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# What do we know about the role of gliotoxin in the pathobiology of *Aspergillus fumigatus?*

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## Abstract

Gliotoxin is a member of the epipolythiodioxopiperazine class of toxins and is both the major and the most potent toxin produced by *Aspergillus fumigatus*. Since the discovery of the putative gliotoxin biosynthetic 12-gene cluster in the genome of *A. fumigatus*, five different laboratories have attempted to determine the role of this toxin in the virulence of *A. fumigatus*. The genes in the cluster that have been disrupted to study the pathobiological importance of gliotoxin include *gliZ* that encodes a transcription factor and *gliP* that encodes a nonribosomal peptide synthase. Two of the five laboratories have reported gliotoxin to be an important virulence determinant of *A. fumigatus*, while the other three laboratories have shown it to be unimportant. Comparisons of the data generated among the five laboratories revealed that the immunosuppressive regimen used for mice was the key factor that contributed to the observed disparity. Regardless of either the mouse strains used or the route of infection, immunosuppression with a combination of cyclophosphamide and corticosteroids (neutropenic mice) showed gliotoxin to be unimportant. The mice immunosuppressed with corticosteroids alone, however, revealed that gliotoxin is an important virulence determinant of *A. fumigatus*. These studies indicate that the neutropenic mice model is inadequate to reveal the pathobiological importance of fungal secondary metabolites in invasive pulmonary aspergillosis.

### Keywords

gliotoxin; virulence determinants; immunosuppressive regimen; invasive aspergillosis

## Introduction

Gliotoxin is an epipolythiodioxopiperazine (ETP) class of toxin produced by *Aspergillus fumigatus* and has been suspected as one of the most likely virulence determinants among various secondary metabolites produced by the species. *A. fumigatus* is the most frequent cause



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of invasive aspergillosis worldwide and over 90% of the strains isolated from invasive aspergillosis cases in tertiary-care cancer centers were found to be gliotoxin producers [1]. Furthermore, the amount of toxin produced by *A. fumigatus* was substantially higher than the amount produced by other less frequent species of pathogenic *Aspergillus* including *A. terreus* and *A. flavus* [1].

In 2004, Gardiner and Howlett discovered a 12-gene cluster in the *A. fumigatus* genome sequence that resembles the gene cluster responsible for the synthesis of sirodesmin, an ETP toxin in *Leptosphaera maculans* [2]. Since gliotoxin is also an ETP toxin, they proposed it to be the putative gliotoxin biosynthetic gene cluster. Similar ETP types of gene clusters were subsequently found in many other fungi belonging to Ascomycetes [3]. The discovery of the putative gliotoxin biosynthetic gene cluster in *A. fumigatus* allowed us for the first time to determine whether this gene cluster was indeed responsible for gliotoxin biosynthesis and whether the toxin plays any role in the pathobiology of *A. fumigatus*. Five laboratories have embarked on the functional analysis of this gene cluster by disrupting either the gene encoding the nonribosomal peptide synthase *gliP* [4–7] or the gene encoding the transcriptional factor *gliZ* [8] in the *A. fumigatus* genomic sequencing strains Af293 and CEA10, or the clinical isolate B-5233. Results have shown that this 12-gene cluster is indeed responsible for gliotoxin production. However, results of the animal experiments were mixed regarding the importance of gliotoxin as a virulence determinant. Three laboratories found gliotoxin to be unimportant while reports from the remaining two laboratories showed it to be important for virulence of *A. fumigatus*. Comparisons between the reports from the five laboratories [4–8] that investigated the function of *gli* genes clearly revealed that the animal host immune status was the key factor responsible for the contradiction. In this mini-review, we sought to summarize the significance of gliotoxin in the pathobiology of *A. fumigatus*.

## The pathogenic species of *Aspergillus* that produce gliotoxin

The most common species of *Aspergillus* that cause invasive aspergillosis world-wide are *A. fumigatus*, *A. terreus*, *A. flavus* and *A. niger* [9]. Although all the species produce gliotoxin, not every strain is a gliotoxin producer [1]. There are several reports that cite the frequency of toxin producing strains that have been isolated from the environment versus clinical specimens from patients. Santos *et al.* [10] reported that only 11% of the *A. fumigatus* strains isolated from moldy silos on Terceira Island in the Azores were gliotoxin producers. In contrast, 93% of the *A. fumigatus* strains ($n$ =40) isolated between 1998 and 2003 from respiratory and tissue samples of cancer patients in MD. Anderson Cancer Center, Houston, Texas, USA were gliotoxin producers compared to 75% and 25% of *A. niger* ($n$ =9) and *A. terreus* ($n$ =27) strains, respectively. Surprisingly, only 4% of the *A. flavus* ($n$ =18) strains produced gliotoxin. Furthermore, the concentrations of gliotoxin produced by *A. fumigatus* were significantly higher than those of other species [1].

