**Murine Infection Models for *Aspergillus terreus***

**Pulmonary Aspergillosis Reveal Long-term Persistence of Conidia and Liver Degeneration**

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(See the editorial commentary by Lass-Floërl, on pages 1192–4.)

*Aspergillus terreus* is emerging as a causative agent of life-threatening invasive aspergillosis. Prognosis for affected patients is often worse than for *A. fumigatus* infections. To study *A. terreus*–mediated disease, we developed 3 infection models. In embryonated hen’s eggs and leucopenic mice, the outcome of invasive aspergillosis was similar to that described for *A. fumigatus*. However, 102- and 103-fold higher conidia concentrations were required for 100% lethality. In corticosteroid-treated mice, only 50% mortality was observed, although bioluminescence imaging revealed transient disease in all infected animals. In surviving animals, we observed persistence of ungerminated but viable conidia. Cytokine levels in these mice were comparable to uninfected controls. In contrast to *A. fumigatus* infections, all mice infected with *A. terreus* developed fatty liver degeneration, suggesting the production of toxic secondary metabolites. Thus, at least in mice, persistence and subclinical liver damage are unique features of *A. terreus* infections.

Invasive pulmonary aspergillosis (IPA) is a major complication in immunocompromised patients, especially in solid organ transplant recipients and those receiving therapy for hematologic malignancies [1, 2]. Despite improvements in diagnosis and antifungal therapy, this life-threatening infection is associated with high mortality rates [3]. Although *Aspergillus fumigatus* is the major cause of IPA [4], *A. terreus* infections are emerging [5, 6]. The frequency of *A. terreus* as the causative agent of IPA varies from 1% to more than 30% [6–8]. Unfortunately, IPA caused by *A. terreus* is even more difficult to treat due to an intrinsic resistance to amphotericin B [9] and high dissemination rates [10]. Although *A. fumigatus* IPA has been extensively studied, little is known about the pathogenesis of *A. terreus*–mediated disease.

Mice are the most commonly used host species for pathogenesis studies because they facilitate genetic manipulation of the host side. For *A. fumigatus*, several murine pulmonary and disseminated infection models have been developed [11–16] that address differences in the immune status of the host, such as leukopenia or long-term corticosteroid application. In contrast, investigations on *A. terreus* in mice have focused on disseminated bloodstream infections. Pulmonary infection has only been described for leucopenic rabbits [17], where IPA caused by either *A. terreus* or *A. fumigatus* progressed at similar rates [17]. Still, it remains unclear whether corticosteroid treatment, often applied in solid organ transplant recipients, affects establishment of *A. terreus* infections.
The aim of our study was to establish and characterize an alternative infection model using chicken embryos and 2 murine models of invasive aspergillosis caused by *A. terreus* to elucidate virulence mechanisms and to facilitate direct comparison with *A. fumigatus*.

**MATERIAL AND METHODS**

**Strains and Culture Conditions**

The *A. terreus* strains SBUG844 (environmental isolate, HKI Strain Collection, Jena, Germany), A1156 (clinical isolate from pulmonary aspergillosis, NIH 2624, Fungal Genetics Stock Center, Kansas City, MO [17]), and SBUG402 (environmental isolate, HKI Strain Collection, Jena, Germany) were routinely grown on malt extract agar slants for 7 days at room temperature. Conidia were harvested in 5 mL sterile phosphate-buffered saline (PBS; PAA Laboratories) and filtered twice through 40-µm cell strainers (BD). Conidia were counted in a Neubauer cell strainers. The model was performed as described previously [19, 20].

Embryonated Egg Infection Model

The model was performed as described previously [19, 20]. In brief, on developmental day 10, the shell was aseptically perforated to generate an artificial air chamber through which 0.1 mL inoculum was applied onto the chorioallantoic membrane (CAM). The holes were sealed with paraffin and embryo viability was checked daily for up to 7 days by candling. For histological analysis, embryos were killed by chilling on ice for 1 hour. The egg surface was disinfected with 70% ethanol and the egg was split in half by longitudinal dissection. The CAM was cut into pieces that were directly fixed in 4% formalin (Histofix). Samples were embedded in paraffin, cut into 5-µm sections, and stained with periodic acid Schiff (PAS) stain using standard protocols. Sections were examined using bright-field microscopy at the indicated magnification.

