotoxin cluster gene expression between *A. fumigatus* wild-type strain AF293 and the gliotoxin mutant ARC2, with or without addition of gliotoxin, was calculated by using 2$^{-\Delta\Delta Ct}$, and all values were normalized to the expression of the *A. fumigatus* actin gene.

Murine virulence assays. Outbred ICR (Harlan Sprague-Dawley) male mice (19 to 21 g in size) were used to assess the role of gliotoxin in fungal virulence. Mice were housed five per cage and had access to food and water ad libitum. All animal research procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio. Mice were immunosuppressed with intraperitoneal (i.p.) injections of cyclophosphamide at 250 mg/kg and with cortisone acetate injected subcutaneously (s.c.) at 250 mg/kg 2 days prior to infection. On day 3 postinfection, repeat injections were given with cyclophosphamide (200 mg/kg i.p.) and cortisone acetate (250 mg/kg s.c.). Mice were given ceftazidime (50 mg/kg s.c.) daily beginning 2 days prior to infection.

Twenty mice per *A. fumigatus* strain (AF293, gliotoxin-deficient mutant ARC2, or ectopic mutant ARC5) were infected with an acrylic aerosol chamber that allows simultaneous challenge of 50 to 60 mice. In this acrylic chamber, *A. fumigatus* conidia were nebulized at 10^9 CFU/ml of fluid for 1 h. Infection inoculum was prepared by growing the *A. fumigatus* isolates on potato dextrose agar plates at 37°C for 7 days. Conidia were harvested by washing the plate surface with sterile phosphate-buffered saline–0.1% Tween 80, dislodging conidia by gentle rubbing with a sterile glass rod. The resultant conidial suspension was centrifuged at 15,000 × g (Sorvall SS-34 rotor) without braking and adjusted to the desired concentration of 10^9 conidia/ml by hemacytometer count. Mice were immunosuppressed, infected, and observed for survival for 14 days after *A. fumigatus* challenge. Any animals showing distress were immediately sacrificed and recorded as deaths within 24 h. Lungs from all mice sacrificed during the experiment were removed for fungal burden assessment.

For tissue burden studies, additional mice were immunosuppressed as described above, infected, and sacrificed at set time points of 1 h and day 7 after *A. fumigatus* challenge. When mice were sacrificed, serum and lungs were removed on that day. Tissues were aseptically removed, weighed, and homogenized with an overhead stirrer in 2 ml of sterile saline with gentamicin at 2.5 mg/liter and chloramphenicol at 400 mg/liter. Tissue homogenates were quantitatively cultured by serial dilution plating on potato dextrose agar incubated at 37°C overnight. Statistical analysis of tissue burdens among the three fungal strains was performed with a Kruskal-Wallis test and Dunn's multiple-comparison test. A log rank test was used for survival comparisons. Statistical significance was defined as $P < 0.05$.

Nucleotide sequence accession number. The nucleotide sequence of the updated *gliP* sequence reported here has been deposited in GenBank under accession number DQ457015.

RESULTS

Disruption of the *gliP*-encoded NRPS. Sequencing of the *gliP* cDNA revealed a 30-nucleotide discrepancy with respect to the previously published sequence. Our cDNA clone had an additional 30 nucleotides starting at position 613 in the cDNA sequence, bringing the total length of the *gliP* cDNA to 6,408 bp. This result is likely due to misannotation of the intron's splice site in the computer-based gene prediction algorithm. This updated *gliP* sequence was deposited in GenBank under accession number DQ457015.

