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RESEARCH PAPER

Ketoacidosis alone does not predispose to mucormycosis by *Lichtheimia* in a murine pulmonary infection model

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ABSTRACT

Mucormycosis is a rare fungal infection; however, the number of cases increased during the last decades. The main risk factors are immunosuppression and uncontrolled diabetes mellitus. Although *Lichtheimia* species represent a common cause of mucormycosis in Europe, virulence and pathogenesis of this genus has not been investigated in detail yet. Using murine pulmonary infection models, we found that immunosuppression is essential for establishment of infection. The disease was characterized by necrosis, angioinvasion, thrombosis, and the lethal course of infection was associated with systemic activation of platelets. Furthermore, dissemination to internal organs was frequently observed. While the virulence potential of individual *L. corymbifera* and *L. ramosa* isolates differed, pathogenicity of both species was comparable. Although ketoacidosis promoted *Rhizopus* infection in mice, it did not predispose mice to infection with *Lichtheimia* in the absence of additional immunosuppression. This might partially explain the dominance of *Rhizopus* as cause of mucormycosis in countries with high prevalence of ketoacidotic patients.

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Introduction

Mucormycosis is a life threatening infection in animals and humans, caused by ubiquitously distributed saprophytic fungi of the order Mucorales. Mucormycoses are rare, but the number of cases has increased during the last decades with mortality rates around 50%.[1] Risk factors for mucormycosis are immunosuppression and uncontrolled diabetes mellitus; cases in immunocompetent patients following trauma have been described.[1-3] Infections are usually initiated by either inhalation of spores, leading to sinusoidal or pulmonary mucormycosis, or are associated with traumatic or surgical wounds, facilitating cutaneous or deep-tissue infections.[1,3,4] Notably, infections can spread hematogenously and by direct invasion of adjacent tissue, leading to rhinocerebral, cerebral and other forms of mucormycosis.[1,3,4]

70–80% of mucormycoses are caused by *Rhizopus*, *Mucor*, and *Lichtheimia* (former *Absidia*) species, with *Lichtheimia* as the second and third most common cause of mucormycosis in Europe and the USA, respectively.[1,3-5] The genus *Lichtheimia* contains at least 6 species,[6] of which only *L. corymbifera*, *L. ramosa* and *L. ornata* were isolated from clinical material,[7] suggesting differences in the virulence potential of the species. This hypothesis is supported by results from a chicken embryo model,[8,9] which furthermore revealed differences in the virulence potential of *L. corymbifera* and *L. ramosa* isolates. Whether these results are transferable to mammalian hosts has however not been determined yet.

Murine models have been successfully used to study pathogenesis[10-12] and to evaluate therapy efficacy[13-17] of mucormycosis caused by *Rhizopus* and *Mucor*. Depending on the localization of mucormycosis to be studied, the route of infection differs; systemic infection via the tail vein to mimic hematogenous dissemination,[13,14,18] and pulmonary infections facilitated by intranasal or intratracheal application of spores are most commonly

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used.[15-17] Chemically-induced diabetic ketoacidosis, leukopenia based on cyclophosphamide treatment and immunosuppression by corticosteroids are used to mimic common risk factors in humans.[12-18] As mucormycosis caused by *Lichtheimia* species has not yet been studied in detail in mammalian models, we established a murine model of pulmonary *Lichtheimia* infection based on published models for *Rhizopus* and *Mucor*. Corticosteroid treatment was confirmed as a risk factor for pulmonary infections by *L. corymbifera* and *L. ramosa*, and infection resembled human disease with necrosis, angioinvasion and thrombosis. However, ketoacidosis did not render mice susceptible to pulmonary *Lichtheimia* infection.

Material and methods

Ethics statement

Animal studies were conducted in accordance with the recommendations of the European Community and were approved by the Animal Care and Usage Committee of the Landesamt Thüringen, Bad Langensalza, Germany (permission no. 03–006/11 and 03–001/14).

Induction of immunosuppression and diabetic ketoacidosis

Outbred female CD-1 mice (Charles River Laboratories Germany) 6 to 8 weeks old were kept under specific pathogen-free conditions in groups of 5 in isolated ventilated cages with sterile food and water provided *ad libitum*. Immunosuppression was established by intraperitoneal injection of 25 mg cortisone acetate (Sigma Aldrich) in 200 μL PBS on day -3 and day 0.[19] To induce insulin-dependent ketoacidosis a single dose of 190 mg/kg streptozotocin (Sigma Aldrich) in 200 μl citrate buffer (0.1 M, pH 4.5, sterile-filtrated) was injected intraperitoneal after 4 h of fasting on day -10. After streptozotocin injection all animals received drinking water supplemented with 10% saccharose (Carl Roth GmbH, Germany) for 24 h. Development of hyperglycemia was evaluated by measuring the blood glucose concentration 4 d before and 7 d after streptozotocin treatment in blood from the lateral tail vain using the Ascensia MICROFILL system (Henry Schein) according to the manufacturer's instructions. Animals that did not develop hyperglycemia (blood glucose levels below 200 mg/dL) were excluded from the experiment before infection. Persistence of hyperglycemia was likewise determined at the end of the experiment (mean blood glucose 421 mg/dL). Untreated animals and mice treated with cortisone acetate analyzed on day 0 served as controls.