**Murine Infection Models**

Female mice 6–8 weeks old (18–20 g, Charles River or Centre d’Elevage R. Janvier) were used for all studies. Animals were housed in individually ventilated cages and cared for in accordance with the principles outlined in the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes ([http://conventions.coe.int/Treaty/en/Treaties/Html/123.htm](http://conventions.coe.int/Treaty/en/Treaties/Html/123.htm)). All animal experiments were approved by the responsible federal/state authority and ethics committee (permit no. 03-002/09; 03-012/10) in accordance with the German animal welfare act.

To induce leukopenia, Balb/C mice were immunosuppressed by intraperitoneal injection of cyclophosphamide (140 mg/kg; Sigma) on days −4, −1, 2, 5, 8, and 11, with an additional subcutaneous dose of cortisone acetate (200 mg/kg; Sigma) on day −1. For the corticosteroid model, Balb/C (bioluminescence imaging) or CD-1 (survival studies) mice received cortisone acetate (25 mg/mouse) intraperitoneally on days −3 and 0. Immunosuppression was confirmed by differential blood cell counts. Mice were challenged intranasally with conidia on day 0 with either 5 × 10⁶ (cyclophosphamide) or 1 × 10⁷ (cortisone acetate) conidia in 20 µL PBS under general anesthesia. Animals were monitored at least twice daily and humanely sacrificed if moribund (defined by severe lethargy, severe dyspnea, or hypothermia).

**Construction of a Bioluminescent *A. terreus* Reporter Strain**

A synthetic codon usage optimized ([http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=5085&aa1=styleN; synthesized by GenScript] Photonis pyralis luciferase gene (protein accession: AAA88784) containing flanking 5’ BamHI and 3’ HindIII restriction sites was cloned into pUC19. The resulting plasmid was linearized with BamHI. The BglII-restricted gpdA promoter from *A. fumigatus* excised from plasmid pET/pgpdAa was inserted [21] and promoter orientation was confirmed by polymerase chain reaction. A synthetic 5’ NotI restriction site in the gpdA promoter enabled the introduction of the pyrithiamine resistance cassette ptrA, which was excised from plasmid NotptrA/pJet [21]. The resulting plasmid PgpDAa/lucOpt/ptrA/UC was used for transformation of *A. terreus* SBUG844 as described previously [18]. For purification, 20 transformants were repeatedly transferred to pyrithiaminc-containing plates. Subsequently, transformant conidia were inoculated into glucose minimal medium in 96 well plates and incubated for 14 hours at 37°C. After addition of luciferin, bioluminescence of transformants was determined in a microplate reader (LumiStar Optima, BMG LabTech) [21]. Strongly luminescent strains were selected for Southern blot analysis. Strain 2/7/15, selected for the murine infection models, contained 2 ectopic lucOpt integrations and did not reveal growth defects under various in vitro cultivation conditions.

**In Vivo Bioluminescence Imaging**

Images were acquired using an IVIS 100 system (Caliper Life Science) as described previously [22]. In brief, 100 µL PBS containing 3.33 mg d-luciferin (Synchem) were injected intraperitoneally 10 minutes prior to imaging. Mice were anesthetized with 2.5% isoflurane (Delta Select GmbH) in medical oxygen using a XGI-8 anesthesia system (Caliper Life Science) as described previously [22]. In brief, 100 µL PBS containing 3.33 mg d-luciferin (Synchem) were injected intraperitoneally 10 minutes prior to imaging. Mice were anesthetized with 2.5% isoflurane (Delta Select GmbH) in medical oxygen using a XGI-8 anesthesia system (Caliper Life Science) as described previously [22]. Mice were anesthetized with 2.5% isoflurane (Delta Select GmbH) in medical oxygen using a XGI-8 anesthesia system (Caliper Life Science) as described previously [22].
Clinical Scoring and Blood Enzymes

Weight and surface body temperature were recorded daily; clinical examinations were performed twice daily. The clinical score was defined by the sum of 3 parameters: fur (normal, 0; ruffled, 1), dyspnea (absent, 0; mild, 1; moderate, 2; severe, 3), and percent loss of body weight ($0.10\%$, 1; $0.25\%$, 2). Thus, healthy animals scored 0, the maximum possible score was 6.

