Mucorin is a ricin-like toxin that is critical for the pathogenesis of mucormycosis

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Fungi of the order Mucorales cause mucormycosis, a lethal infection with an incompletely understood pathogenesis. We demonstrate that Mucorales fungi produce a toxin, which plays a central role in virulence. Polyclonal antibodies against this toxin inhibit its ability to damage human cells in vitro and prevent hypovolemic shock, organ necrosis and death in mice with mucormycosis. Inhibition of the toxin in *Rhizopus delemar* through RNA interference compromises the ability of the fungus to damage host cells and attenuates virulence in mice. This 17 kDa toxin has structural and functional features of the plant toxin ricin, including the ability to inhibit protein synthesis through its N-glycosylase activity, the existence of a motif that mediates vascular leak and a lectin sequence. Antibodies against the toxin inhibit *R. delemar*- or toxin-mediated vascular permeability in vitro and cross react with ricin. A monoclonal anti-ricin B chain antibody binds to the toxin and also inhibits its ability to cause vascular permeability. Therefore, we propose the name 'mucoricin' for this toxin. Not only is mucoricin important in the pathogenesis of mucormycosis but our data suggest that a ricin-like toxin is produced by organisms beyond the plant and bacterial kingdoms. Importantly, mucoricin should be a promising therapeutic target.

Mucormycosis is a lethal fungal infection that usually afflicts immunocompromised hosts, such as patients with diabetic ketoacidosis (DKA) or neutropenia, patients undergoing haematopoietic-cell or solid-organ transplants and patients receiving high-dose corticosteroids1–6. Immunocompetent patients with severe trauma are also at risk of contracting mucormycosis by direct inoculation of open wounds1–4. The overall mortality rate of mucormycosis is >40% and approaches 100% in patients with disseminated disease, persistent neutropenia or brain infection1,11. The two most common forms of the disease are rhino-orbital/cerebral and pulmonary mucormycosis. In both forms of the disease, infection is initiated by the inhalation of spores that germinate in the host to form hyphae, which are capable of invading host tissues while avoiding phagocytic killing4,6.

A characteristic feature of mucormycosis is the propensity of Mucorales to invade blood vessels, resulting in thrombosis and subsequent tissue necrosis4. The massive tissue necrosis associated with mucormycosis compromises the delivery of antifungal drugs to the infected foci, thereby necessitating radical surgical intervention to improve the outcome of therapy. We have previously determined that Mucorales fungi invade human umbilical vein endothelial cells (HUVECs) by expressing the fungal invasin CotH3, which interacts with the 78 kDa host receptor glucose regulated protein (GRP78). The interaction between CotH3 and GRP78 induces the endothelial cells to endocytose the fungi10–12. However, the mechanisms by which Mucorales damage host cells and cause necrosis are unknown.

While studying the capacity of *Rhizopus delemar*, the most common cause of mucormycosis, to damage HUVECs, we observed that killed hyphae of this organism and other Mucorales caused considerable damage to host cells13. This experimental finding and the clinical observation of the extensive tissue necrosis observed in patients with mucormycosis led us to speculate that a fungal-derived toxin may be involved in the pathogenesis of this disease.

Here we identify and characterize a hyphal-associated and secreted/shed toxin produced by Mucorales. This toxin damages host cells in vitro by inhibiting protein synthesis. The toxin is required for the pathogenesis of mucormycosis in mice, where it induces inflammation, haemorrhage and tissue damage resulting in apoptosis and necrosis. Suppression of toxin production in *R. delemar* by RNA interference (RNAi) attenuates virulence in DKA mice, and polyclonal anti-toxin antibodies (IgG anti-toxin) protect mice from mucormycosis by reducing tissue inflammation and damage. Thus, the toxin is a key virulence factor of Mucorales fungi and a promising therapeutic target. Because this toxin shares

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Results
Mucorales damage host cells through a hyphal-associated toxin.

