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# *Galleria mellonella* as a host model to study *Aspergillus terreus* virulence and amphotericin B resistance

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**Keywords:** amphotericin B resistant and susceptible *A. terreus* strain cluster, *Galleria mellonella*, invertebrate *in vivo* model

The aim of this study was to investigate if the alternative *in vivo* model *Galleria mellonella* can be used (i) to determine differences in pathogenicity of amphotericin B (AMB) resistant and susceptible *A. terreus* isolates, (ii) to evaluate AMB efficacy *in vivo* (iii) and to correlate outcome to *in vitro* susceptibility data. Larvae were infected with 2 *A. terreus* AMB resistant (ATR) and 3 AMB susceptible (ATS) isolates and survival rates were correlated to physiological attributes and killing ability of larval haemocytes. Additionally, infected larvae were treated with different concentrations of L-AMB. Haemocyte density were ascertained to evaluate the influence of L-AMB on the larval immune cells. Larvae were sensitive to *A. terreus* infection in an inoculum-size and temperature dependent manner. *In vitro* susceptibility to L-AMB correlated with *in vivo* outcome of antifungal treatment, defining an AMB susceptible strain cluster of *A. terreus*. Susceptibility to L-AMB increased virulence potential in the larval model, but this increase was also in accordance with faster growth and less damage caused by larval haemocytes. L-AMB treatment primed the larval immune response by increasing haemocyte density. *G. mellonella* provides a convenient model for the *in vivo* screening of *A. terreus* virulence and treatment options, contributing to the generation of a hypothesis that can be further tested in refined experiments in mammalian models.

## Introduction

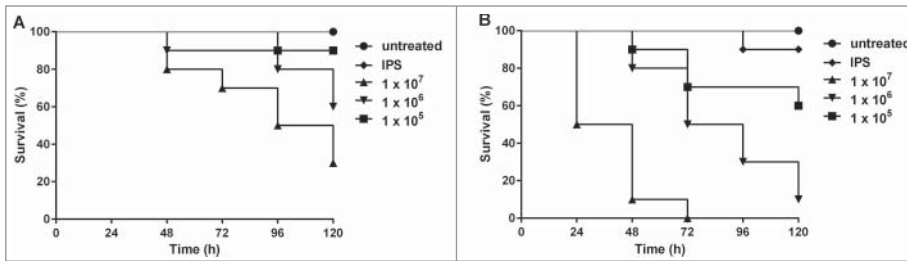
Invasive aspergillosis (IA) is associated with considerable morbidity and mortality in patients with compromised immunity due to cancer chemotherapy, haematopoietic stem-cell transplantation and solid organ transplantation.<sup>1–3</sup> While *A. fumigatus* is the most commonly cause of IA worldwide, *A. terreus* appears to be frequently isolated in some institutions; e.g. the Medical University Hospital of Innsbruck, Austria, where approximately 15% of all *Aspergillus* infections are caused by this pathogen, and the M.D. Anderson Cancer Center in Houston, Texas, USA.<sup>4,5</sup> In general, mold infections remain difficult to manage because of late diagnosis and complication during treatment due to drug toxicities.<sup>6,7</sup> Due to the high side effects caused by AMB such as nephrotoxicity and infusional toxicity, lipid formulations of AMB (L-AMB) are the reference formulation in the treatment of invasive fungal infections.<sup>8,9</sup> *In vivo* and *in vitro* data indicate that *A. terreus* isolates are intrinsically resistant to amphotericin B (AMB) (MIC  $\geq 2 \mu\text{g/mL}$ ).<sup>10,11</sup> The mechanisms involved in AMB resistance are only partly elucidated and special focus has been put on comparing cellular and metabolic differences in AMB resistant isolates (ATR) to one susceptible isolate (ATS)

from the University of Milano (MIC of  $0.5 \mu\text{g/mL}$ ).<sup>12,13</sup> Currently, 2 further *A. terreus* isolates from the USA have been found to be susceptible against AMB.