Cultivation of Lichtheimia *and intranasal infection*

The strains used in this study are listed in Table 1 and are deposited in the Jena Microbial Resource Collection (www.jmrc.hki-jena.de). Spore suspensions were prepared as described previously[8,9]; in brief, strains were revived from soil cultures (double-sterilized sand-loam-garden soil) and precultivated at 37°C in 10 mL modified liquid SUP medium (55 mM glucose, 30 mM potassium dihydrogen phosphate, 20 mM ammonium chloride, 5 mM di-potassium hydrogen phosphate, 1 mM magnesium sulfate and 0.5% yeast extract; all reagents from Carl Roth GmbH). The mycelium was further cultivated on modified SUP plates at 37°C for 7 d to allow sporulation. Spores were harvested, washed twice with sterile PBS (Dulbeccos, Thermo Fisher) and diluted in sterile PBS to the indicated concentrations. Intranasal infection was performed with 20 μL of the spore suspension as described previously[19] under general anesthesia (0.05 mg/kg fentanyl, 0.5 mg/kg medetomidin, and 5 mg/kg midazolam). Anesthesia was terminated by subcutaneous application of antidote (1.2 mg/kg naloxone, 2.5 mg/kg atipamezol, and 0.5 mg/kg flumazenil). Mice were monitored at least twice daily for development of clinical symptoms and humanely killed if they met predefined humane endpoints ($\geq$ 25% weight loss, severe lethargy, severe dyspnea, inability to move coordinately) or at the end of the experiment. To assess the pathological alterations caused by the infection, all animals were necropsied and lung, liver, heart, kidneys and brain were analyzed by histology.

Table 1. Strains used in this study.

Isolate	Designation in strain collection	Designation in this study	Source
L. corymbifera	JMRC:FSU:09682	Reference strain	Environment
L. corymbifera	JMRC:FSU:10164	LCJ5	Environment
L. corymbifera	JMRC:FSU:10061	LCJ3	Human
L. corymbifera	JMRC:FSU:10240	LCJ9	Human
L. ramosa	JMRC:FSU:11788; Luo *et al.* (25)	LCJ22	Human
L. ramosa	JMRC:FSU:06197	LRJ17	Environment
L. ramosa	JMRC:FSU:10238	LRJ14	Human
L. ramosa	JMRC:FSU:10251	LRJ13	Human

Histology

Lung, kidneys, a lobe of the liver, heart and brain were aseptically collected during necropsy and fixed in neutral-buffered formalin. Paraffin-embedded sections were stained with either periodic acid Schiff (PAS) or hematoxylin and eosin (H&E) and microscopically analyzed using the Axiocam from Zeiss. A scoring system was used to quantify tissue alterations (fibrosis, necrotic tissue, immune cell infiltration, and presence of fungal elements, respectively) as follows: 0 = no alterations; 1 = single fields of small areas (< 50 μm) of alterations; 2 = multiple fields and/or larger areas (100 – 200 μm); 3 = severe tissue alterations, with the majority of the organ structure impaired. For quantification, each type of alteration (fibrosis, necrotic tissue, immune cell infiltration, and presence of fungal elements, respectively) was first scored individually. Then, single scores for the different types of alterations were added. The use of this additive scoring system was considered to be more precise as e.g. in lungs of mice who survived the infection, no fungal cells can be found but areas of necrosis and immune cell infiltration are still detectable.

Collection of blood and determination of platelet activation

100 μl of blood were collected under terminal anesthesia by retro-orbital bleeding, immediately transferred into a tube containing 10 μl EDTA solution (1.6 mg/ml), and gently mixed. 20 μl of the sample were analyzed on a BC-5300Vet (Mindray) configured for murine blood, providing 23 parameters, including platelet counts and mean platelet volume. Platelet-rich plasma (RPP) was prepared from whole-blood by centrifugation at 135 g for 15 min at room temperature. To detect platelet activation, platelets were stained for 30 min with fluorescence-labeled antibodies (BioLegend) directed against CD41 as platelet marker and CD63 as activation marker, followed by fixation with 1% formaldehyde. Half of the sample was used directly for flow cytometry (FC)-based quantification of CD41+ microparticles, which were gated according to their size. The rest of the sample was analyzed by FC for CD63 expression on the surface of CD41-positive platelets. Fibrinogen binding, which indicates activation of surface-bound GPIIb-IIIa complex, was quantified using fluorescence-labeled antibodies (BioLegend) as described for CD63. Plasma concentrations of soluble CD62P were determined using the Quantikine® ELISA Mouse sP-Selectin/CD62P kit (R&D Systems, USA) performed according to manufacturer instructions. Plasma was prepared by centrifugation of whole-blood at 1500 g for 15 min at room temperature.