Kosalec and Pepeljnjak [11] reported very different frequencies of gliotoxin producing *A. fumigatus* strains recovered from immunocompromised patients with various diseases in the hematology unit at the University of Zagreb in Croatia. Of the 50 clinical isolates, only 18% produced gliotoxin, while no environmental strains of an equivalent number screened were found to produce the toxin. Although the data are fragmentary, the results suggest that the frequency of gliotoxin producing *A. fumigatus* strains varies depending on the geographic region and/or type of patients that yielded the fungal strains. It appears, however, that the frequency of finding gliotoxin producing *A. fumigatus* strains is higher among clinical isolates than among environmental isolates.

The Ascomycete species that produce secondary metabolites, especially of ETP, are largely ubiquitous fungi such as *Penicillium*, *Trichoderm*, *Leptosphaera* and others besides

*Aspergillus* [3]. Secondary metabolites may render the species more fit for survival in all types of soil throughout the world. The recent report by Rohlfs and colleagues has shown that the fungivorous springtails, a soil insect, preferred to consume conidia of *laeA* mutant over wild type conidia of *A. nidulans* [12]. Although *A. nidulans* does not produce gliotoxin, it produces an array of toxic secondary metabolites controlled by the global regulator, *laeA*. *LaeA* also regulates secondary metabolism in *A. fumigatus*. Deletion of *laeA* in *A. fumigatus* not only resulted in the abolition of gliotoxin production but attenuated fungal virulence [13,14]. Unlike ubiquitous fungi, no genomic evidence of ETP-like gene clusters were found in endemic species such as *Coccidioides immitis, Histoplasma capsulate* [3] and *Blastomyces dermatitidis* [15], which are confined to a certain environmental niche. Considering the presence of 2,000–18,000 different genomes per gram of soil [16], it is easy to understand how fungi not producing secondary metabolites would be at a disadvantage for competition with other organisms in soil.

## Gliotoxin exerts detrimental effects on mammalian cells *in vitro*

Gliotoxin is a dipeptide characterized by the presence of a disulfide bridge across the piperazine ring [17]. The disulfide bridge allows the cross linking with proteins via cysteine residues and generate deleterious reactive oxygen species (ROS) through the redox cycling between the reduced and oxidized form. This mechanism of ROS generation is believed to be responsible for the toxicity of gliotoxin [18,19]. The toxin is known to be immunosuppressive: inhibits phagocytosis [20,21], inhibits the transcription factor NF-κB thereby blocking inflammatory response and cytokine production [22] and blocks mast cell degranulation [23]. Furthermore, the toxin is known to cause apoptotic cell death in professional immune cells such as macrophages and monocytes [24,25], as well as in non-immune cells such as EL4 thymoma cells [6] or mouse embryonic fibroblast (MEF) cells [26]. Recent work by Pardo *et al.* [26] demonstrated that gliotoxin directly activated the proapoptotic Bcl-2 family member Bak, a constitutive mitochondrial protein in MEF cells. The ROS generated as a result was reported to facilitate the release of cytochrome c and apoptosis inducing factors from mitochondria, leading to caspase activation, as well as other events that mediate cell death [26]. Unlike the effects of gliotoxin observed in macrophages or monocytes, neutrophils from healthy humans are reported to be resistant to gliotoxin-mediated apoptosis in vitro [27]. Neutrophil functions, however, were significantly blunted by gliotoxin, with suppressed ROS production and impaired phagocytic capacity. Methylprednisone, a corticosteroid commonly used to suppress immunity in the stem cell transplant recipient, reversed the suppression of ROS production by Neutrophils. These results suggest that gliotoxin inhibits important neutrophil functions in normal individuals, while increasing PMN-mediated inflammation in immunocompromised hosts; this differential effect may contribute to tissue destruction in patients with invasive aspergillosis [27].