To definitively study the role of gliotoxin in *A. fumigatus* physiology and pathogenesis, we created a mutation in the *gliP* NRPS gene predicted to be involved in gliotoxin biosynthesis (Fig. 1A). Transformation of *A. fumigatus* protoplasts with the *gliPKO* PCR disruption construct yielded 25 transformants. Transformant ARC2 had no detectable PCR amplicon with primers (5′GlioPReplace and 3′GlioPReplace) designed to amplify the targeted replacement region of the *gliP* locus (Fig. 1B).
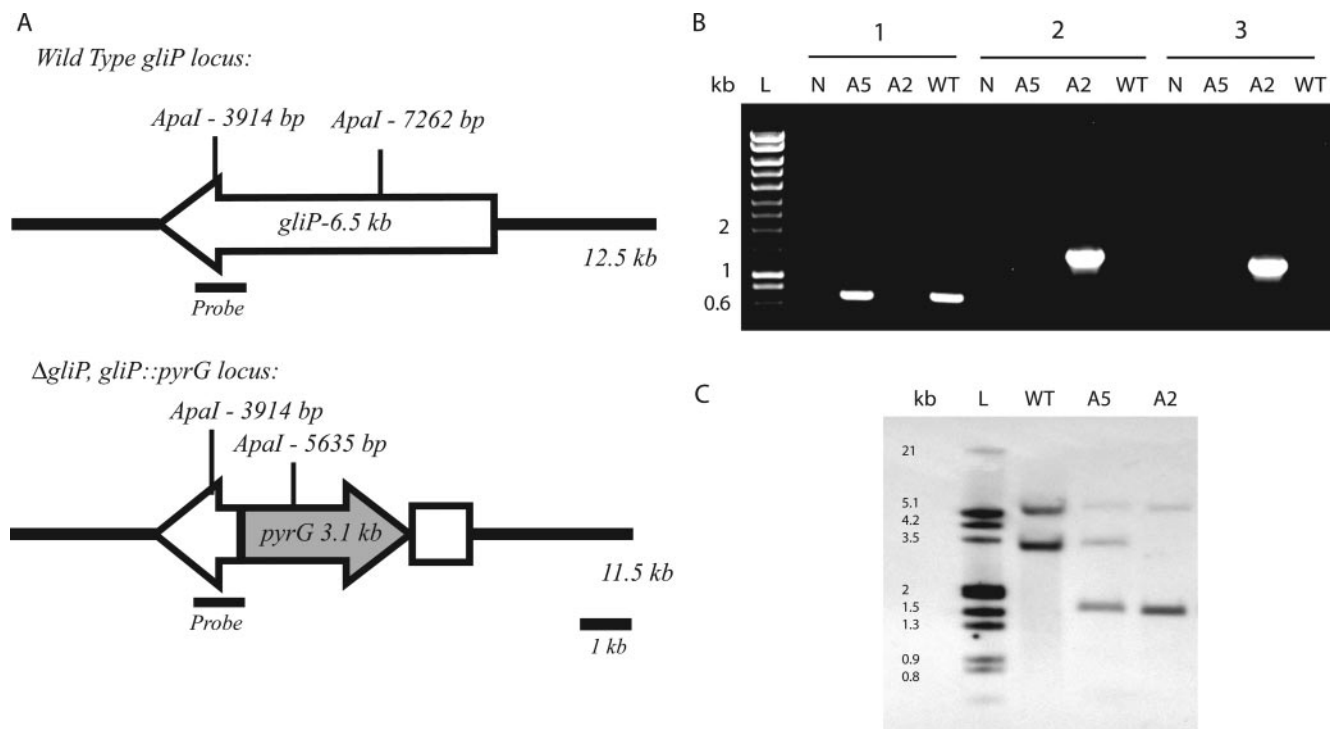
FIG. 1. Disruption of *A. fumigatus* NRPS gene *gliP*. (A) Representation of the *gliP* locus in wild-type and Δ*gliP* mutant strains. In Δ*gliP* mutant strain ARC2, a 3.8-kb internal section of the *gliP* coding region was replaced with the 3.1-kb *pyrG* gene from *A. parasiticus*. The ApaI restriction enzyme was used to digest chromosomal DNA, which was probed with the 1.0-kb probe as indicated. (B) PCR confirmation of *gliP* gene disruption. Lanes: L, DNA ladder; N, water control; A5, ectopic transformant strain ARC5; A2, Δ*gliP* mutant strain ARC2; WT, wild-type strain AF293. Group 1, amplification of internal *gliP* amplicon absent in Δ*gliP* mutant strain ARC2 because this sequence was replaced with *pyrG* via homologous recombination. Groups 2 and 3, 5′ and 3′ flanking regions, respectively. Amplification products in Δ*gliP* mutant strain ARC2 indicate successful homologous recombination at the *gliP* locus. (C) Southern analysis of *A. fumigatus* transformants. Lanes are the same as those in panel B.

ARC2 had detectable PCR amplicons with primers (5′GliPKOLF and 3′PyrGKOLF; 5′PyrGKORT and 3′GliPKORT) designed to amplify the predicted Δ*gliP* disruption locus after homologous recombination (Fig. 1B). These results suggested that the *gliP* locus had been precisely replaced with the Δ*gliP::pyrG* mutant allele in transformant ARC2.

By Southern analysis with ApaI-digested genomic DNA (Fig. 1A and C), we confirmed disruption of the *gliP*-encoded NRPS in transformant ARC2. Transformant ARC2 has a single insertion of the disruption construct into the *gliP* locus replacing the 3.7-kb internal fragment of the *gliP*-encoded NRPS, as indicated by the absence of the 3.3-kb wild-type band and the presence of a single 1.7-kb disruption construct band. Transformant ARC5 had a single ectopic integration of the disruption construct into the *A. fumigatus* genome, as indicated by the single 1.7-kb band corresponding to the disruption construct. This transformant was used as a *gliP* wild-type control since it complemented the *pyrG* auxotrophic defect in the recipient AF293.1 strain.