Statistical analysis

The Graph Pad Prism 6 software (GraphPad Software Inc.) was used for statistical analysis. Survival data were analyzed using the Log-rank Mantel-Cox test. Gaussian distribution of the histological scores and flow cytometry data was confirmed by the D'Agostino & Pearson omnibus normality test. Histological scores were analyzed by one-way ANOVA with Tukey's multiple comparison test. Platelet activation markers were analyzed by unpaired, 2-sided t-test. Statistical significance was considered for p-values < 0.05.

Results

Immunosuppression is essential for the development of pulmonary Lichtheimia *infection in mice*

As inhalation of spores is the most common route of infection with mucormycetes, we performed intranasal infection. To determine whether this leads to reproducible numbers of spores reaching the lung, mice were killed 10 minutes after infection and pulmonary tissue (after removal of trachea and major bronchi) was homogenized and quantitatively cultured on SUP plates. Similar amounts of spores were recovered from all mice infected with the same dose (Fig. 1A), indicating reliable delivery of spores to the lung.

Immunosuppression is a risk factor for human mucormycosis,[1] and was shown to facilitate pulmonary infection by other mucormycetes in mice. To determine whether immunosuppression was necessary to establish infection, immunocompetent and mice immunocompromised with corticosteroids were infected in parallel with 10^5 to 5×10^6 spores (n = 5 per dose). While immunocompetent mice remained healthy, corticosteroid-treated mice infected with either 5×10^6 or 10^6 spores succumbed to infection (median survival time 6 and 8 days, respectively). 80% of the corticosteroid-treated mice receiving 10^5 spores survived the infection and were clinically healthy 58 d after infection (Fig. 1B). The susceptibility of corticosteroid-treated mice to pulmonary *L. corymbifera* infection was confirmed in 2 additional independent experiments (5×10^6 spores/mouse, n = 20). Parallel experiments with immunocompetent mice (n = 10) did neither lead to clinical symptoms nor histological tissue alterations, indicating that immunosuppression is essential for pulmonary *Lichtheimia* infections in mice.

Murine pulmonary Lichtheimia *infection is characterized by thrombosis, tissue necrosis and dissemination*

In *L. corymbifera*-infected corticosteroid-treated mice the first clinical symptoms occurred 3 to 4 d post

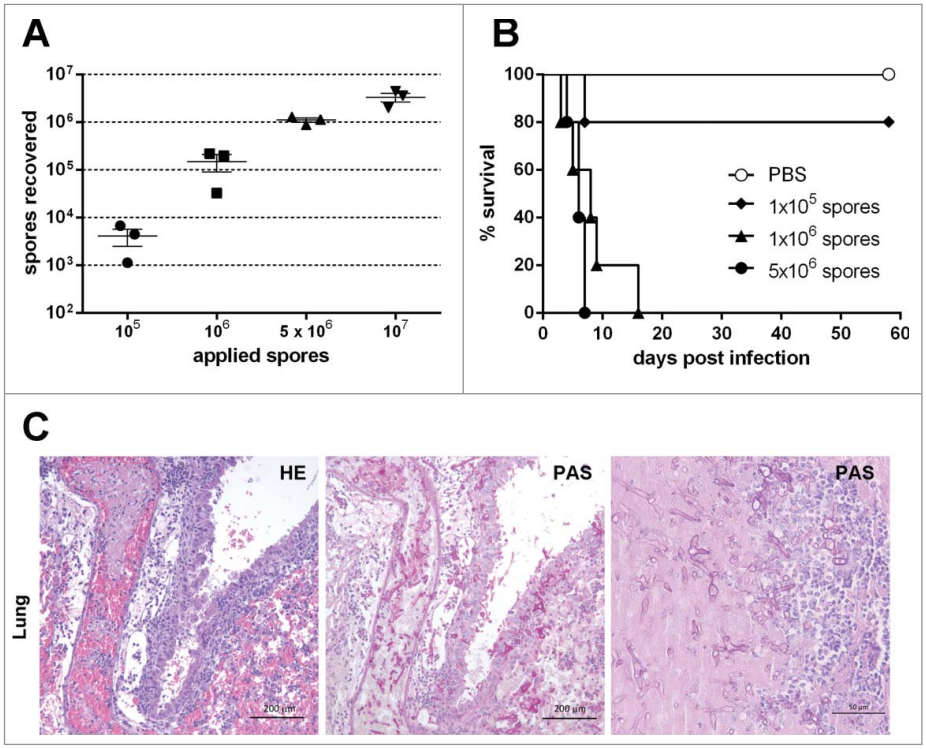

Figure 1. Susceptibility of corticosteroid-treated mice to pulmonary *Lichtheimia* infection and histopathological alterations in the lung. (A) Number of spores reaching the lung after intranasal application. Female CD-1 mice (n = 3 mice/group) were killed 10 min after intranasal application of *L. corymbifera* FSU 9682 spores. (B) Dose-dependent survival of corticosteroid-treated mice intranasaly infected with *L. corymbifera* FSU 9682 (n = 5 mice/group). Log rank (Mantel-Cox) test was used to compare each infection dose to PBS treated control mice ($^{*}p = 0.05$, $^{**}p = 0.01$). (C) Representative lung sections from mice infected with 5×10^6 spores of the *L. corymbifera* reference strain. Slides were stained with hematoxylin/eosin (HE) or Periodic Acid Schiff (PAS) as indicated in the upper right corner of each image. Representative HE and PAS stained slides from 2 consecutive sections are shown in the left and middle column, the right column represents an enlarged area.