In contrast to helvasin or tremogerin, other known secondary metabolites produced by *A. fumigatus*, gliotoxin inhibited oxidative burst of human neutrophils [28]. Gliotoxin also causes damage to the ciliated respiratory epithelium in vitro and this property might assist *A. fumigatus* in the colonization of the respiratory mucosa [29]. Furthermore, Nierman and colleagues have recently shown by the genome-wide gene expression profile analysis that gliotoxin genes are upregulated in germlings during initiation of infection in mice [30]. All of these findings implicate gliotoxin in playing a role in guarding *A. fumigatus* in the host environment.

## Functional studies on gliotoxin biosynthetic genes

Using a comparative genomics approach, Gardiner and Howlett *et al.* [2] discovered the putative cluster of 12 genes involved in gliotoxin biosynthesis (Fig. 1) in *A. fumigatus*. Putative

ETP gene clusters similar to that of *A. fumigatus* are also found in the genomes of other pathogenic aspergilli, such as *A. terreus* and *A. flavus* [3]. The gene cluster in *A. terreus* is identical to the gliotoxin gene cluster in *A. fumigatus* except for the absence in the former of the gene for the methyl transferase *gliN* in Fig. 1 [Howlett, B. Personal communication]. The gliotoxin gene cluster of *A. fumigatus* spanned 28 kb in the genome of the *A. fumigatus* strain Af293 and contained eight genes homologous to those in the gene cluster responsible for the synthesis of sirodesmin in *L. maculans*. Based on the sequence of the gene cluster and the time course of gliotoxin production, Gardiner *et al.* [2] proposed a pathway for the synthesis of the toxin with six enzymatic steps. The cluster is composed of genes encoding a putative zinc finger transcription factor (*gliZ*), an aminocyclo-propane carboxylic acid synthase (*gliL*), a dipeptidase (*gliJ*), a peptide synthase (*gliP*), two cytochrome p450 monooxygenases (*gliC* and *gliF*), an O-methyltransferase (*gliM*), a glutathione S-transferase (*gliG*), a hypothetical protein (*gliK*), a transporter (*gliA*), a methyltransferase (*gliN*) and a thioredoxin reductase (*gliT*). Conclusive evidence that the 12-gene cluster is responsible for gliotoxin synthesis was obtained by the functional studies of *gliZ* and or *gliP* in three strains of *A. fumigatus* Af293, B-5233 and CEA10. The *gliZ* gene controls gene expression of the remaining 11 genes in the cluster [8] while *gliP* encodes a multimodular nonribosomal peptide synthase that catalyzes the condensation of serine and phenylalanine, the first step of the pathway making diketopiperazine scaffold of the toxin [31]. The evidence that the gene cluster is indeed responsible for gliotoxin synthesis include: (i) Deletion of either *gliP* or *gliZ* in the strain Af293 and *gliP* deletion in the strains CEA10 and B-5233 abolished synthesis of the toxin. Reconstitution of the deletants with their respective wild type genes restored the production of gliotoxin to wild type level [5–7]; (ii) Deletion of *gliZ* in the strain Af293 resulted in the absence of gene expression in the remaining 11 genes of the cluster [8]; and (iii) Over expression of *gliZ* in the strain Af293 enhanced the production of gliotoxin above the wild type level [8]. These results confirmed that the 12-gene cluster is indeed responsible for the biosynthesis of gliotoxin.

## Pathobiological significance of gliotoxin in *A. fumigatus*

Deletants of either the *gliP* or *gliZ* gene showed no difference in morphology or growth rate compared to wild type strains except that mutants produced no gliotoxin [5–8]. These results were expected since, unlike primary metabolism, the secondary metabolism, which includes the biosynthesis of gliotoxin, does not affect growth of the organism. Secondary metabolism that is associated with fungal development, such as polyketide synthesis, is commonly associated with sporulation or pigmentation of spores [32 and, reviewed in 33]. Interestingly, deletion of *gliP* in Af293 caused a down regulation of gene expression in the 12-gene cluster. Addition of exogenous gliotoxin restored the gene expression level suggesting that gliotoxin regulates its own production [7]. *In vitro* analysis showed that culture filtrates from gliotoxin-negative mutants failed to inhibit oxidative burst in human neutrophils [6], failed to cause apoptosis or cell detachment in various mammalian cells [6,8] and also failed to inhibit mast cell degranulation [7].