Measurement of gliotoxin production. Previous bioinformatic and gene expression analyses suggested that *gliP* encodes the NRPS responsible for gliotoxin production in *A. fumigatus* (9, 10). To test this hypothesis, HPLC was used to determine if disruption of the *gliP*-encoded NRPS eliminates gliotoxin production. HPLC analysis of culture extracts from wild-type strain AF293 and transformation control strain ARC5 revealed the presence of a peak with a retention time (8.3 min) identical to that of a gliotoxin standard (Fig. 2A to C). This peak at 8.3 min is absent in the Δ*gliP* mutant ARC2, indicating that disruption of the *gliP*-encoded NRPS eliminated gliotoxin production (Fig. 2D). This result definitively confirms that *gliP* encodes the NRPS responsible for gliotoxin production. Quantification of gliotoxin production in strains ARC5 and AF293 revealed that the ectopic transformant ARC5 still produced wild-type levels of gliotoxin (13.1 ± 1.71 mg of toxin/g of fungus versus 10.96 ± 1.96 mg of toxin/g of fungus, respectively) and that the transformation procedure itself did not disturb or alter gliotoxin biosynthesis. We observed an additional peak (retention time of 7.05 min) with unknown identity produced in equal concentrations by all three strains.

Phenotypic characteristics. No detectable morphological or development differences were observed among strains ARC2, ARC5, and AF293. Conidia of ARC2 and ARC5 displayed normal pigmentation in culture, and scanning electron microscopy revealed no structural defects (Fig. 3). Conidiation was not affected in the gliotoxin-deficient mutant ARC2 or the ectopic transformant ARC5 (Table 2). Colony growth, as measured by the rate of increasing colony diameter, was not affected in any of the strains, regardless of the medium type (glucose minimal medium versus Sabouraud's medium), at 37°C (Table 2). Thus, we conclude that elimination of gliotoxin production has no effect on the overall growth and physiology of *A. fumigatus*.
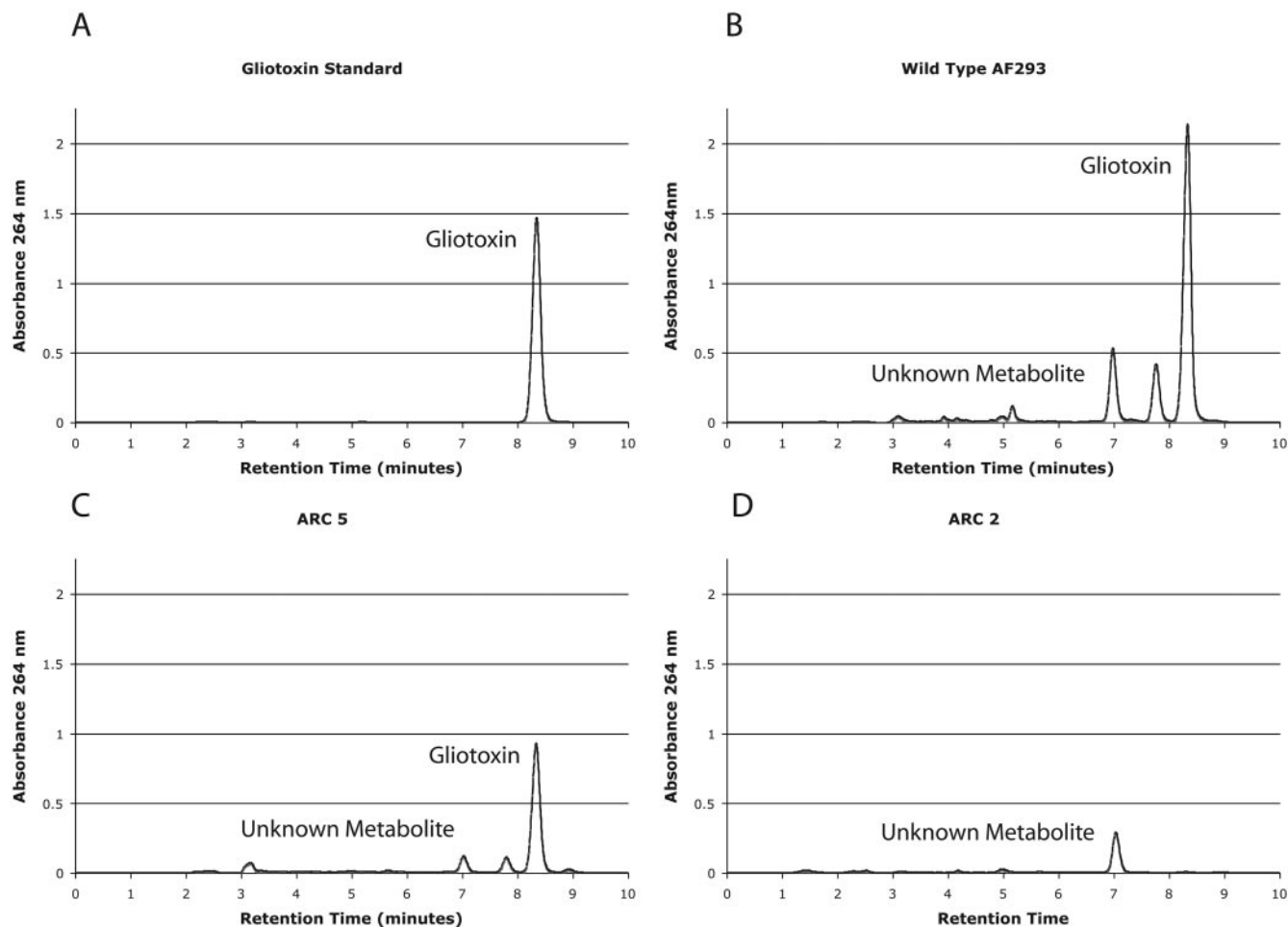
FIG. 2. HPLC analysis of the *A. fumigatus* strains used in this study. Chloroform extracts of CFs from Czapek Dox broth cultures grown for 72 h at 37°C. Gliotoxin and unknown metabolite peaks are identified. Panels: A, gliotoxin standard, 1 mg/ml; B, wild-type AF293; C, ectopic transformant ARC5; D, Δ*gliP* mutant strain ARC2 (lacks the peak at approximately 8.3 min, which demonstrates elimination of gliotoxin production in this mutant).