infection (p. inf.), characterized by ruffled fur and mild dyspnea. In these animals dyspnea commonly progressed to severe respiratory distress warranting euthanasia within 12 h to 72 h (11/35 mice). In animals with later onset of clinical disease, dyspnea was less prominent and lethargy and hypothermia led to euthanasia (7/35 mice). Individual animals presented with ataxia, torticollis or tremor and were killed due to their inability to ambulate (6/35 mice) between day 6 and 9 p. inf.. Mild clinical symptoms that occurred later than day 10 p. inf. (ataxia, lethargy or dyspnea) did not progress and were usually transient (11/35 mice).

Consistent with the clinical manifestation, the lungs of mice with overt dyspnea showed extensive dark red to black areas histologically characterized by hemorrhage, tissue destruction, fungal growth, angioinvasion and thrombosis (Fig. 1C), reminiscent of typical mucormycosis in human patients.[20] Comparable pathology was also observed in the kidneys of some animals killed after day 5 p. inf. (16/35 mice, see Fig. S1). Occasionally, whitish areas were also observed in the liver (8/35), heart (4/35), or diaphragm (1/35), corresponding with tissue necrosis but not necessarily detection of fungal elements by histology (see Fig. S1). The neurologic symptoms observed in some mice suggested dissemination to the brain; this was histologically confirmed in some, but not all cases (see Fig. S1). Notably, the lung histopathology in animals that developed clinical disease later or at slower progression was more moderate than in acute cases and characterized by infiltration of lymphoid cells; it was however also frequently associated with dissemination into other organs.

Diabetic ketoacidosis alone does not promote pulmonary Lichtheimia *infection*

Uncontrolled diabetes mellitus leading to ketoacidosis is an important risk factor for mucormycoses in humans and has been shown to promote pulmonary *Rhizopus* infections in mice.[1,5,17,21,22] Insulin-dependent ketoacidosis occurs in genetically modified mice, in certain mouse strains fed with a special diet,[23] or can be achieved by treatment with streptozotocin, an antibiotic that causes pancreatic β-cell destruction and thereby insulin

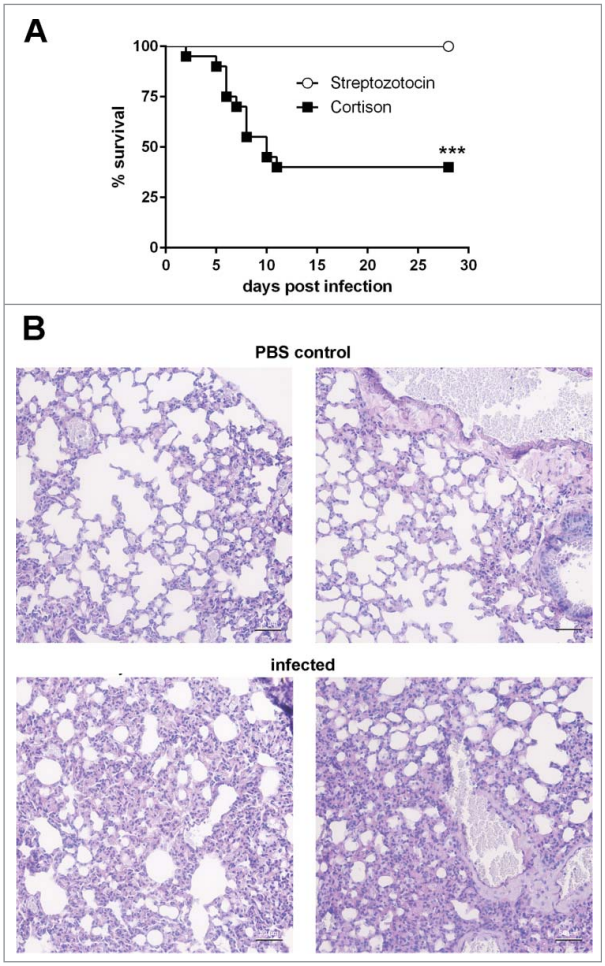

Figure 2. Corticosteroid-treated but not diabetic ketoacidotic mice succumb to infection with *Lichtheimia corymbifera*. (A) Survival of corticosteroid-treated and diabetic ketoacidotic mice intranasaly infected with 5×10^6 spores of *L. corymbifera* FSU 9682. Shown are the combined survival data from 2 independent experiments with n = 7–10 mice/group/experiment. Log-rank (Mantel-Cox) test was used to compare both groups, $^{**}p < 0.01$. (B) Representative PAS stained histological sections from lungs of PBS control mice (upper row) and infected mice 28 d p. inf. Black scale bar = 50 μm.