The importance of gliotoxin in virulence of *A. fumigatus* in mice, however, was equivocal. Five laboratories used various mouse strains that were either immunosuppressed by the administration of cyclophosphamide plus cortisone acetate [4,5,7,8] or cortisone acetate alone [4,6] in order to study the effect of gliotoxin on virulence of the three *A. fumigatus* strains (Table 1). The two laboratories that showed gliotoxin to be an important virulence determinant used mice that were immunosuppressed only with cortisone acetate. Mice infected with *gliPΔ* strains survived significantly longer than those infected with wild type or *gliP* reconstituted strains [4,6], as shown in Fig. 2. Histopathological analysis of lung sections confirmed the attenuated virulence of *gliPΔ* strains in 129/Sv mice [6]. Interestingly, the fungal burden of *gliPΔ* strain in the lung of BALB/c mice was not lower than those of the wild type or reconstituted strain [4]. Fragmentation of neutrophils at the infection foci, however, was

much more pronounced in the lungs of mice infected with wild type or reconstituted strains than the *gliPΔ* strain [4]. The other three laboratories that showed no effect of the toxin in invasive infection invariably used neutropenic mice that resulted from immunosuppression by combination of cyclophosphamide and cortisone acetate. All succumbed to aspergillosis by the wild type and *gliPΔ* or *gliZΔ* strains at a similar rate. This suggested that one of the important targets of gliotoxin is neutrophils and as a result, gliotoxin is less important for *A. fumigatus* virulence in neutropenic mice that lack one of the toxin's targets. The survival data shown in Fig. 3 directly support this notion.

With these results, one should ask about the clinical relevance of the gliotoxin effect in the cortisone-treated mouse model of invasive aspergillosis. Invasive aspergillosis occurs most commonly in neutropenic patients [34]. However, invasive aspergillosis is being increasingly reported in allogeneic stem cell transplant (SCT) recipients who have been treated with immunosuppressive regimens, most commonly corticosteroids, because of the graft-versus host disease [35–38]. These patients are no longer neutropenic but susceptible to invasive fungal infection. Gliotoxin would play an important role in the pathogenesis of *A. fumigatus* in these patients.

In addition to mice immunosuppressed by cortisone acetate, pathobiological importance of gliotoxin was also demonstrated in *Drosophila melanogaster* [4]. Toll-deficient *D. melanogaster* was significantly less susceptible to *gliPΔ* of the *A. fumigatus* Af293 strain than the wild type or the reconstituted strain (Fig. 4). This suggests that Toll-deficient *D. melanogaster* is useful model for the assessment of the pathobiological importance of fungal secondary metabolites.

## Conclusions

Gliotoxin is an important factor contributing to the virulence of *A. fumigatus* in mouse model. Its pathobiological importance, however, has only been demonstrated in non-neutropenic mice suggesting that gliotoxin plays an important role in the pathogenesis of aspergillosis in patients immunosuppressed, but not neutropenic. The disparate result published on the importance of gliotoxin as a virulence determinant was neither due to differences in the fungal strains, the inoculation method, nor the differences in the mouse strain used. It is clear that the immune status of the mice used was the key factor which contributed to the controversy. Mice immunosuppressed by a combination of cyclophosphamide and cortisone acetate should not be considered as the standard model applicable for every situation in which the potential virulence determinants are being evaluated.

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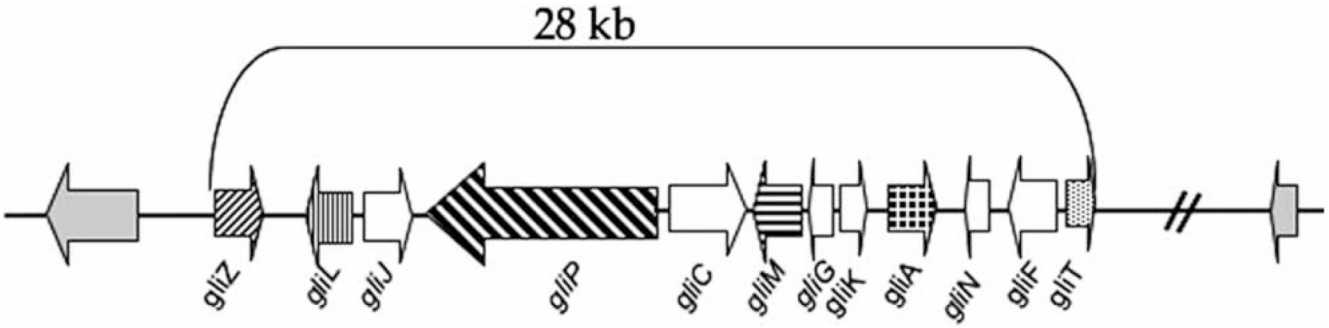


**Fig. 1.**
Genomic organization of the 12 *gli*-gene cluster responsible for gliotoxin biosynthesis in *Aspergillus fumigatus*.