Expression of gliotoxin biosynthesis gene cluster. Genes involved in the production of fungal secondary metabolites are often physically located together in the genome in gene clusters. We used real-time RT-PCR to examine the expression of genes in the gliotoxin biosynthesis cluster in the wild-type and *gliP* mutant strains (Table 3). These data demonstrated a significant reduction at 48 h of incubation in the expression of all genes in the gliotoxin biosynthesis cluster in Δ*gliP* strain ARC2 relative to that in wild-type strain AF293 (Table 3). As expected, expression of *gliP* was eliminated (0.00007) in strain ARC2 compared to that in strain AF293. Expression of the predicted gliotoxin transporter gene *gliA* and the transcription factor gene *gliZ* was also virtually eliminated.

This result suggested two possible competing mechanisms of gene regulation in the gliotoxin biosynthetic cluster. First, the *gliP* gene product itself could somehow regulate expression of the gene cluster. Alternatively, gliotoxin itself might control the induction of these genes. To distinguish between these two models and rule out damage to transcriptional regulators found in the gene cluster, we examined whether addition of gliotoxin to the ARC2 culture could restore expression of

genes in the gliotoxin biosynthetic cluster. Addition of exogenous gliotoxin at concentrations of 500 ng/ml and 5 µg/ml had little effect on the expression of gliotoxin cluster genes in ARC2, and a direct relationship between gliotoxin concentration and gene transcription was not seen for all of the genes in the cluster. Interestingly, however, when gliotoxin was added to a final concentration of 20 µg/ml, expression of genes in the cluster was restored to wild-type or higher levels (Table 3). Addition of 20 µg/ml gliotoxin to a wild-type AF293 culture did not significantly alter cluster gene expression. These results suggest that disruption of *gliP* affects the expression of other genes in the gliotoxin biosynthetic cluster by eliminating production of gliotoxin, which subsequently positively regulates its own production by a dose-dependent mechanism.