The larvae of the greater wax moth, *Galleria mellonella*, have been widely used as alternative models to evaluate the virulence of microbial pathogens, including fungal pathogens, and to evaluate the efficacy of antimicrobial drugs.<sup>14,15</sup> Of particular importance is the strong correlation to data obtained in murine studies.<sup>16</sup> Insects possess a cellular and humoral innate immune system which bears similarities to innate immune response of vertebrates.<sup>17</sup> The cellular immune response is mediated by haemocytes, which show strong structural and functional similarities with mammalian phagocytes.<sup>18,19</sup> Six different types of haemocytes are defined in *Galleria* which are involved in phagocytosis, encapsulation and melanization.<sup>20</sup> Because their density is not stable, examining changes in haemocyte populations can be used to estimate immune responses to fungal infections or drug treatment.<sup>14,21</sup>

The aim of this study was to establish and characterize a simple alternative *in vivo* model to study infections due to *A. terreus*. Two ATR isolates and 3 ATS isolates were used to determine differences in virulence potential and survival rates were

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**Figure 1.** Dose- and temperature dependent mortality of *G. mellonella* larvae infected with *A. terreus*. Ten larvae each were infected with different conidial concentrations of *A. terreus* (T90) and incubated at 30°C (A) or 37°C (B), respectively. Experiments were performed at least 3 times. Kaplan-Meier curves represent one out of 3 experiments. Significant difference ( $p \leq 0.05$ ) was observed within different inocula tested at each temperature.

correlated to physiological attributes and to *in vitro* AMB susceptibility profile. The wax moth larvae were further used to evaluate L-AMB efficacy *in vivo* and to compare outcome to *in vitro* susceptibility data. Additionally, the influence of L-AMB on haemocyte density was determined.

## Results

### Killing of *Galleria mellonella* larvae by *A. terreus* isolates is dose- and temperature dependent

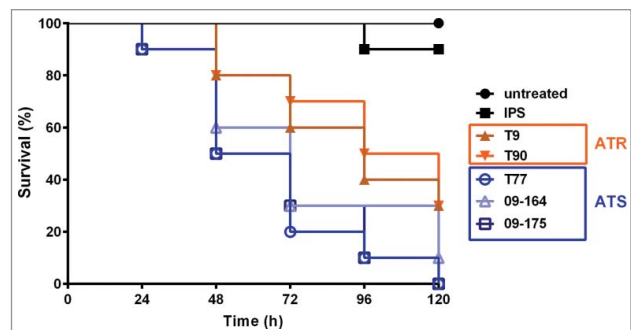
To establish *G. mellonella* larvae as a model for *A. terreus* infections, first, the optimal conidial dosage for virulence studies was determined. Therefore, larvae were infected with  $1 \times 10^5 - 1 \times 10^7$  conidia per larva of the ATR isolate (T90) and survival was monitored in parallel at 30°C or 37°C. As expected, killing of the larvae depended on the number of conidia injected and the incubation temperature (Fig. 1). Larvae were killed significantly faster ( $p \leq 0.05$ ) when incubated at 37°C compared to 30°C at each inoculum density tested. Significant difference between all 3 inocula was observed at both temperatures. At 37°C, all larvae infected with  $1 \times 10^7$  conidia died within 72 h, while survival still reached 70% in those incubated at 30°C and it took 6 days before all larvae died. Infection with  $1 \times 10^6$  conidia at 37°C resulted in 100% mortality at day 6, while almost 50% survival was monitored at 30°C. Lower inoculum density ( $1 \times 10^5$ ) resulted in 50% mortality at day 6 at 37°C, whereas larvae kept at 30°C survived the infection (less than 10% mortality). Heat-inactivated conidia of T90 were not able to cause mortality and survival was more than 80% after 120 h incubation at either 30°C or 37°C (data not shown). The dose and temperature dependency of this killing indicates that *G. mellonella* larvae have the potential to serve as a non-vertebrate animal host model for this group of *Aspergilli*.

### *A. terreus* isolates exhibit different virulence potential

Distinct morphological variations were observed between the ATR and ATS isolates. In order to see if the various isolates exhibit differences in their virulence potential and to check if this could be linked to morphological characteristics or AMB susceptibility, the virulence potential of the 5 isolates was

compared. Significant differences in survival rates of larvae were determined, when larvae were infected with  $1 \times 10^7$  conidia of each strain and incubated at 30°C (Fig. 2). In summary, the ATR strains (T9 and T90) showed lower virulence potential compared to the ATS strains (T77, 09-164 and 09-175), indicated by significant differences in the median survival of each strain, which was 60–72 h for the ATS strains and 96–108 h for ATR isolates. T77, the isolate exhibiting fastest killing of larvae, showed significantly higher virulence potential compared to T9 ( $p = 0.0310$ ) and T90