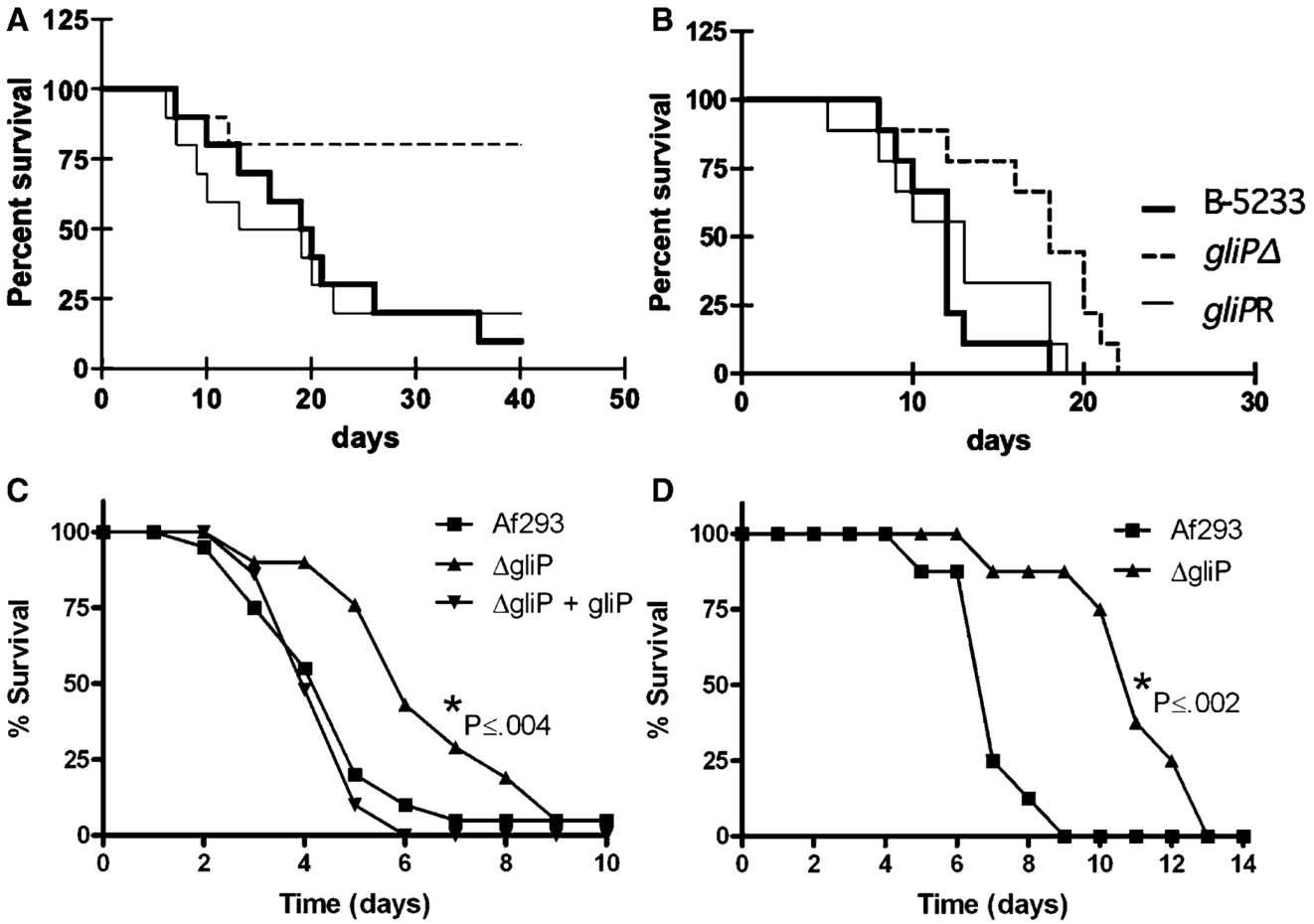


**Fig. 2.**

Virulence of the wild type, *gliPΔ* and reconstituted strains of *Aspergillus fumigatus* inoculated into two different mouse strains immunosuppressed with cortisone acetate. (A) and (B) Survival of 129/Sv (A) and BALB/c (B) mice infected intranasally with conidia of B-5233, *gliPΔ* and *gli*PR (*gliPΔ* + *gliP*) strains (from Sugui *et al.* 2007) [6]. (C) Survival of BALB/c mice infected intranasally with conidia of Af293, *gliPΔ* and *gliPΔ* + *gliP*. (D) Survival of BALB/c mice infected by inhalation