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, and pancreatic amylase were determined from serum samples using the EuroLyser CCA 180 Vet system (QinLAB Diagnostik) according to standard methods recommended by the International Federation of Clinical Chemistry. Sterile blood samples were taken from mice on the day of sacrifice under general anesthesia by heart puncture.

Pathology and Histology

Gross pathological changes were recorded during postmortem analysis. For histological analysis, representative parts of lung, liver, kidney, and spleen were fixed in buffered formalin and embedded in paraffin; 3-μm sections were stained with hematoxylin-eosin or PAS stain using standard protocols [23].

Quantification of Myeloperoxidase and Cytokines From Tissue Homogenates

Organs were homogenized in tissue lysis buffer (200 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 10 mM Tris, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 μg/mL leupeptide, and 28 μg/mL aprotinin) and centrifuged twice (1500g, 10 minutes, 4°C). The supernatants were stored at $-80°C$ until use. Myeloperoxidase (MPO) and cytokine levels were determined using commercially available murine enzyme-linked immunosorbent assays (ELISA; MPO, Hycult Biotechnology; cytokine ELISA, eBioscience; and mouse inflammation antibody array G series, RayBiotech) according to the manufacturers’ recommendations.

Statistical Analyses

Data were plotted and analyzed statistically using GraphPad Prism 5.0 (GraphPad Software). Survival data were evaluated by Kaplan-Meier curves and log-rank test. Cytokines, MPO, and blood enzyme data were analyzed using 1-way analysis of variance followed by Tukey-Kramer test.

RESULTS

Strain Selection by Virulence Studies in Embryonated Eggs

Embryonated hen’s eggs have been shown to provide a suitable screening model for virulence of A. fumigatus wild-type and mutant strains [20, 24]. Thus, to select a strain and estimate the infectious dose for murine virulence studies, we investigated the virulence of A. terreus wild-type strains A1156, SBUG402, and...
SBUG844 in embryonated eggs. Dose-dependent killing was observed with \(1 \times 10^{6}\) to \(1 \times 10^{7}\) conidia per egg (Figure 1A). For comparable mortality rates, \(10^3\) times more \textit{A. terreus} conidia were needed compared with \textit{A. fumigatus} wild-type CBS144.89 (Figure 1B and 1C). All 3 \textit{A. terreus} strains showed similar virulence potential. However, strain SBUG844 displayed the highest reproducibility (Figure 1B and 1C) in these experiments and was thus selected for subsequent studies.

Histologically, conidia were detected inside or closely attached to the upper epithelial layer of the CAM 8 hours and 24 hours after infection (Figure 1E). Forty-eight hours after infection, hyphae penetrated the underlying tissue (Figure 1F). These hyphae induced recruitment of immune cells (Figure 1G). Embryos that succumbed to infection showed massive destruction of the CAM by fungal mycelia. In contrast, surviving embryos displayed granuloma formation, which likely restricted tissue invasion (Figure 1H). Although \textit{A. terreus} grew invasively, these experiments implied that compared with \textit{A. fumigatus}, a significantly higher infectious dose might be required in murine infection models for pulmonary aspergillosis by \textit{A. terreus}.

**Figure 2.** Bioluminescence imaging of leucopenic and corticosteroid-treated mice infected with \textit{Aspergillus terreus} SBUG844. \(A\), Representative time course of a single leucopenic mouse infected with \(1 \times 10^7\) conidia. Bioluminescence strongly increases from day 6 onward until the mouse succumbs to infection on day 9. \(B\), Representative time course of 2 corticosteroid-treated mice infected with \(1 \times 10^7\) conidia. The mouse on the left-hand side succumbed on day 5. Thus, pictures for day 5 to day 10 show the surviving mouse (right-hand side on previous pictures) only. \(A\) and \(B\), The graphs on the right show quantification of light emission.