Mast cell ionomycin chase β-hexosaminidase release assay. To begin to examine the potential effects of gliotoxin on *A. fumigatus* opportunistic parasitism, the effects of gliotoxin loss on effector cells of the innate immune system were examined. Fungal CFs from ARC2 and AF293 grown for 48 h in Czapek Dox broth were examined for their effects on RBL-2H3 mast cells. CFs from wild-type AF293 inhibited ionomycin-induced
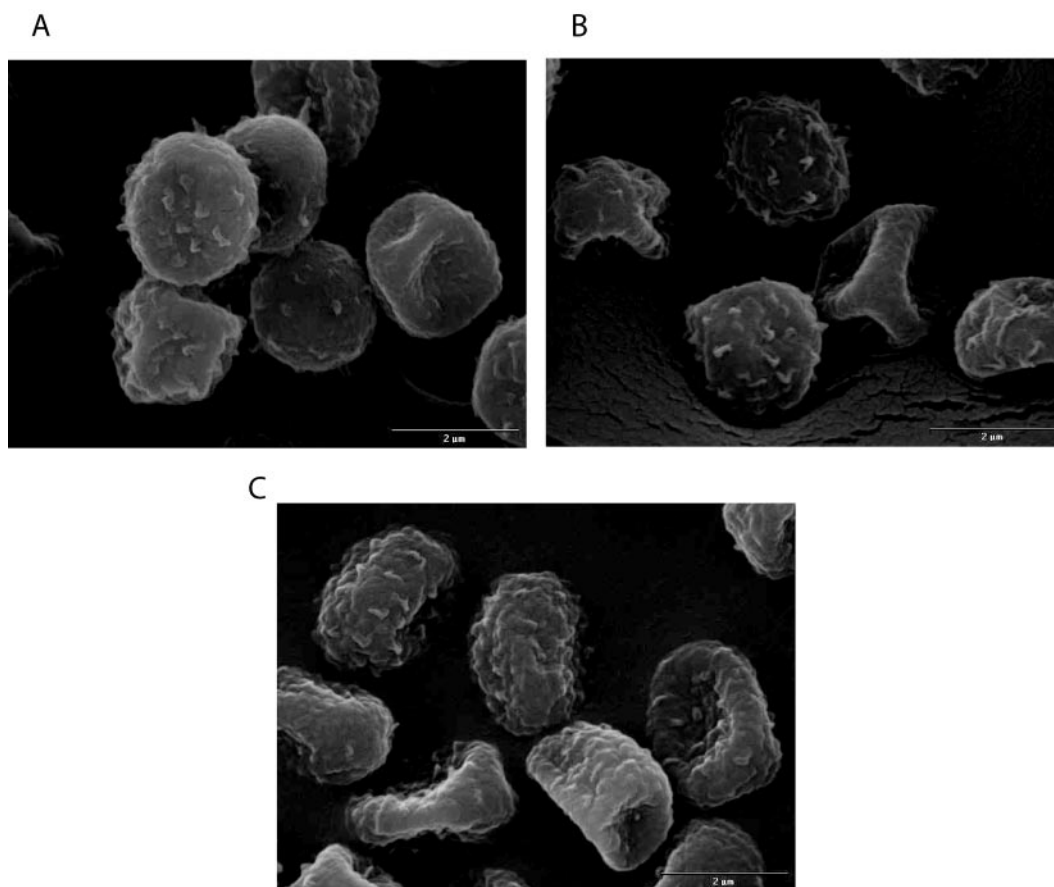
FIG. 3. Scanning electron microscopy of conidia from the *A. fumigatus* strains used in this study. Panels: A, ectopic transformant ARC5; B, Δ*gliP* mutant strain ARC2; C, wild-type strain AF293. No change in conidial surface morphology is apparent.

degranulation of the RBL-2H3 mast cells, while CFs from the gliotoxin mutant ARC2 did not (Fig. 4). Czapek Dox broth itself did not induce spontaneous degranulation. Light microscopy of the mast cells during treatment revealed that cells treated with wild-type CFs displayed morphological changes including rounding of the cells, which may indicate the beginning of cell death. However, these cells did not detach from the substrate, indicating that they were still viable. Trypan blue exclusion also revealed that the cells were still viable. Morphological changes in the cells were not observed with CFs from gliotoxin mutant strain ARC2. While CFs contain numerous fungal secondary metabolites, these results seem to confirm

results previously reported by Niide et al. (24) that showed gliotoxin inhibition of mast cell functions. However, it should be noted that comparison of levels of all secondary metabolites in the CFs from the wild-type strain and strain ARC2 was not conducted, and thus changes in other secondary metabolite levels in the CFs may potentially account for this result. What is clear is that removal of gliotoxin production by disruption of the biosynthetic NRPS-encoding gene *gliP* significantly affects the interaction between *A. fumigatus* and RBL-2H3 mast cells.

Inhalational neutropenic murine model of IA. The potential inhibition of mast cell degranulation by gliotoxin suggested a possible role for gliotoxin in the development of IA. To test this hypothesis, the virulence of Δ*gliP* mutant strain ARC2 was compared to that of ectopic transformant ARC5 and wild-type strain AF293 in an inhalational neutropenic model of IA (Fig. 5). In this model, *Aspergillus* conidia are inhaled by immunosuppressed mice in a sterile chamber. This experimental model most closely reflects clinical *Aspergillus* infections in severely immunocompromised patients. Infection with wild-type strain AF293 in this model consistently results in mortality rates of 70 to 100%. In this study, no significant difference in the survival of mice infected with the wild-type strain or Δ*gliP* mutant strain ARC2 was seen ($P > 0.05$) (Fig. 5). The tissue burden in mouse lungs 1 h after inoculation revealed that all of the mice received approximately equal amounts of conidia from the

TABLE 2. Growth and conidiation of *A. fumigatus* strains used in this study[a]

A. fumigatus strain	Conidiation on glucose minimal medium (mean no. of conidia harvested/ culture ± SD)	Mean diam increase (cm)/ 24 h ± SD	
		Growth on Sabouraud's agar	Growth on glucose minimal medium
AF293	$2.87 \times 10^{10} \pm 1.31 \times 10^{10}$	2.13 ± 0.13	1.30 ± 0.07
ARC5	$2.67 \times 10^{10} \pm 2.40 \times 10^{9}$	2.13 ± 0.32	1.31 ± 0.24
ARC2	$2.87 \times 10^{10} \pm 1.68 \times 10^{9}$	2.13 ± 0.22	1.32 ± 0.04

[a] Growth for all experiments was conducted at 37°C.