We previously observed that *R. delemar* causes considerable damage to HUVECs within 8 h of infection. This organism also damages the A549 alveolar epithelial cell line and primary alveolar epithelial cells, but only after 30 h of incubation. *R. delemar*-mediated damage to both HUVECs and alveolar epithelial cells is associated with the formation of extensive hyphae, suggesting that the hyphal form of this organism produces one or more factors that damage host cells. We compared the extent of damage to A549 cells caused by live and heat-killed hyphae to investigate whether viability is required for *R. delemar* hyphae to damage host cells. We found that although heat-killed hyphae caused less damage to these cells than live hyphae, the extent of host cell damage was still significant (Extended Data Fig. 1b). These findings suggested that a hyphal-associated heat-stable toxin may be partially responsible for host cell damage. To explore this hypothesis, we compared the ability of aqueous extracts from dead *R. delemar* spores and/or hyphae to damage host cells. Extracts from either hyphae alone or a mixture of spores and hyphae damaged the A549 cells, whereas an extract from spores alone caused no detectable damage (Extended Data Fig. 1c). We also found that killed cells and pelleted hyphal debris from four different Mucorales fungi, but not the yeast

structural and functional features with ricin produced by the castor bean plant, *Ricinus communis*, we named it ‘mucoricin’.

Fig. 1 | The *R. delemar* toxin is sufficient to cause damage in vitro and in vivo. a, Effect of the *R. delemar* toxin on different cell lines (*n* = 7 wells per time point, pooled from three independent experiments). Statistical analyses were performed using the non-parametric Mann–Whitney (two-tailed) test to compare HUVECs with primary alveolar epithelial or A549 cells. b, Damage caused by the extracted and recombinant toxin (approximately 500 μg ml⁻¹ or 29.4 μM) to epithelial cells at different time points (*n* = 3 wells per time point). Data are representative of three independent experiments. c, Weight loss (c) and per cent survival (d) of mice following intravenous injection with 0.1 mg ml⁻¹ (5.9 μM) toxin every other day (×3; *n* = 3 mice per group). a–c, Data are the median ± interquartile range. e, H&E histomicrographs showing the effects of the toxin on mouse organs. Livers (top) showed necrosis (white arrow), infiltration and calcification of PMNs (black arrow) due to inflammation and a cluster of mononuclear cells (cyan arrow). Lungs (bottom) showed megakaryocytes (black arrow) and haemorrhage (yellow arrow). Data in each group are representative of two mice. Top: scale bars, 50 μm (leftmost image) and 100 μm (all other micrographs). Bottom: scale bars, 50 μm.
Candida albicans, caused substantial damage to HUVECs (Extended Data Fig. 1d). Collectively, these results suggest that Mucorales produce a hyphal-associated toxin that damages mammalian cells.

**Purification and activity of the toxin.** To purify the hyphal-associated toxin, R. delemar spores were cultured in a liquid medium for 4–7 d to generate a hyphal mat. The mat was ground in liquid nitrogen, extracted with sterile water, concentrated and analysed using size-exclusion chromatography. When the different fractions were analysed for their ability to damage A549 cells, activity was found in the fractions with molecular masses of 10–30 kDa (Extended Data Fig. 2a). The concentrated water extract was then subjected to three dimensional (3D) chromatographic fractionations, yielding a fraction that caused notable damage to A549 cells (Extended Data Fig. 2b–g). This fraction was further subjected to high-performance liquid chromatography (HPLC) using hydrophobic interaction chromatography. The purified sample was trypsinized and sequenced using liquid chromatography.
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with tandem mass spectrometry, which identified a 17 kDa protein (RO3G_06568).

The open reading frame encoding the 17 kDa protein is widely present in other Mucorales that we and others have previously sequenced16-19 and that are reported to cause disease in humans (Mucor, Cunninghamamella and Lichtheimia), animals (Mortierella) or plants (Choanephora cucbiturbarum). Orthologues were also found in the arbuscular mycorrhizal fungus Rhizophagus species as well as the bacterial genera Streptomyces and Paenibacillus (Supplementary Table 1). Given that orthologues were detected in other Mucorales known to cause mucormycosis, we examined the ability of unfraccionated hyphal extracts from various Mucorales fungi to damage A549 cells relative to that induced by hyphal extracts from the R. delemar 99-880 reference strain. Hyphal extracts from R. oryzae, another strain of R. delemar, Lichtheimia corymbifera and Cunninghamamella bertholletiae all caused substantial damage to A549 cells (Extended Data Fig. 1e).