**Dose-Dependent Survival of Leucopenic and Corticosteroid-Treated Mice Infected With \textit{A. terreus}\)**

A bioluminescent \textit{A. fumigatus} strain has been shown to be useful for investigating the progression of pulmonary aspergillosis in mice [21, 22]. Therefore, we constructed bioluminescent \textit{A. terreus} strains in which expression of a codon-optimized luciferase gene was driven by the \textit{A. fumigatus} gpdA promoter. The strain SBUG844_2/7/15_lucOPT was chosen for mouse experiments because it displayed high bioluminescent signals under various in vitro growth conditions, no growth defects, and virulence comparable to the parental strain in the embryonated egg model (data not shown).
Mice were either rendered leucopenic or immunosuppressed with corticosteroids prior to infection. Immunosuppression was confirmed by differential blood counts (Supplementary Figure S1A). Groups of 5 corticosteroid-treated mice were infected intranasally with $1 \times 10^6$, $5 \times 10^6$, or $1 \times 10^7$ conidia per animal. Similarly, groups of leucopenic animals were infected with $5 \times 10^5$, $1 \times 10^6$, or $1 \times 10^7$ conidia. Leucopenic mice developed luminescence signals 4–6 days after infection when challenged with $1 \times 10^7$ conidia. Signal intensity steadily increased until mice succumbed to infection (Figure 2A). This result was similar to that obtained for A. fumigatus infections [22]; however, lethal infections can be induced with only 1–5 $\times 10^4$ A. fumigatus conidia [25]. Significantly higher conidia concentrations were needed with A. terreus, and luminescence appeared 3–4 days later [22].

Corticosteroid-treated mice only developed lethal disease if infected with $1 \times 10^7$ conidia, which is 100-fold higher than for A. fumigatus [26]. These animals displayed weak luminescence within the first 48 hours. The signal then increased significantly and remained at high levels up to day 4 (Figure 2B) if animals succumbed. In surviving animals, the signal intensity declined and reached basal levels 7–10 days after infection. These animals recovered and showed no clinical symptoms at the end of the experiment. In contrast, a decline of the signal in A. fumigatus-infected corticosteroid-treated mice correlated with strong inflammation, tissue necrosis accompanied by physical distress, and severe loss of body weight [21].

To confirm the challenge doses required for lethal infections and to ensure that the use of a bioluminescent A. terreus strain did not influence the results, 3 additional independent experiments per immunosuppression regimen were performed. Within every experiment, 10 mice were infected with A. terreus and 5 animals received PBS, serving as the mock infected control. Leucopenic mice were infected with $5 \times 10^6$ conidia, leading to 100% mortality within 14 days (Figure 3A). Nearly all infected corticosteroid-treated mice ($1 \times 10^7$ conidia) showed clinical symptoms of disease (ruffled fur, weight loss, dyspnea). However, only 50% succumbed to infection (Figure 3B).
clinical status of the remaining 50% improved from day 6 onward, and mice fully recovered toward the end of the experiment at day 14 (Figure 3C). Survival curves were reproducible between experiments (Supplementary Figure S2A and S2B). Similar results were obtained with strain A1156 (data not shown).

To further characterize A. terreus IPA in both immuno-suppression models, we performed histological and cytokine
Figure 5. Analysis of corticosteroid-treated mice infected with Aspergillus terreus SBUG844. A–G, Representative lung histology, periodic acid Schiff staining. The right panel shows a magnification of the boxed area marked in the respective left panel. Inlays in the right panel (B–D, G) show additional features.
analyses of moribund animals from survival experiments. In addition, in an independent experiment, 5 mice each were sacrificed for analysis 48 hours, 72 hours, and 5 days after infection or mock infection (controls).