TABLE 3. Analysis of gliotoxin biosynthesis cluster gene expression in Δ*gliP* mutant strain ARC2[a]

Gene	Product	ARC2	ARC2 + 500 ng/ml gliotoxin	ARC2 + 5 μg/ml gliotoxin	ARC2 + 20 μg/ml gliotoxin	Wild type + 20 μg/ml gliotoxin
gliA	MFS transporter	0.00 ± 0.00	0.01 ± 0.00	0.33 ± 0.05	2.10 ± 0.27	1.37 ± 0.69
gliC	Cytochrome P450 monooxygenase	0.29 ± 0.01	0.57 ± 0.05	0.79 ± 0.07	1.29 ± 0.41	1.04 ± 0.49
gliF	Unknown	0.11 ± 0.01	0.55 ± 0.10	0.39 ± 0.07	2.30 ± 0.52	1.28 ± 0.63
gliG	Glutathione *S*-transferase	0.04 ± 0.01	0.05 ± 0.15	0.10 ± 0.3	3.18 ± 0.62	1.01 ± 0.50
gliI	Aminocyclopropane carboxylate synthase	0.30 ± 0.04	2.96 ± 0.24	1.02 ± 0.15	2.70 ± 1.10	1.22 ± 0.63
gliJ	Dipeptidase	0.45 ± 0.02	1.23 ± 0.27	0.66 ± 0.09	1.18 ± 0.84	2.12 ± 1.20
gliK	Unknown	0.09 ± 0.03	0.34 ± 0.03	0.15 ± 0.02	3.43 ± 0.51	1.57 ± 0.75
gliM	*O*-Methyltransferase	0.03 ± 0.00	0.27 ± 0.09	0.19 ± 0.05	4.81 ± 0.72	1.22 ± 0.63
gliN	Methyltransferase	0.16 ± 0.00	1.00 ± 0.15	0.65 ± 0.14	2.70 ± 1.28	1.85 ± 0.88
gliP	Peptide synthetase	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.88 ± 0.91
gliT	Thioredoxin reductase	0.09 ± 0.00	0.85 ± 0.47	0.32 ± 0.21	2.7 ± 1.34	0.75 ± 0.61
gliZ	Zinc finger transcription factor	0.03 ± 0.00	0.12 ± 0.02	0.14 ± 0.06	4.29 ± 0.24	1.82 ± 1.18

[a] The wild-type strain and Δ*gliP* mutant strain ARC2 were incubated in Czapek Dox broth for 24 h, when various concentrations of gliotoxin (0, 500 ng/ml, 5 μg/ml, and 20 μg/ml) were added and the cultures were incubated for an additional 24 h before total RNA extraction. Total RNA (500 ng) was reverse transcribed and used in a real-time RT-PCR. Data are presented as the mean fold change in expression ($2^{-\Delta\Delta Ct}$) from wild-type AF293 (fold change = 1) ± the standard deviation. Expression of each gene under each condition is normalized to that of the gene for actin. Addition of 20 μg/ml gliotoxin restored expression of the gliotoxin biosynthesis gene cluster in Δ*gliP* mutant strain ARC2 but did not repress expression when added to the wild type.

wild-type strain and Δ*gliP* mutant strain ARC2 ($P > 0.05$) and the wild-type strain and ectopic transformant strain ARC5 ($P > 0.05$) (mean $\log_{10}$ CFU per gram ± the standard error of the mean: AF293, 4.34 ± 0.08; ARC2, 3.96 ± 0.06; ARC5, 4.50 ± 0.03). The tissue burden in mouse lungs was also measured 7 days after *A. fumigatus* infection, and no significant difference in CFU counts was seen among ARC2, ARC5, and wild-type AF293 ($P > 0.05$ for all pairwise comparisons) (mean $\log_{10}$ CFU per gram ± the standard error of the mean: AF293, 4.29 ± 0.11; ARC2, 4.56 ± 0.07; ARC5, 4.39 ± 0.11). Therefore, we conclude that gliotoxin production is not required for the development of IA in this murine model.

DISCUSSION

Gliotoxin has long been speculated to be a potential virulence factor of *A. fumigatus* (30). Gliotoxin is a strong immunosuppressant with effects on mature lymphocytes and macro-

phages and recently was detected in the serum of patients with IA (14, 21, 22, 29, 33). The disulfide bridge that characterizes ETP toxins like gliotoxin is responsible for the diverse array of biological activities of these toxins (31). The disulfide bridge can redox cycle, generating reactive oxygen species that can damage cells of the immune system, and additionally it can form mixed disulfide bonds with proteins that contain susceptible thiol residues (2, 34). Recently, it was shown that gliotoxin exerts much of its immunosuppressive biological activity through inhibition of the transcription factor NF-κB by inhibition of proteasome-mediated degradation of IκBα (12).