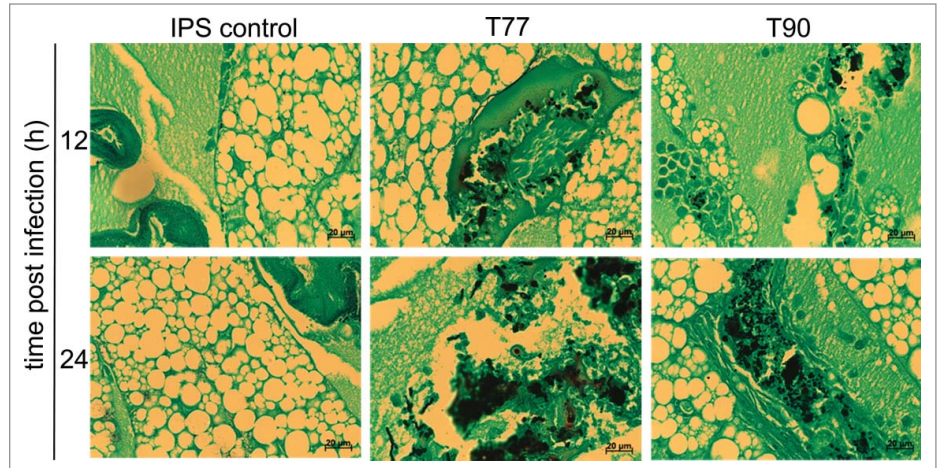
( $p = 0.0157$ ), and isolate 09-175 displayed significantly higher virulence compared to T9 ( $p = 0.0444$ ) and T90 ( $p = 0.0214$ ) (Fig. 2). No significant difference compared to ATR isolates was detected for the ATS isolate 09-164 ( $p > 0.05$ ). As total mortality is reached earlier at 37°C, we performed virulence studies with  $1 \times 10^6$  conidia of each strain and incubated larvae at 37°C (Fig. 4, solid lines). Difference in virulence potential was less prominent at 37°C than at 30°C. Still, the difference is reflected in the median survival of each strain. While larvae infected with any of the ATS strains demonstrated a median survival of 72 h, for groups infected with ATR strains median survival was 84 h. The correlation between virulence potential and physiological attributes of the infectious agent, or the size of the infecting agent was already discussed before.<sup>22</sup> Here too, higher virulence potential correlated with higher germination rates and increased growth rates *in vitro* (Figs. S1 and S2). For further support, tissue sections of infected larvae embedded in paraffin were performed and revealed that *A. terreus* is able to proliferate within the host (Fig. 3). Additionally, faster growth ability, connected with higher virulence potential, was obvious *in vivo* too, as for the ATS strain T77 faster development of fungal hyphae within the host tissue was observed.



**Figure 2.** Survival of *G. mellonella* larvae following inoculation with different *A. terreus* isolates. Larvae were infected with 20  $\mu$ L IPS containing  $1 \times 10^7$  conidia of the respective *A. terreus* isolates and incubated at 30°C. Survival was monitored every 24 h over 144 h (6 days). (ATR: T9 and T90; ATS: T77, 09-164 and 09-175). Kaplan-Meier curves represent one out of 3 experiments.

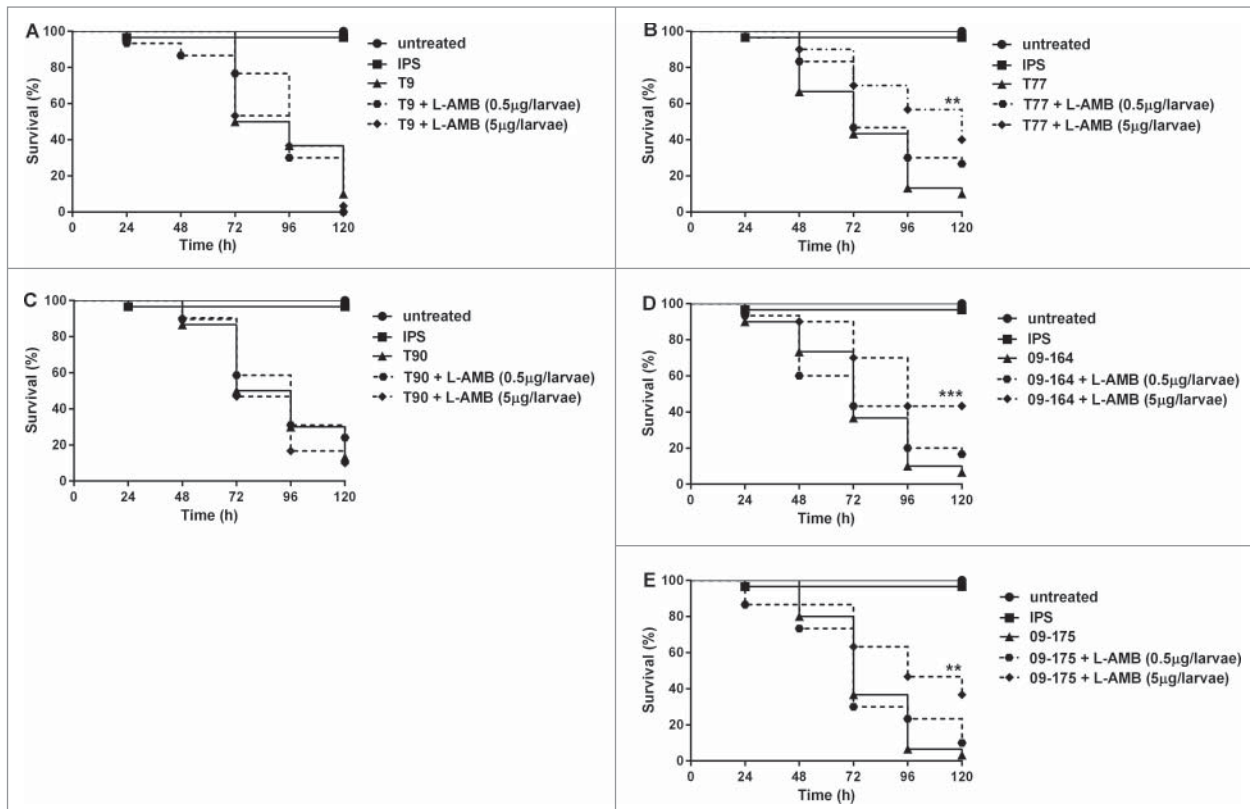
**AMB efficacy against *A. terreus* infections in *G. mellonella* larvae correlates with *in vitro* susceptibility**