of conidia from the strains Af293 and *gliP* deletant (C and D from Spikes *et al.* 2008) [4].

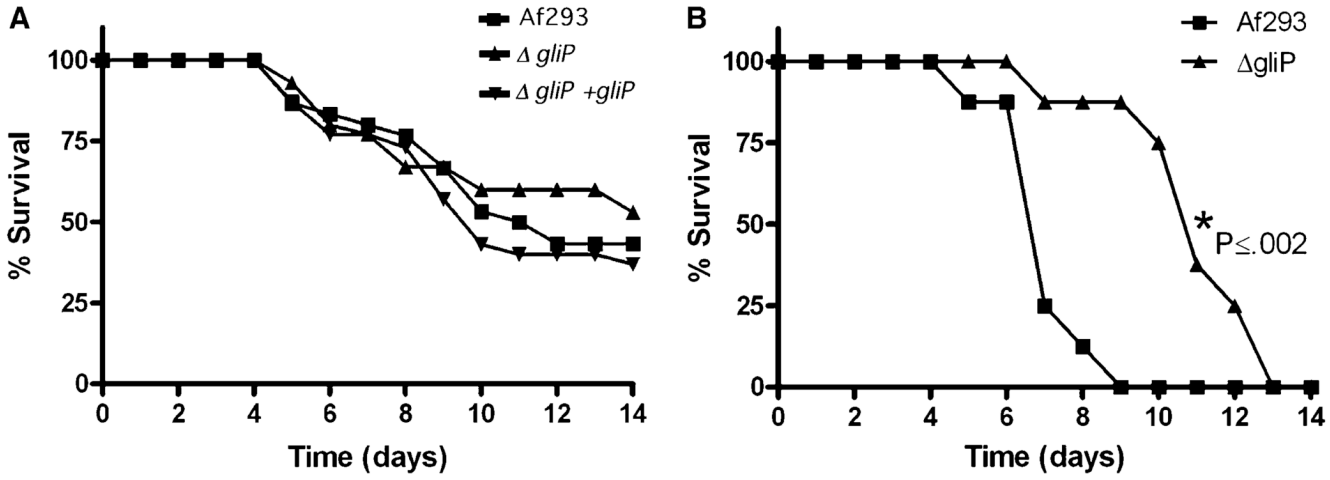


**Fig. 3.**

Survival of BALB/c mice infected by inhalation of Af293 and its *gli* strains. (A) Mice immunosuppressed by combination of cyclophosphamide and cortisone acetate showing no virulence difference between the strains. (B) Mice immunosuppressed by cortisone acetate alone showing statistically significant difference in virulence between the wild type and *gliP* deletant (from Spikes *et al.* 2008) [4].