Given these biological effects on cells of the immune system, it has been hypothesized that gliotoxin is a significant factor in the pathogenesis of IA. However, direct genetic evidence in support of this hypothesis has been lacking, mainly because of
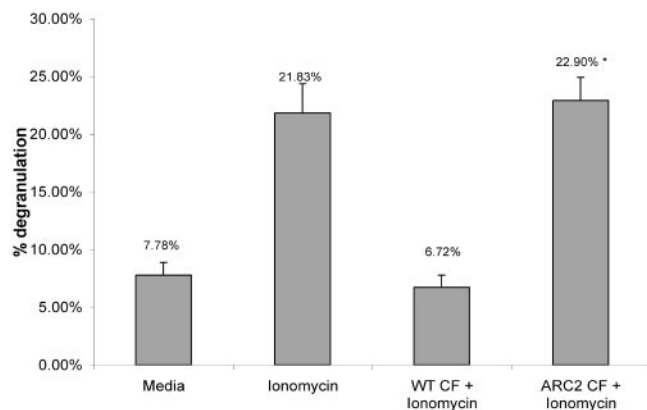
FIG. 4. Inhibition of ionomycin-dependent degranulation of RBL-2H3 mast cells by wild-type CF. CFs were added to RBL-2H3 mast cells, which were then incubated for 2 h. Mast cells were then stimulated for 1 h with 0.1 ml of 1 μM ionomycin. Significant inhibition of ionomycin-dependent degranulation is shown in wild-type CF versus the CF from Δ*gliP* mutant strain ARC2. *, $P < 0.05$ compared to wild-type (WT) CF plus ionomycin and a medium-alone control.
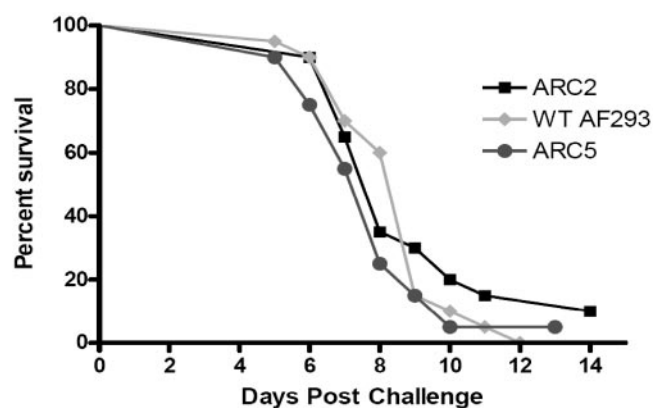
FIG. 5. Inhalational neutropenic murine model of IA. Outbred ICR mice were used to assess the role of gliotoxin in fungal pathogenesis. Mice were immunosuppressed by i.p. injection of cyclophosphamide (250 mg/kg) and s.c. injection of cortisone acetate (250 mg/kg) 2 days prior to infection and injection of 200 mg/kg cyclophosphamide and 250 mg/kg cortisone acetate 3 days postinoculation. Mice were inoculated by exposure to a 10^9-conidium/ml suspension for 1 h in an inhalation chamber. A log rank test was used for pairwise comparisons of survival levels among the strain groups. P value for comparison between ARC2 and wild-type (WT) AF293, 0.642.

the unknown identity of the gene or genes involved in the biosynthesis of gliotoxin. While many biologically active fungal secondary metabolites have been found, identifying the genes responsible for their complex biosynthesis can be extremely difficult. However, the completion of the *A. fumigatus* genome project has made it possible to identify candidate genes involved in secondary metabolism based on sequence similarity (23). Recently, identification of genes involved in the biosynthesis of the ETP toxin sirodesmin, from the plant pathogen *Leptosphaeria maculans*, made it possible to use sequence similarity to predict genes involved in gliotoxin production in *A. fumigatus* (9).

In this study, we definitively confirm the bioinformatic prediction that gliotoxin is synthesized by a two-module system consisting of NRPS and associated genes located in a gene cluster on chromosome 6 in the genome of *A. fumigatus*. Disruption of the NRPS *gliP* gene resulted in elimination of gliotoxin production, confirming the role of *gliP* in the biosynthesis of gliotoxin. Unexpectedly, we found that gliotoxin positively regulated the expression of genes in the biosynthetic cluster. Transcription of genes in the gliotoxin biosynthetic cluster was dramatically reduced in the gliotoxin mutant ARC2 (Table 3). Addition of gliotoxin to the culture medium restored expression of the genes in the biosynthetic cluster in a dose-dependent manner, with the threshold being approximately 20 μg/ml of gliotoxin (Table 3). A direct relationship between gliotoxin concentration and gene expression was not seen for all of the genes in the cluster (Table 3), although all of the genes were repressed without gliotoxin addition and expression was restored when the gliotoxin concentration reached 20 μg/ml. Thus, we cannot rule out the possibility that this apparent self-regulation is an indirect effect attributed to other not apparent effects on *A. fumigatus* physiology. Genome-wide expression profiling with various gliotoxin concentrations and time points may help clarify the underlying mechanism of this interesting regulation of secondary metabolite production. Examples of secondary metabolites regulating their own production, while rare, have been previously found in filamentous fungi. For example, the oxylipins produced by *Aspergillus nidulans* also seem to regulate their own production (32). However, to our knowledge, this is the first report of a fungal toxin involved in the regulation of its own production.