*In vitro* susceptibility of ATR and ATS isolates against AMB and L-AMB was determined with microbroth dilution assay according to the EUCAST guidelines.<sup>23</sup> No significant differences in MICs between the different AMB formulations were determined (data not shown). Antifungal susceptibility testing demonstrated a MIC range of 2 – 4 µg/mL for both ATR isolates (geometric mean of 3.125 µg/mL), while all 3 AMB susceptible strains had a MIC range of 0.25 – 0.5 µg/mL (geometric mean of 0.378 µg/mL). As L-AMB is the therapeutic of choice, we used this formulation for all *in vivo* assays. For assessing AMB efficacy *in vivo*, a single dose of L-AMB at a concentration of 0.5 µg and 5 µg per larvae, chosen according to the MIC values of ATS strains plus a concentration 10 times higher, was administered to each larva, previously infected with  $1 \times 10^6$  conidia of



**Figure 3.** Histopathology of *G. mellonella* infected with *A. terreus* ATS strain T77, and ATR strain T90. Larvae were infected with IPS (left panel),  $1 \times 10^7$  spores per larvae of ATS T77 (middle panel) and of ATR T90 (right panel). Larvae were sacrificed at 12 h and 24 h after incubation at 37°C, conserved in formalin, embedded in paraffin. To identify the fungus more easily, tissue sections were stained with Grocott's silver stain.

the respective *A. terreus* isolate and incubated at 37°C. In addition to untreated and IPS controls, further control groups were included receiving L-AMB (0.5 or 5 µg per larva) to rule out



**Figure 4.** Ability of L-AMB to increase the time to death of *G. mellonella* challenged with ATR (A, C) and ATS strains (B, D, E). Larvae were infected with  $1 \times 10^6$  spores 2 h before L-AMB (0.5 µg and 5 µg per larvae) was administered and incubated at 37°C. Survival was monitored every 24 h. Graphs represent one out of 3 single experiments. Results are significantly different (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ; Mantel Cox test) to the untreated infected larvae.



putative toxic effects of L-AMB. Survival was not less than 80% in any of the controls, indicating that L-AMB itself had no effect on survival at the concentrations used (data not shown).

Treatment with L-AMB only showed success in the test groups infected with ATS isolates (T77, 09-164, 09-175) (Fig. 4B, D, and E), reflecting *in vitro* data. In groups that received 5 µg of L-AMB, survival of larvae was significantly ( $p \leq 0.01$ ) prolonged compared to control groups that received IPS only. Although less pronounced, even the lower dose of 0.5 µg L-AMB per larva seemed to have a positive effect on survival, although statistically not significant. No significant increase in survival due to L-AMB application could be detected for the 2 ATR isolates T9 and T90 (Fig. 4A, C).