deficiency and hyperglycemia.[24] We chose the streptozotocin model as it has been used by others to investigate *Rhizopus* infections in mice.[17,25,26] Development of hyperglycemia was confirmed by determination of blood glucose, and animals infected with *R. arrhizus* (formerly *R. oryzae*; CBS 126971) as a positive control succumbed to infection (data not shown). To our surprise, in 2 independent experiments all diabetic ketoacidotic (DKA) mice survived *L. corymbifera* infection without developing clinical symptoms (Fig. 3A) and no fungal elements could be detected in the lungs and kidneys of DKA mice at the end of the experiments. However, infiltration of lymphoid cells in the lungs of DKA mice indicated a local immune response that likely mediated fungal clearance (Fig. 3B). It should however be noted that DKA mice in this study were not additionally treated with corticosteroids, in contrast to previous studies by others.[17] Thus, we cannot exclude an exacerbating effect of DKA on the susceptibility of immunocompromised mice to *Lichtheimia* infections.

Virulence varies between different L. corymbifera *isolates*

Using a chicken embryo model, we previously observed comparable overall virulence potential of *L. corymbifera* and *L. ramosa* but also substantial variation in the virulence between distinct strains.[8] To determine whether this finding was transferable to infection of a mammalian host, we tested the virulence of 3 additional *L. corymbifera* and 4 *L. ramosa* strains in corticosteroid-treated and DKA mice.

None of the tested *Lichtheimia* strains was able to cause clinical symptoms in DKA mice (data not shown), confirming that, in contrast to *Rhizopus*, ketoacidosis does not significantly promote the development of pulmonary *L. corymbifera* infection in mice. However, infection of immunosuppressed mice revealed differences in the virulence potential of the *L. corymbifera* strains tested (median survival times ranging from 8 to 13.5 days; Fig. 4A). Whereas the reference strain and LCJ9 showed average virulence (median survival 10 and 8 days, respectively), LCJ5 was found to be strongly attenuated. Only LCJ3 was significantly ($p < 0.01$, Log-rank test) more virulent than the reference strain, with a median survival time of 6 d and higher absolute mortality. Additionally, quantification of the histological alterations using an additive scoring system for lung and kidneys supported the higher virulence of LCJ3 (Fig. 3B, C and see Fig. S2). In contrast, both survival and lesion scores were comparable for the *L. ramosa* strains tested (Fig. 3B, C) and comparable to the *L. corymbifera* reference strain used for the establishment of the model (Fig. 3A). In all cases of clinical infections, the pathological alterations resembled those described above for the *L. corymbifera* reference strain. Based on the previous experience that the initial onset of symptoms occurred no later than 12 d after infection, the experiment was terminated on day 16; all remaining mice at this time point were free of clinical symptoms and on necropsy showed no or only minor pulmonary and/or renal alterations characterized by lymphocytic infiltrates. The histological lung score correlated with the time of survival for both fungal species (see Fig. S3), suggesting that histological alterations and virulence are quantitatively associated. It should however be noted that histological samples from different time points after infection were used which limits the