We expressed the putative toxin gene for the 17 kDa protein in S. cerevisiae and used the purified recombinant toxin to raise polyclonal anti-toxin antibodies in rabbits. Although the IgG fraction of the antisera (IgG anti-toxin) had no effect on the growth or germination of R. delemar in vitro (Extended Data Fig. 3), it resulted in approximately 50–70% inhibition of the damage caused by heat-killed hyphae of several Mucorales to A549 cells (Extended Data Fig. 1f). From these findings we concluded that the putative toxin is responsible for host cell damage caused by most, if not all, members of the Mucorales fungi.

We also used quantitative PCR with reverse transcription (RT–qPCR) to study the expression of this open reading frame in R. delemar. In agreement with data showing a lack of toxin activity in spores (Extended Data Fig. 1c), there was minimal expression of this gene during the first 3 h of incubation (before germination13). Expression of this gene began to increase when the spores germinated at 4 h (ref. 13), peaked by 5 h and plateaued for at least 16 h of hyphal formation (Extended Data Fig. 4a). Protein expression in gernlings and hyphae, but not spores, was confirmed by immunostaining using the IgG anti-toxin (Extended Data Fig. 4b). The expression of the putative toxin gene was high in hyphae cultured under aerated conditions but not in hyphae cultured in the absence of aeration (Extended Data Fig. 4c). In addition, RNA expression was five- to tenfold higher following 2–5 h of co-culture with A549 cells compared with co-culture with HUVECs or human erythrocytes. (Extended Data Fig. 4d).

The toxin is capable of damaging host cells in vitro and in vivo. We compared the ability of the purified toxin to damage primary lung epithelial cells, A549 cells and HUVECs. After 1 h, the toxin caused notable damage to all of the host cells and especially to HUVECs (Fig. 1a). After 3 h, there was almost 100% damage to all host cells. We also compared the ability of the purified and recombinant toxins to damage A549 cells. Both caused substantial damage (Fig. 1b). Therefore, the purified and recombinant proteins damage A549 cells in a similar and time-dependent manner.

We next tested the activity of the toxin in vivo. Toxin purified from R. delemar was intravenously injected into mice every other day for a total of three doses and the mice were monitored for behavioural changes, weight loss and survival. We observed behaviour that is highly suggestive of sudden circulatory hypovolemic shock—including rapid and shallow breathing, weakness and cold skin—within 10–30 min following the injection of 0.1 mg ml⁻¹ (5.9 μM) purified toxin. The mice lost >25% of their original body weight (Fig. 1c); most of the mice eventually died. These events were similar to those observed in mice infected with live R. delemar spores (Fig. 1d). Finally, histopathology of organs collected from the mice showed pathological changes that included necrosis, haemorrhage and infiltration of the pulmonary interstitium by macrophages in the lungs. Liver changes included necrosis, clusters of mononuclear cells and the presence of megakaryocytes, polymorphonuclear cell (PMN) infiltration and tissue calcification indicative of uncontrolled inflammation, haemorrhage and necrosis (Fig. 1e). These data suggest that the toxin is sufficient to cause clinical symptoms often associated with disseminated mucormycosis.

RNAi knockdown and antibody-mediated neutralization of the toxin reduces the virulence of R. delemar in vitro and in vivo. To further confirm the role of this toxin in the pathogenesis of mucormycosis, we used RNAi20 to downregulate gene expression of the toxin. The extent of downregulation of the toxin was measured through RT–qPCR using toxin-specific primers and through western blotting or immunostaining of R. delemar with the IgG anti-toxin. This IgG anti-toxin specifically recognized the toxin by enzyme-linked immunosorbent assay (ELISA) and western blotting. RNAi knockdown of the toxin caused approximately 90% inhibition in gene expression (Extended Data Fig. 5a). Furthermore, RNAi knockdown resulted in a reduction in protein expression of >80% (Extended Data Fig. 5b) and negligible staining of toxin-RNAi R. delemar gernmlings compared with gernmlings of a control strain that had been transformed with an empty plasmid (Extended Data Fig. 5c). In agreement with the lack of effect on the growth and germination of R. delemar by the IgG anti-toxin, RNAi knockdown of the toxin had no effect on fungal germination or growth (Extended Data Fig. 6).

We next assessed the effect of downregulation of toxin expression on the ability of R. delemar to damage A549 cells. Damage to epithelial cells induced by R. delemar cells with RNAi targeting of the toxin gene was reduced by approximately 40% relative to both the wild-type strain and R. delemar transformed with the empty plasmid (Fig. 2a). Similarly, the IgG anti-toxin protected alveolar epithelial cells from wild-type R. delemar-induced injury by about 40% in vitro, whereas normal rabbit IgG did not (Fig. 2b).