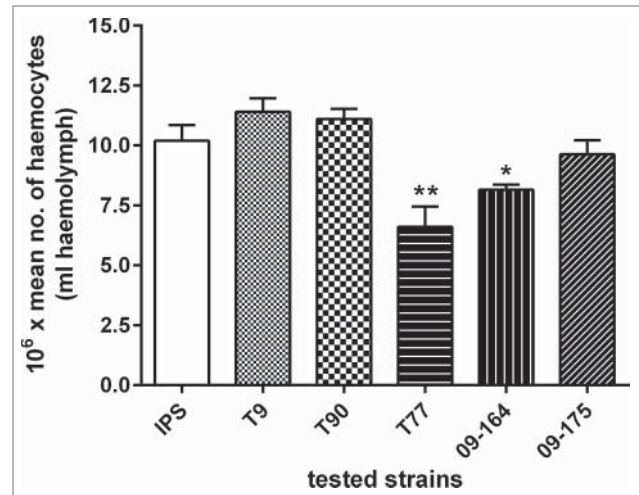
#### *A. terreus* strains induce different cellular immune responses in *G. mellonella*

To determine if differences in survival rates could be attributed to different killing activity of haemocytes against hyphae of the different isolates, the extent of fungal damage induced by larval haemocytes was measured by XTT-assay. Fungal damage caused by isolated haemocytes was higher in the 2 ATR isolates (T9 and T90) than in the ATS strains (T77, 09-164 and 09-175) (data not shown). These results correspond to the data obtained in virulence assays (Fig. 2), where ATS strains demonstrated higher killing ability of larvae compared to ATR strains, which might be due to less damage by haemocytes. Highest fungal damage was measured for ATR isolate T90 (65.26%; SD ± 12.36), that also showed the lowest virulence potential. Interestingly, all ATS strains exhibited 20% (SD ± 6.3) less damage caused by larval haemocytes compared to the resistant strain T90. Furthermore, haemocyte densities of infected larvae were determined by calculating the concentration of haemocytes per mL of haemolymph. Haemocyte densities of infected larvae revealed comparable numbers for larvae infected with ATR strains and the control, while larvae infected with the ATS strains exhibited a decreased density of circulating haemocytes in the haemolymph (Fig. 5). This reduction was most prominent in larvae infected with the ATS strain T77 (35.29%, SD ± 10.94), suggesting that infection with T77 induced haemocyte destruction.

Melanization is one of the first humoral responses of the larvae and occurs quickly after infection.<sup>24</sup> Larvae infected with ATS strains showed higher melanization than ATR strains (Fig. 6). Quantification of melanization revealed significant differences ( $p < 0.05$ ) in melanin accumulation between ATS and ATR strains 24 h post infection, the effect being apparent at 37°C, resulting in a 3 times higher melanization rate for T77 than the IPS control. ATR strains T9 and T90 showed only little difference in melanization compared to the IPS control (Fig. 6). These data strengthen the hypothesis that *A. terreus* ATR and ATS strains induce different cellular immune responses in *G. mellonella*, which might contribute to virulence potential.

#### Effect of L-AMB administration on haemocyte density in *G. mellonella* larvae

Previous work demonstrated that administration of caspofungin to larvae primes the larval immune response by elevating



**Figure 5.** Larval haemocyte densities in response to *A. terreus* infections. Larvae were injected with a non lethal dose of different *A. terreus* spores (ATR: T9, T90; ATS: T77, 09-164, 09-175) or IPS buffer and incubated at 30°C for 48 h and haemocyte counts were carried out relative to the IPS control. Results are significantly different (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; Students t-test, 2 tail) to the IPS control.

haemocyte density.<sup>21</sup> L-AMB administration of 5 µg resulted in a significant ( $p < 0.05$ ) increase of 38.38%; SD ± 5.14 in haemocyte density 24 h after administration. Additionally, administration of 0.5 µg of L-AMB demonstrated same increase in haemocyte density after 24 h injection (35.61%; SD ± 14). Interestingly, no significant alterations in haemocyte densities could be observed 4 h post-treatment (Fig. 7).