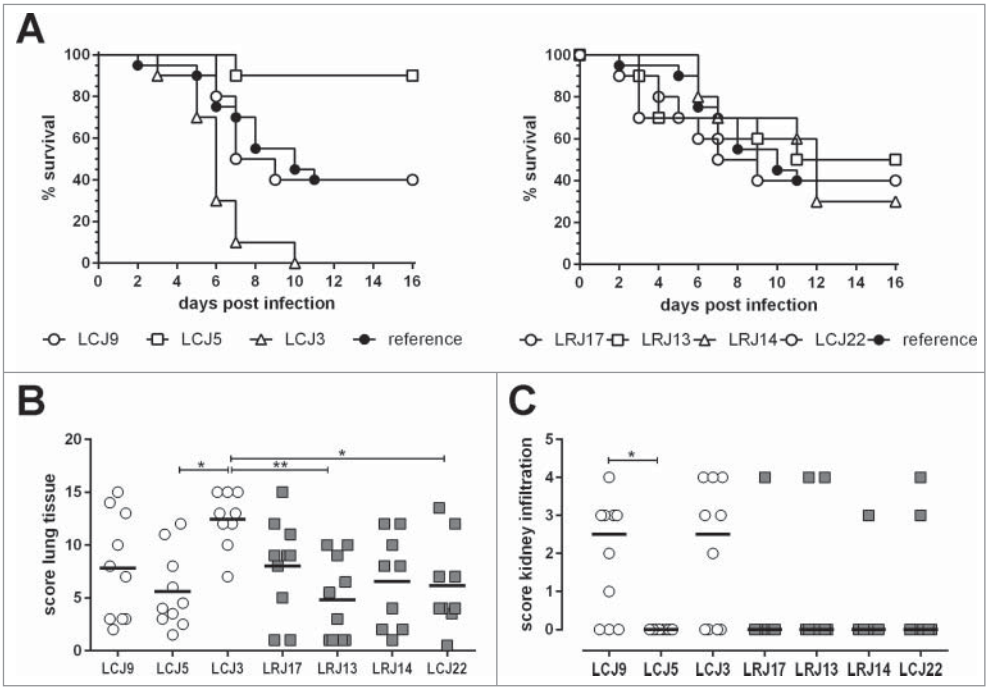

Figure 3. Strain specific virulence of different *Lichtheimia* isolates in a mouse model of pulmonary infection. (A) Survival of immunocompromised female CD-1 mice intranasaly infected with 5×10^6 spores of the indicated *L. corymbifera* and *L. ramosa* strains. Combined data from 2 independent experiments (n = 5 mice/group/experiment). $^{**}p < 0.01$, determined by Log-rank (Mantel-Cox) test. (B) Analysis of histological scores from lung and kidneys (C) (n = 8–10 mice/group). Statistical significance was calculated using one-way ANOVA with a Tukey's multiple comparison test, $^{*}p < 0.05$ and $^{**}p < 0.01$.

extend of the conclusions that can be drawn from this correlation.

Lethal Lichtheimia *infection is accompanied by increased platelet activation and reduced platelet numbers*

Angioinvasion and thrombosis were commonly observed in infected tissues; to determine whether systemically platelet activation occurred, peripheral blood samples from PBS control mice, moribund animals and surviving mice 16 d p. inf. were analyzed. Moribund animals showed reduced numbers of circulating platelets (Fig. 4A) and increased mean platelet volume (Fig. 4B), consistent with increased consumption of platelets during thrombus formation and increased release of young platelets from the bone marrow. Furthermore, the platelets circulating in the periphery were significantly more activated in moribund than in surviving mice as shown by enhanced CD63 expression on platelet surfaces (Fig. 4C), implying dense granule release. Similarly, fibrinogen binding (Fig. 4D) on the platelet surface was higher in moribund than in surviving mice, suggesting enhanced tendency of clot formation and thrombosis.[27] Additionally, levels of secreted CD62P (Fig. 4E) and formation of microparticles (Fig. 4F) were increased in the plasma of moribund animals. These platelet-derived proinflammatory factors might contribute to excessive inflammation and pathophysiology of infected animals.[28]

Discussion

Mucormycoses are rare but life-threatening fungal infections that usually affect patients with immunosuppression due to neutropenia, corticosteroid treatment, or diabetes.[1,29] Therapeutic interventions are often difficult and require a combination of surgical removal of infected foci and antifungal therapy, still resulting in overall high mortality.[1,20] Thus, there is a need to better understand these infections and murine models are an essential tool to investigate pathogenesis and assess treatment efficacy. In contrast to *Rhizopus, Lichtheimia* infections have not been systematically studied in mice yet. We therefore established a pulmonary infection model that is based on models widely used for *Rhizopus, Aspergillus* and other fungi, thereby allowing future comparative studies. Outbred mice were chosen for this study to avoid any possible influence of specific genetic backgrounds in inbred mouse lines on the results.

Similar to infection with *Rhizopus*,[12] immunosuppression significantly increased susceptibility of mice to pulmonary *Lichtheimia* infection. This is consistent with