Finally, we evaluated the effects of RNAi inhibition of toxin production on the virulence of R. delemar in our model of pulmonary...
mucormycosis. The DKA mice infected with R. delemar harbouring the empty plasmid had a median survival time of 6 d and 90% mortality by day 21 post intratracheal infection, whereas the mice infected with the toxin-attenuated expression strain had a median survival time of 21 d and mortality of 30% (Fig. 2c). The surviving mice had no residual fungal colonies in their lungs when the

### Table: Protein sequence homology

| NCBI sequence ID | Name                        | Length (amino acids) | Protein sequence homology | Vascular leak motif | Predicted molecular functions |
|------------------|-----------------------------|----------------------|---------------------------|-------------------|-------------------------------|
| NP_001310630.1   | Ricin precursor (R. communis) | 565                  | 26.6                      | 105               | Yes                           |
|                  |                             |                      |                            |                   | 87%                           |
|                  |                             |                      |                            |                   | 29%                           |
| EIE81863.1       | R. delemar toxin            | 147                  |                            |                   | Yes                           |
|                  |                             |                      |                            |                   | Yes                           |
|                  |                             |                      |                            |                   | Yes                           |
|                  |                             |                      |                            |                   | Yes                           |
|                  |                             |                      |                            |                   | Yes                           |
|                  |                             |                      |                            |                   | Yes                           |
|                  |                             |                      |                            |                   | Yes                           |

### Figure Panel:

**b**
- R. delemar toxin
- Ricin B chain (amino acids 304–437)
- Ricin B chain (amino acids 438–565)
- Superimposed (R. delemar toxin versus Ricin B chain (amino acids 304–437))
- Superimposed (R. delemar toxin versus Ricin B chain (amino acids 438–565))

**c**
- Detection antibody concentration (µg ml⁻¹) vs. Absorbance (OD450)
- IgG anti-toxin
- Normal rabbit IgG
- R. delemar toxin

**d**
- Ricin (µg per dot)
- Normal mouse IgG
- Anti-ricin toxin B chain (8A1)
- Normal rabbit IgG
- IgG anti-R. delemar toxin

**e**
- Marker
- R. delemar toxin
- Ricin

**f**
- Anti-A549 cell damage relative to ricin isotype-matched IgG (%)
- Detection antibody concentration (µg ml⁻¹)
experiment was terminated on day 21. Inhibition of toxin production seemed to have minimal effects on the early stages of infection because the fungal burdens of the lungs and brains (the primary and secondary target organs, respectively) of the mice after four days of infection with the R. delemar toxin-attenuated strain and R. delemar harbouring the empty plasmid were similar (Extended Data Fig. 7a). Reduced virulence without affecting the fungal burden in tissues has been reported for non-neutrophic mice infected with an Aspergillus fumigatus null mutant that does not produce the Asp f1 ribotoxin\(^2\), representing a classical feature of disease tolerance\(^3\). Collectively, our results indicate that although the toxin is dispensable for the initiation of mucormycosis, it plays a central role in the lethality of this disease.

IgG anti-toxin protects mice from mucormycosis. To further verify the role of the toxin in the pathogenesis of mucormycosis, we infected DKA mice intratracheally with wild-type R. delemar and then treated them with a single 30-μg dose of either the IgG anti-toxin or normal rabbit IgG 24 h later. Whereas mice treated with normal IgG had a mortality rate of 95%, treatment with the IgG anti-toxin resulted in a long-term survival of approximately 70% (Fig. 2d). The surviving mice seemed healthy and had no detectable fungal colonies in their lungs when the experiment was terminated on day 21. In agreement with the data on the fungal burden in the tissues of mice infected with the toxin-attenuated strain, the antibody treatment had no effect on the fungal burden in the lungs and brains that were harvested 4 d post infection (Extended Data Fig. 7b). These data further confirm the role of the toxin in the pathogenesis of mucormycosis and point to the potential of using anti-toxin antibodies to treat the disease.