## Discussion

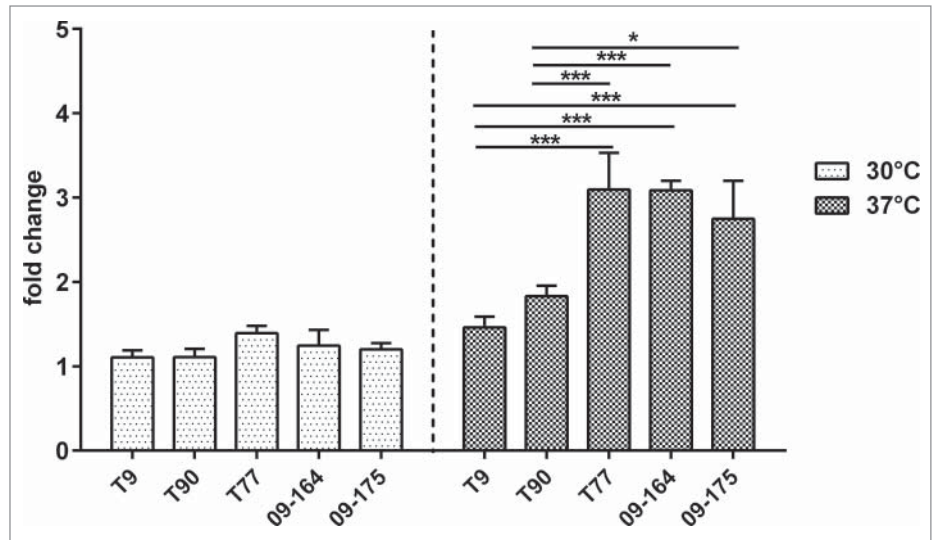
The use of *G. mellonella* larvae to study the pathogenicity of various microbial pathogens, including fungi, has been widely reported in the recent years.<sup>15,25</sup> Dose-dependent mortality observed in this study was expected, as it has also been shown in studies carried out with *A. fumigatus*.<sup>25</sup> This, plus the obvious development of hyphae inside the larval body, indicates that *A. terreus* kills larvae through an active infection mechanism rather than a merely physical effect of the injected conidia. Similar to what has been shown in 2 different mouse models and embryonated chicken eggs, higher doses of *A. terreus* conidia were necessary to cause same lethality compared to *A. fumigatus* in larvae.<sup>26</sup> The differences in virulence potential of *A. terreus* in *G. mellonella* as observed here, correlate closely with previously published data obtained with Balb/c mice, in which the ATS strain T77 also exhibited higher pathogenicity than the ATR strain T90.<sup>13</sup> These findings make *G. mellonella* a valid model for *A. terreus* infections.

Adaptation of a fungal pathogen to different micro-environmental stresses is necessary to allow germination and growth within the host environment and strongly influences virulence. Petraitis et al.<sup>22</sup> demonstrated a correlation between virulence

potential *in vivo* with species-dependent differences in germination rate *in vitro*. In contrast, investigating putative differences in virulence potential of *A. fumigatus* color mutants did not reveal any correlation between faster germination and higher pathogenicity.<sup>25</sup> Different outcome of infection is also linked to variable response of immune cells, such as killing of intruding pathogens by phagocytic cells, as shown in this study. Reasons therefore could be explained by variations in the outer layers of the isolates or to a difference in resistance to killing by reactive oxygen species.

Another factor contributing to virulence is the secretion of secondary metabolites such as mycotoxins, that influence host immune system or harm host tissue. A well studied example is *A. fumigatus* gliotoxin which is known to induce apoptosis and prevents NF- $\kappa$ B activation by inhibition of the proteasome and suppression of angiogenesis.<sup>27-31</sup> Additionally, fumagillin is also produced by *A. fumigatus* during hyphal growth and has been identified as an inhibitor of angiogenesis through a covalent interaction with methionine aminopeptidase-2 and suppresses tumor growth.<sup>32,33</sup> The production of gliotoxin and fumagillin was also proved in infected *G. mellonella* larvae.<sup>31,34</sup> A study by Lewis et al.<sup>35</sup> detected gliotoxin in culture medium of *A. terreus*, but as we did not test gliotoxin production, or any other secondary metabolites, of the 5 different isolates we can only speculate its contribution to differences in virulence potential. The same effect may account for the variable production of proteases by the ATR and ATS strains. A linear relationship between the haemocyte density and the survival rate of the larvae infected with *Candida* spp. was demonstrated by Bergin et al.<sup>36</sup> and these findings correlate with the results presented here, demonstrating lower haemocyte density in larvae infected with ATS. Additionally, our results agree with those presented previously,<sup>37</sup> demonstrating correlation between high increase in melanin and higher mortality rates in larvae infected with different *Klebsiella* strains. Together, the observed differences in resistance to haemocyte killing and melanin production might explain the differences in virulence potential in addition to physiological attributes of the tested strains.