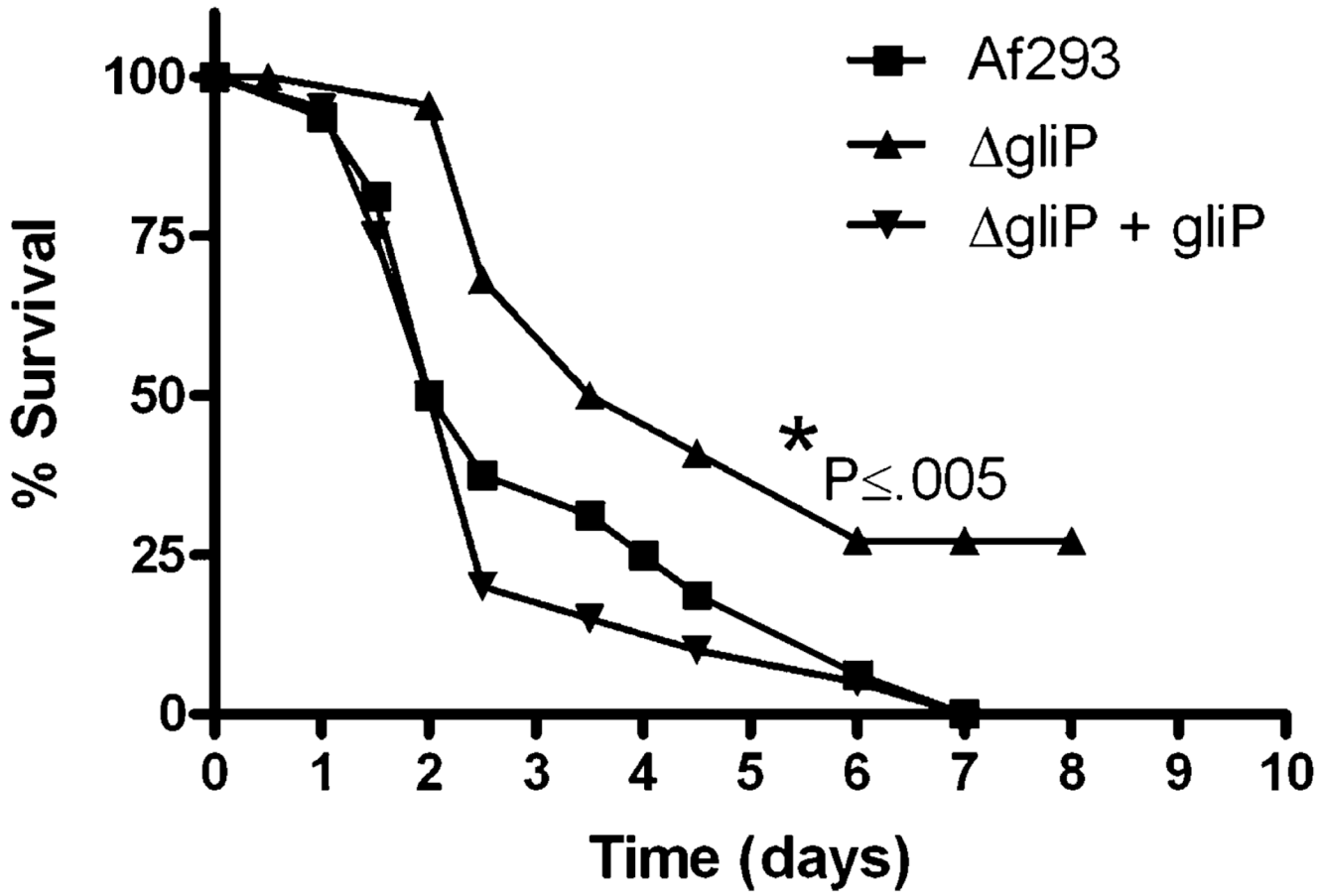


**Fig. 4.**

Toll deficient *Drosophila melanogaster* infected with Af293 and its derivative *gli* strains showing attenuated virulence in *gliP* deletant compared to the wild type and *gliP* deletant strain reconstituted with the wild type *gliP* gene (from Spikes *et al.* 2008) [4].

**Table 1**

Role of gliotoxin in pathobiology of *Aspergillus fumigatus* studied in five laboratories.

| Strain background | Gene deleted | Mouse strains | Immunosuppressive regimen | Inoculation method | Virulence | Reference |
|---|---|---|---|---|---|---|
| Af293 | *gliP* | ICR | Cyclophosphamide+Cortisone | Inhalation | No effect | [7] |
| Af293 | *gliP* | BALB/C | Cyclophosphamide+Cortisone | Inhalation | No effect | [4] |
| Af293 | *gliP* | BALB/C | Cortisone | Inhalation | Attenuated | [4] |
| CEA10 | *gliP* | BALB/C | Cyclophosphamide+Cortisone | Intranasal | No effect | [5] |
| B-5233 | *gliP* | BALB/C | Cortisone | Intranasal | Attenuated | [6] |
| B-5233 | *gliP* | 129/Sv | Cortisone | Intranasal | Attenuated | [6] |
| Af293 | *gliZ* | ICR | Cyclophosphamide+Cortisone | Intranasal | No effect | [8] |