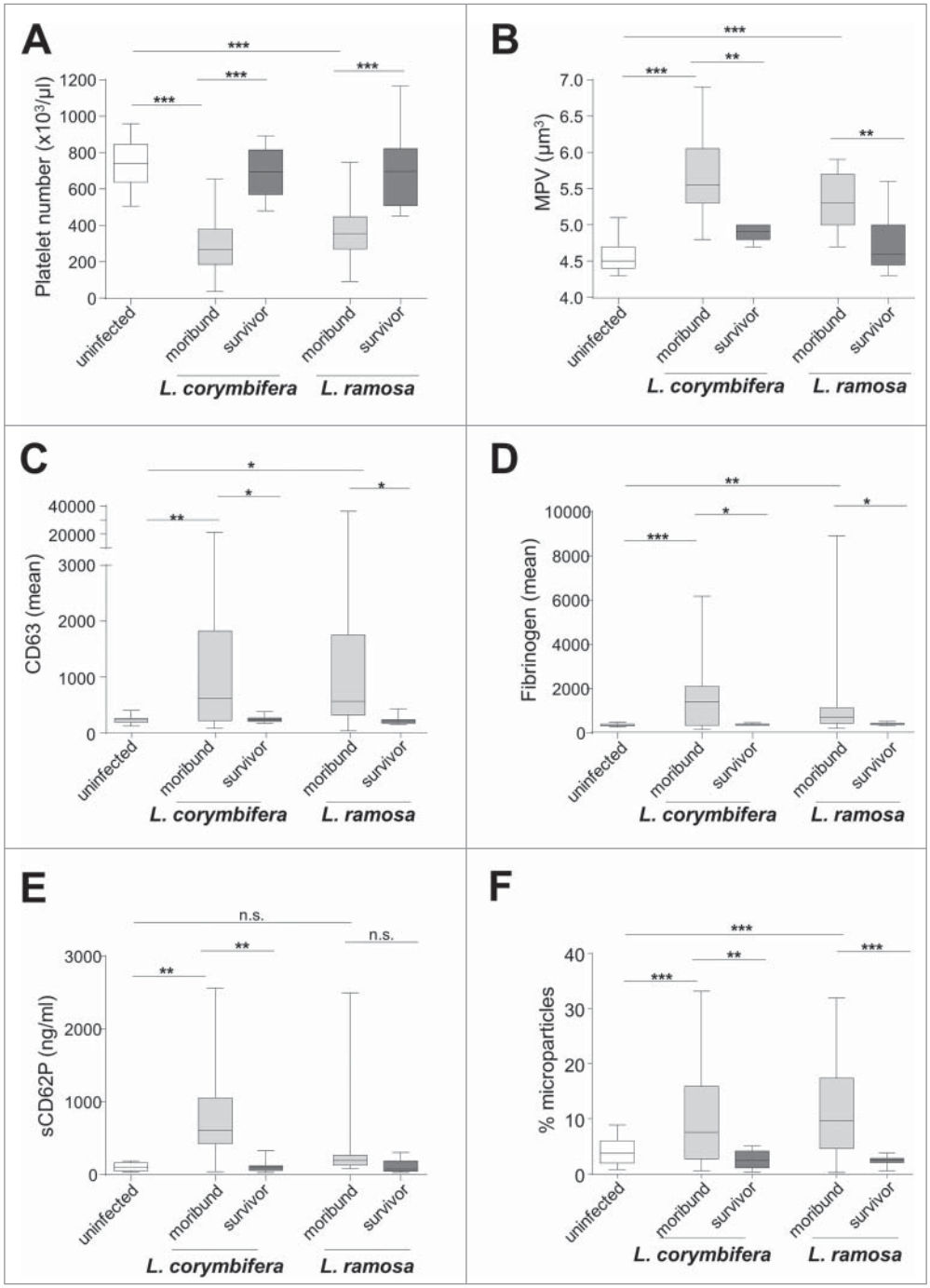

Figure 4. Platelet activation during *Lichtheimia* infection in corticosteroid-treated mice. (n = 6 mice/group/experiment). (A) Number of circulating platelets. (B) Mean platelet volume. (C) Surface expression of CD63 on circulating platelets. (D) Binding of fibrinogen to circulating platelets. (E) Quantification of soluble CD62P (sCD62P) in plasma. (F) Formation of platelet-derived microparticles circulating in the plasma. Statistical significance was calculated using 2-sided unpaired t-test, $^{*}p < 0.05$ and $^{**}p < 0.01$

epidemiological studies in humans, in which only a minority of mucormycosis patients were identified as immunocompetent[1,3-5] and these patients commonly had severe trauma as a predisposing factor,[30] which likely allowed introduction of spores directly into deeper tissues. In contrast to our results, intravenous infection of immunocompetent mice resulted in severe systemic infection.[31] However, this route of inoculation leads to direct dissemination and thus likely mimics traumatic inoculation rather than pulmonary exposure. Consistent with this hypothesis, mucormycosis induced by intravenous infection affected mainly brain and kidneys but rarely the lung, reflecting observations in human patients with mucormycosis following trauma.[1,3,4]

The pathological alterations that developed following intranasal challenge of corticosteroid-treated mice accurately mimicked clinical hallmarks of mucormycosis: vascular invasion associated with thrombosis and tissue infarction/necrosis.[20] Angioinvasion has been associated with the ability of the pathogen to hematogenously disseminate from the primary site of infection toward other organs,[20] which we also frequently observed following intranasal infection and establishment of pulmonary mucormycosis. Interestingly, we also observed strong systemic activation of platelets in mice with severe infection. The observation of necrotic areas in various organs in the absence of overt fungal growth might be the result of thrombotic ischemia, either mediated by general platelet activation or fungal growth in other parts of the organ. Whether platelets activation was a result of angioinvasion alone or whether specific fungal factors contribute to this phenomenon remains to be elucidated. It is however tempting to speculate that platelet activation with subsequent clotting and thrombosis as well as formation of pro-inflammatory factors such as sCD62P and microparticles contribute to pathogenesis and worsened outcome of infection. Overall, the clinical disease following intranasal infection of corticosteroid-treated mice accurately reflected the most common manifestation of mucormycosis in immunocompromised patients.

In our hands, around 50% of mice immunosuppressed with cortisone acetate reproducibly succumbed to infection with virulent *L. corymbifera* and *L. ramosa* strains, with some variation between experiments. It appears likely that more severe forms of immunosuppression, e.g., neutropenia or leucopenia, would further increase susceptibility as previously observed for pulmonary aspergillosis caused by *A. terreus*[19]; there, 100% mortality could be achieved in leukopenic, but not cortisone acetate-treated mice. This suggests that the residual host defense system might be sufficient to clear the infection in some cortisone acetate-treated mice. Importantly, some strain-specific virulence differences may depend on the type of immunosuppression used, as has been demonstrated for the role of gliotoxin in the pathogenesis of aspergillosis.[32] Thus, it should be noted that the results presented here based on corticosteroid-treatment are not necessarily transferable to other modes of immunosuppression.