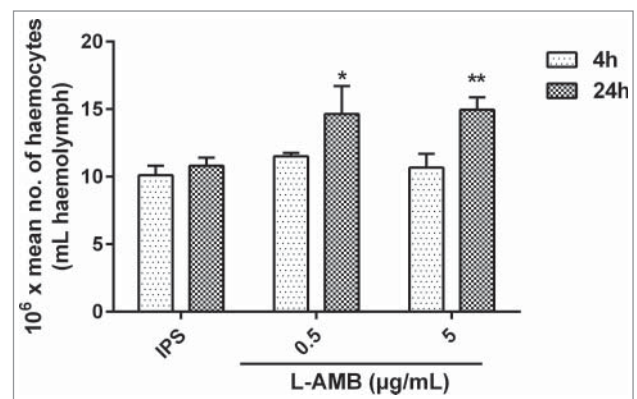
*A. terreus* isolates are known to show an intrinsic resistance to AMB with MIC values  $\geq 2$   $\mu$ g/mL.<sup>10,11,38</sup> No clinical breakpoints for AMB are defined for *A. terreus* species, but regarding the EUCAST breakpoints for other *Aspergillus* spp, isolates with MIC values  $> 2$   $\mu$ g/mL are considered to be clinically resistant against AMB.<sup>39</sup> Data from *in vitro* studies correlate well with murine studies.<sup>12</sup> Only few *A. terreus* isolates have been found so far that showed lower MIC values, making them AMB susceptible from a clinical point of view. One of these isolates has already been tested in a murine model, and treatment success was consistent with *in vitro*



**Figure 6.** Melanization of *G. mellonella* larvae infected with *A. terreus*. (A) Fold change of melanization rate (OD 405nm) normalized to the IPS control at 30°C (white) and 37°C (gray). (B) Visual appearance of larvae infected with different *A. terreus* strains at a dose of  $1 \times 10^7$  spores after 24 h. Error bars represent standard deviation out of 3 independent experiments. (\*  $p \leq 0.05$ ; \*\*\*  $p \leq 0.001$ ; 2 way ANOVA).

data.<sup>12</sup> In the larval model, the same outcome was shown for the 3 ATS strains and 2 ATR isolates, indicating that the larval model is a useful tool to test for L-AMB efficacy *in vivo*, and could also be employed with other fungal pathogens.

Administration of external agents into the insect haemocoel was shown to result in an unspecific antimicrobial immune response.<sup>21,34,40</sup> A study by Kelly et al.<sup>21</sup> demonstrated that caspofungin administration primes the immune response of *G. mellonella* larvae and induces a non-specific antimicrobial response. Increased haemocyte numbers due to L-AMB seem not to be enough to cure the infection, otherwise one would expect



**Figure 7.** Effect of L-AMB on the haemocyte density in *G. mellonella*. Haemocyte density in larvae injected with IPS or L-AMB (0.5 or 5  $\mu$ g/mL) and incubated at 37°C was assessed after 4 h and 24 h. Gray bars, 4 h; black bars, 24 h. Results are significantly different (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; Students t-test, 2 tail) to the IPS control.

increased survival rates also in larvae infected with ATR strains. Nevertheless, for ATS the positive effect on haemocyte numbers might be an additional effect in prolonging survival.

In conclusion, this study demonstrates for the first time that the *G. mellonella* larval model is useful to investigate the *in vivo* antifungal efficacy of L-AMB against *A. terreus* isolates and it is also an effective tool to screen for differences in virulence potential. Immuno-stimulatory effects of antifungal agents should be taken into consideration when interpreting results from *in vivo* treatment studies employing *Galleria*, but as they were shown to be marginal for AMB, *G. mellonella* larvae can be employed as a rapid, inexpensive and reliable way to evaluate AMB efficacy *in vivo*.

## Materials and methods

### Fungal strains, growth conditions and susceptibility testing

All five *A. terreus* isolates tested were derived from clinical specimens of either the University Hospital Innsbruck, Austria, the University of Milan and Rome, Italy or the Stanford University, USA, ATS strains are named T77, 09-164 and 09-175 and ATR strains T9 and T90. Species identification was performed by sequencing the internal transcribed spacer region (ITS 3–4), gene regions of  $\beta$ -tubulin, calmodulin, enolase and cytochrome C. Fungi were grown on *Aspergillus* Complete Media<sup>41</sup> for 7–14 days at 37°C to obtain conidia. Minimum inhibitory concentration (MIC) of AMB and L-AMB were determined for all strains following EUCAST guidelines 9.2.<sup>23</sup> Antifungal susceptibility testing was performed in triplicates on 3 separate occasions. MIC was defined as the minimal concentration of AMB resulting in complete growth inhibition.