Characterization of Infection in Leucopenic Mice
Up to 72 hours after infection, only swollen conidia, but no germings, were observed within the lung. Conidia resided within epithelial cells and macrophages (Figure 4B and 4C). Germings became visible at day 5 (Figure 4D), coinciding with the late appearance of bioluminescent signals in imaging experiments. Similar to pathology in rabbits [17], lung alterations in moribund mice were characterized by invasive growth of A. terreus hyphae, angioinvasion, and necrosis (Figure 4E). Furthermore, aleuroconidia were regularly observed in infected tissue (Figure 4F). Consistent with leucopenia, immune cell infiltrations were sparse and MPO content, which is a neutrophil marker, did not increase (data not shown). The levels of tumor necrosis factor (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 10 (IL-10), IL-17A, interferon γ (IFN-γ), IL-6, and IL-22 were undistinguishable between infected and control animals at all time points. Only IL-1β increased moderately (Figure 4G), possibly resulting from the interaction of A. terreus with epithelial cells.

Spleen and kidneys were macroscopically and histologically unaltered. In contrast, 48 hours after infection and later, livers of infected mice appeared pale and histologically revealed vacuolar, fatty degeneration of hepatocytes (Figure 4H and 4I; Supplementary Figure S2D–F), mainly in the lobular periphery. However, neither conidia nor mycelium was detected. Furthermore, no influx of leucocytes was visible histologically and MPO levels were comparable to PBS controls (data not shown). The serum levels of the liver marker enzymes ALT and AST were not significantly increased (data not shown).

Characterization of Infection in Corticosteroid-Treated Mice
Similar to the leucopenic model, conidia residing within host cells were observed in corticosteroid mice within the first 48 hours (Figure 5B and 5C). Swelling of conidia started on day 3 (Figure 5D) and hyphae were visible on day 5 (Figure 5E). Influx of neutrophils and monocytes accumulating at the site of infection were observed earliest at 48 hours after infection and increased over time (Figure 5C–F). In moribund animals, accumulations of hyphae and surrounding immune cells blocked the main air passages, likely leading to the observed severe respiratory distress and corresponding with severe lung hepatization observed at necropsy. The recruitment of neutrophils to the lung was reflected by increased MPO levels, which were highest in moribund animals (Figure 5H). Aleuroconidia were regularly observed in infected tissue. Based on cytokine arrays (Figure S3), we selected TNF-α, IL-1β, GM-CSF, IFN-γ, IL-6, IL-10, IL-17, and IL-22 for quantification by ELISA. TNF-α, IL-1β, and IL-6 increased over time and were highest in moribund mice (Figure 5I). Interestingly, the regulatory cytokine IL-10 was significantly reduced in infected animals. All other determined cytokine levels were unaltered compared with controls. On day 14 after infection, surviving animals showed only sparse immune cell infiltrates within the lungs and intracellular conidia were observed (Figure 5J). To determine whether these conidia were viable, lung homogenates were plated for isolation of the fungus. Surprisingly, A. terreus could be isolated in large numbers from 87% of the survivors (Figure S2C), suggesting persistence of viable fungal cells despite clinical convalescence. MPO, TNF-α, IL-1β, and IL-6 levels of surviving animals were similar to uninfected controls (Figure 5H and 5I).

Similar to leucopenic mice, corticosteroid-treated animals infected with A. terreus showed fatty degeneration of hepatocytes (Figure 5L), mainly in the lobular periphery, in the absence of both mycelium and significant neutrophil recruitment from day 2 onward (Figure 5L). ALT serum levels were unaltered and AST levels were increased in moribund animals only, accompanied by significantly reduced liver weight (Figure 5N). Thus, hepatocyte degeneration was observed in infected animals independent of the immunosuppressive regimen but not in mock-infected control mice.

DISCUSSION
The aim of this study was to develop infection models for A. terreus based on well-established models for A. fumigatus. The alternative infection model using chicken embryos and both the leucopenic and corticosteroid-treated murine models were highly reproducible. However, significantly higher infectious doses were required for lethal infections by A. terreus compared with A. fumigatus [11, 20], consistent with finding of others [17, 27–29].

Bioluminescence imaging and histology revealed that germination of A. terreus conidia within the host is strongly delayed.