While we did not expect development of clinical mucormycosis in immunocompetent mice, we were surprised that none of the DKA mice developed clinical symptoms. Diabetic ketoacidosis has been described as an important clinical risk factor for mucormycosis[1-4] and DKA mice readily succumb to infection with *R. arrhizus* (formerly *R. oryzae*),[12,17,33] (and data not shown). Diabetic ketoacidosis is associated with increased expression of the endothelial heat shock host receptor glucose-regulated protein 78 (GRP78).[26] *R. arrhizus* CotH binds to GRP78 thereby mediating endocytosis.[26,34,35] *R. arrhizus* CotH has been shown to be essential for *R. arrhizus* virulence in a ketoacidotic mouse model.[34] As CotH genes are also present in the sequenced *Lichtheimia* genomes,[36,37] it could be assumed that they likewise contribute to infection. However, their function and expression *in vivo* has not been analyzed so far and it appears possible that functional differences in the CotH genes explain why DKA mice are less susceptible to pulmonary *Lichtheimia* infection. Furthermore, differences in the interaction with innate immune cells could contribute to the differential susceptibility of DKA mice to *Rhizopus* and *Lichtheimia*: *L. corymbifera* has been shown to be more susceptible to killing by neutrophilic granulocytes,[38,39] and this may explain the resistance of DKA mice to intranasal *Lichtheimia* infection. Finally, strain-specific differences might contribute to these findings: We showed that *L. corymbifera* strains differ in their virulence potential in immunocompromised mice, and thus, our findings do not exclude that specific *Lichtheimia* strains are capable of inducing infections in ketoacidotic animals. Reinhardt *et al.* used ketotic rabbits and found that 13/13 *Rhizopus* strains but only 1/13 *L. corymbifera* (*Absidia corymbifera*) strains were pathogenic in this model.[40] Of note, treatment with cortisone acetate also led to elevated blood glucose (175 ± 37 mg/dL on day 0 compared with 134 ± 11 mg/dL in untreated control animals). However, glucose levels were significantly lower than in DKA mice at day -3 and at the end of the experiment (421 ± 138 mg/dL and 499 ± 158 mg/dL; $p < 0.0001$ compared with cortisone acetate treated mice, Mann-Whitney test) and did not exceed 240 mg/dL. It therefore is unlikely that cortisone acetate treated mice in our model developed ketoacidosis.

An important difference between models used previously by others and our study is the lack of additional immunosuppression by corticosteroids in this study. Thus, while our data shows that DKA alone is not sufficient to promote *Lichtheimia* mucormycosis, it yet appears possible that DKA affects susceptibility to *Lichtheimia* in hosts with underlying immune dysfunction. This still needs to be determined experimentally. Furthermore, mucormycosis in diabetic hosts typically manifests as rhinocerebral infection.[1,3,4] We did not observe any clinical symptoms suggestive of affection of sinuses or the brain, e. g. torticollis, in DKA mice in our study; however, this does not exclude subclinical manifestations that could progress to life-threatening disease in the presence of other risk factors such as immunosuppression.

It is tempting to speculate that the differences in risk factors also affect the relative prevalence of *Lichtheimia*

infections: In Europe, where *Lichtheimia* is a relatively common cause of mucormycosis, ketoacidosis was reported in less than 20% of the patients with mucormycosis.[3-5] In a global survey based on published data, ketoacidosis was present in 36% of the cases and *Lichtheimia* was less frequently isolated.[1] Furthermore, diabetic patients appear to be more frequently developed rhinocerebral mucormycosis, whereas pulmonary infections are more commonly associated with hematological malignancies, corticosteroid treatment or other forms of immunosuppression.[1,3,4] Studies linking risk factors, localization of mucormycosis and the fungal species involved are sparse; however, one study found that *R. arrhizus* was responsible for 85% of all rhinocerebral cases but only isolated from 17% of non-rhinocerebral forms.[3] This could imply that fungal-specific differences in the preferred route of entry and manifestation of mucormycosis exist. However, the available epidemiological data on mucormycosis is limited both regarding the number of studies, the number of cases included, and the methodology used in individual studies. Thus, no final conclusions can be drawn from the available data.

In summary, we established a murine pulmonary infection model which revealed that immunosuppression was essential for establishment of infection. The disease was characterized by necrosis, angioinvasion and thrombosis. Dissemination from the lung to other internal organs, especially the kidneys, was frequently observed. Although ketoacidosis induced by streptozotocin treatment promotes *Rhizopus* infection in mice, it did not predispose mice to infection with *L. corymbifera* or *L. ramosa*. This finding might partially explain the dominance of *Rhizopus* as causative agent of mucormycosis in countries with high prevalence of ketoacidotic patients. However, more comprehensive epidemiological studies are needed to elucidate whether associations between route of infection, distinct risk factors and the species causing mucormycosis do indeed exist.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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