### Virulence assay and antifungal treatment studies in *G. mellonella*

Sixth instar larvae of *G. mellonella* (Biologische Wurmzucht, Langenzersdorf, Austria) were stored in wood shavings in the dark at 18°C prior to use. Larvae weighing 0.3 – 0.4 g were selected for experimental use. Inoculum preparation, larval infection and monitoring of survival were carried out as described previously.<sup>42</sup> Larvae injected with sterile insect physiological saline (IPS; 150 mM NaCl, 5 mM KCl, 10 mM EDTA and 30 mM sodium citrate in 0.1 M Tris-HCl, pH 6.9)<sup>18</sup> and untouched larvae served as controls. To utilize *G. mellonella* larvae as a model for *A. terreus* infections to compare killing ability of different *A. terreus* isolates, larvae were first infected with different concentrations of conidia ( $1 \times 10^5$ ,  $1 \times 10^6$  and  $1 \times 10^7$  conidia per larva) of an *A. terreus* patient isolate. Survival was monitored in parallel at 30°C or 37°C over 144 h. To determine different virulence potential of ATR and ATS, larvae were infected with  $1 \times 10^7$  conidia per larvae and survival was monitored over 120 h at 30°C. Additionally, survival rates were determined at 37°C with an infection dose of  $1 \times 10^6$  conidia per larvae. To verify that mortality is due to active growth, larvae were infected with heat-inactivated spores and survival was monitored at 30°C and 37°C.

To assess efficacy of L-AMB *in vivo*, larvae were infected with  $1 \times 10^6$  conidia per larva, and injected with 0.5 (1.6 mg/kg),

5  $\mu$ g L-AMB (16.6 mg/kg) per larva, or IPS, respectively, 2 hours post-infection. For evaluation of antifungal treatment larvae were incubated at 37°C to make comparison to vertebrate hosts possible.

### Haemocyte isolation and determination of haemocyte density

*G. mellonella* larvae were infected and treated as described above. Haemocytes were isolated as described in<sup>21</sup> from 3 larvae of each group, 4 h, 24 h or 48 h post injection of either pathogen or L-AMB. Larvae were incubated at 30°C or 37°C. Cell density was determined by enumeration using a haemocytometer.

### Determination of hyphal damage caused by haemocytes

*A. terreus* conidia ( $1 \times 10^4$  conidia per 100  $\mu$ L) were incubated in RPMI<sub>1640</sub> at 37°C for 16 h to allow formation of hyphae, before adding freshly isolated haemocytes in a ratio of 1:1 and further incubation at 30°C. Wells containing either fungus or haemocytes alone served as controls. After 4 h haemocytes were lysed by ice-cold 2% Triton X-100 and the viability of remaining hyphae was quantified by the use of the In Vitro Toxicology Assay Kit, (XTT assay; Sigma Aldrich) according to the manufacturer's description. The percentage of fungal damage was calculated with the following formula:  $(1 - [\text{OD}_{450} \text{ of fungi with haemocytes} - \text{OD}_{450} \text{ of haemocytes alone}] / [\text{OD}_{450} \text{ of fungi alone}]) \times 100$ .

### Histology of larvae

Infected larvae plus control larvae (injected with IPS), incubated at 37°C, were conserved as a whole in formalin for 10 days, embedded in paraffin, cut at 5  $\mu$ m and stained with Grocott's silver stain to detect fungal elements.<sup>43</sup>

### Quantification of melanization

Larvae were infected with  $1 \times 10^7$  conidia per larva, and incubated at either 30°C or 37°C, before haemolymph was collected at 3 h and 24 h post infection. Haemolymph was diluted 1:10 in IPS buffer and 100  $\mu$ L of each sample were placed in 96 well microdilution plates and quantification of melanization assays were performed.<sup>14</sup> Optical density of haemolymph from infected larvae was normalized to that of control larvae.

### Statistical analysis

All experiments were performed on at least 3 independent occasions. Results are expressed as the mean  $\pm$  SD. Survival rates were evaluated using Kaplan Meier survival curves and analyzed with the log rank (Mantel-Cox) method utilizing GraphPad Prism software. Further, Tukey Test (one way ANOVA) or Students t-test were used to determine differences. Differences were considered significant at  $*p \leq 0.05$ .

### Disclosure of Potential Conflicts of Interest

C. L-F has received grant support from the Austrian Science Fund (FWF), MFF Tirol, Astellas Pharma, Gilead Sciences, Pfizer, Schering Plough, and Merck Sharp & Dohme. She has been an advisor/consultant to Gilead Sciences, Merck Sharp &



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### Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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