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compared with *A. fumigatus* [21], thus explaining the comparatively delayed onset of mortality in the leucopenic mouse model [25]. In cell culture media, germination of *A. terreus* was likewise slower than *A. fumigatus* ([17] and data not shown), but no difference could be observed in brain heart infusion broth (data not shown). Thus, slower germination does not seem to be an intrinsic feature of *A. terreus* but depends on environmental conditions. Because nongerminated *A. terreus* conidia were mainly observed within host cells, it appears possible that germination is specifically delayed for intracellular conidia. In corticosteroid-treated mice, the immune system is impaired, but immune cells are still recruited to the site of infection [11, 21]. Immune cell recruitment and increase of proinflammatory cytokines coincided with the appearance of germings and hyphae, suggesting that germination is necessary to trigger immune cell infiltrations. Although bioluminescence imaging revealed successful initial establishment of infection in all infected animals, only 50% of mice succumbed to infection. Nongerminated conidia were found frequently in surviving mice, suggesting that only part of the inhaled conidia germinated in these animals. Thus, if fewer conidia germinate, fewer immune cells are recruited and disease is less severe, allowing animals to survive. No hyphae were observed in surviving mice, suggesting that they were successfully eliminated by the immune response. However, nongerminating conidia resided in the lung and were successfully isolated at high numbers from most surviving animals. Interestingly, these conidia did not trigger an immune response because surviving animals showed only slightly different MPO and cytokine levels compared with mock-infected controls. Persistence solely occurred within cellular structures such as macrophages or epithelial cells in which resting conidia can hide from the immune responses.

If so, *A. terreus* infections are unlikely to be acquired nosocomially. It is tempting to speculate that high-risk patients who develop *A. terreus*–mediated disease are carriers of persisting *A. terreus* conidia and an outbreak of infection is induced under severe immunosuppression.

Furthermore, under corticosteroid treatment we observed reduced IL-10 levels in infected animals compared with mock-infected mice. This contrasts the infection with *A. fumigatus*, showing an increase in IL-10 under steroid treatment [30]. IL-10 usually induces a T-helper 2 (Th2) cell response, activates macrophages, and silences the T-helper 1 (Th1) immune response [31]. Moreover, we investigated IL-17/IL-22 production in the lung in both infection models. An increase of these cytokines was not observed in either model. This is obverse to *A. fumigatus* infections, where the induction of a T-helper 17 (Th17)–dominated immune response is regularly observed and this response has been assumed important for the clearance of the fungus [32–35]. Thus, the lack of IL-17 induction may contribute to the persistence and limited clearance of *A. terreus* resting conidia in the lung. The Th1 cytokines TNF-α, IL-1β, and IL-6 showed similar increase over time compared with an *A. fumigatus* infection, resulting in fatal or positive outcome of infection due to the severity and location of immune cell recruitment in the early acute phase of disease [30, 36, 37].

In addition to differences in cytokine response, animals infected with *A. terreus* developed a fatty degeneration of liver cells regardless of the immunosuppression regimen. Such alterations have not been described for mice succumbing to *A. fumigatus* infections. Because fungal elements were neither detected by histology nor bioluminescence imaging and the degeneration appeared in the periphery of the hepatic lobes, it is likely that the effect derives from *A. terreus*–specific secondary metabolites. Given that the degeneration was only mildly reflected in marker enzymes in the blood, these metabolites probably affect hepatocyte metabolism instead of directly lysing the cells. Whether these metabolites play a role in human IPA caused by *A. terreus* and could be used as diagnostic markers needs to be investigated in the future. However, our models were highly reproducible and provided hints to substantial differences in pathogenicity compared with *A. fumigatus*.

In conclusion, *A. terreus* can cause fatal invasive pulmonary aspergillosis in the immunocompromised host. Our data indicate a possible long-term persistence of *A. terreus* conidia that needs further investigation. Additionally, to elucidate the nature of liver degeneration in more detail, we currently identify secondary metabolites from *A. terreus* that show a hepatotoxic capacity and aim at the identification of secondary metabolites produced in vivo.

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://www.oxfordjournals.org/our_journals/jid/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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