In an attempt to mechanistically examine the potential impact of gliotoxin on opportunistic fungal parasitism, we studied the impact of *gliP* loss on macrophage and mast cell interactions. Preliminary experiments with macrophage cell lines in our laboratory and CFs from wild-type AF293 and the gliotoxin mutant ARC2 suggested that loss of *gliP* does not affect the initial conidium-macrophage interaction but may impact macrophage cell viability later in the interaction when gliotoxin is present (data not shown). These results confirm previous reports of substantial effects of gliotoxin on macrophages and other phagocytes (8, 20). Next, we examined the potential effects of gliotoxin production on the *A. fumigatus*-mast cell interaction.

The role of mast cells in allergic disease is well established, but studies have also demonstrated a critical role for mast cells in the host defense against microorganisms (1, 17). Mast cells contain immunomodulating molecules that can accelerate the production of inflammation to clear invading microbes but can paradoxically lead to chronic inflammatory disease. Although the role of mast cells in invasive fungal infections has not been extensively explored, it was recently shown that gliotoxin can inhibit mast cell functions, including degranulation (24). Our results obtained with CFs from *A. fumigatus* confirm these results and suggest that gliotoxin is the main secondary metabolite responsible for inhibition of mast cell degranulation. Although it may be possible that changes in the presence or absence of other secondary metabolites in the CFs may account for the results seen with ARC2 CFs, it is clear from these results and other studies that gliotoxin has significant biological activity which may stimulate or depress immune system responses.

Since mast cells serve as sentinels of the immune system, we hypothesized that inhibition of degranulation by gliotoxin would contribute to the inhibition of neutrophil recruitment to sites of *A. fumigatus* infection and affect survival. However, we found no significant difference in mortality between mice infected with gliotoxin-deficient strain ARC2 and mice infected with gliotoxin-producing wild-type AF293 in an inhalational model of IA (Fig. 5). This result suggests that, despite its known immunosuppressant activities, including inhibition of mast cell degranulation, gliotoxin is not required for development of IA in immunosuppressed mice. This result is in agreement with a recent study by Lewis et al. (15) that found no correlation with gliotoxin production and the severity and dissemination of *Aspergillus* infections in patients at a tertiary cancer center. However, there are important additional issues to consider with regard to animal models and the pathophysiology of IA that may affect the ability to precisely define the role of gliotoxin in disease development.

The first issue for consideration is the immunosuppression regimen required for IA to develop in animal models. These regimens lead to profound immunosuppression in order to establish disease, which is an attempt to reproduce the immune status of patients with IA. Thus, it is questionable whether additional immunosuppression by gliotoxin would significantly alter the outcome of the fungus-host interaction. Furthermore, the timing of gliotoxin production in vivo may also be a critical factor in its possible role in IA. It is likely that *Aspergillus* infection is already established in immunocompromised hosts by the time gliotoxin production begins and gliotoxin accumulates to levels capable of affecting the already depleted host immune system. In vitro studies at 37°C generally first detect gliotoxin production 24 h after culture inoculation, with maximum production occurring between 48 and 72 h (10). Further studies are needed to address the possibility that gliotoxin production is altered somehow in vivo, which could affect its impact on disease development. However, our results with an inhalational murine model of IA strongly suggest that gliotoxin does not significantly affect the outcome of infection in an immunosuppressed murine model.

However, since fungal toxins like gliotoxin have inhibitory and lethal effects on cells of the immune system, their role in noninvasive diseases such as allergic bronchopulmonary aspergillosis and invasive disease in other at-risk patient populations such as patients with chronic granulomatous disease needs further examination. Although we have shown that gliotoxin itself is not essential for fungal virulence in an invasive pulmonary infection, aspergillosis is a multifaceted disease

characterized by a continuum of overstimulation of host responses in allergic bronchopulmonary aspergillosis and immune system repression in IA. Thus, we cannot rule out the possibility that gliotoxin contributes to the morbidity seen in patients with other forms of aspergillosis. Finally, we also cannot rule out the possibility that the immunosuppressive properties of gliotoxin contribute to the development of additional secondary infections in immunocompromised patients.

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