We also performed histopathological examinations of the tissues of all groups of mice, sampled at the same time of the tissue fungal burden studies (day 4), to gain insight into the mechanism of action of the toxin. Whereas the uninfected mice had normal lung architecture with no signs of inflammation or infection (Fig. 2e), the lungs of the mice infected with R. delemar transformed with the RNAi empty plasmid (control) showed fungal and granulocyte infiltration (Fig. 2f, left) as well as angiogenesis with thrombosis (Fig. 2f, right). In contrast, the lungs of the mice infected with the toxin-attenuated mutant showed only mild signs of inflammation with no angiogenesis (Fig. 2g). Importantly, the lungs of the mice infected with wild-type R. delemar and treated with the IgG anti-toxin showed lung architecture similar to the lungs of the uninfected control mice; there were no signs of inflammation or infiltration with R. delemar (Fig. 2h).

Downregulation of the toxin gene and treatment with IgG anti-toxin attenuates R. delemar-mediated host cell damage in vivo. To determine whether the toxin contributed to host cell damage in vivo, we used an ApopTag in situ apoptosis kit to stain the lung tissues of the infected mice. Whereas extensive lung damage was observed in the mice infected with wild-type R. delemar, the lungs harvested from the mice infected with the toxin-attenuated mutant (Extended Data Fig. 8a) as well as those infected with wild-type R. delemar and treated with the IgG anti-toxin (Extended Data Fig. 8b) had almost no detectable damage.

Finally, we have previously reported on a mucormycosis case in a patient with disseminated mucormycosis\(^4\). Haematoxylin and eosin (H&E) staining of the tissues of this patient showed broad aseptate hyphae that caused necrosis and massive infiltration of tissues compared with thinner septated hyphae present in a patient suffering from invasive pulmonary aspergillosis (Extended Data Fig. 9a,b). Subsequent immunohistochemistry of the lungs of the patients using the IgG anti-toxin (versus control IgG) showed association of the toxin with the fungal hyphae and the surrounding tissues in the patient with mucormycosis and lack of staining in tissues of the patient with aspergillosis (Extended Data Fig. 9c,d). These results show that the toxin is also involved in human mucormycosis, is cell-associated as well as secreted/shed into the surrounding tissues, and confirm the specificity of the antibody used in these studies given that the putative toxin does not have an orthologue in Aspergillus (Supplementary Table 1).

To confirm the secretion/shedding of the toxin, we cultured R. delemar spores in a 96-well plate with or without aphorbinic B and assayed the cell-free supernatants for the presence of the toxin using a sandwich ELISA with an IgG anti-toxin monoclonal antibody that we raised in mice as a capture antibody and the rabbit IgG anti-toxin as the detecting antibody. The toxin was detected in the cell-free supernatants of wild-type R. delemar (26.7 ± 0.87 nM) and R. delemar transformed with the empty plasmid (23.0 ± 2.04 nM) but not in R. delemar transformed with RNAi targeting the toxin. In agreement with secretion/shedding of the toxin by live hyphae, supernatants collected from wild-type R. delemar hyphae in which further growth was hampered by aphorbinic B (100 nM) concentrations ≥2 μg ml\(^{-1}\) showed little to no secretion/shedding of the toxin (Extended Data Fig. 10). These results confirm that the toxin is secreted/shed into the growth medium.

The hyphae-associated toxin has the structural features of ricin. Given the critical role of the toxin in the pathogenesis of mucormycosis, we performed structural and bioinformatics studies to understand its mechanism of action. As reported in Supplementary Table 1, many of the toxin orthologues found in other organisms are
annotated as ricin domain-containing proteins or ricin B chain-like lectins. Further detailed bioinformatic analysis of the \( \text{R. delemar} \) toxin sequence showed a two-domain structure similar to that of ricin (Sequence ID: NP_001310630.1; ref. 24; Fig. 3a). Specifically, the \( \text{R. delemar} \) toxin harboured a small region (amino acids 198–289) that resembled a sequence in ricin chain A known to be involved in inactivating ribosomes (that is, ribosome-inactivating protein (RIP)) and two domains (amino acids 304–437 and 438–565) of the lectin-binding ricin chain B. Moreover, the \( \text{R. delemar} \) toxin contained an LDV motif (Fig. 3a), which is present in ricin (Fig. 3a, red.
The **R. delemar** toxin is immunologically crossreactive with ricin. To further explore the similarities between the **R. delemar** toxin and ricin, we used the IgG anti-**R. delemar** toxin in an ELISA to determine whether the toxin and ricin were immunologically crossreactive. Plates were coated with ricin or the **R. delemar** toxin and then incubated with the IgG anti-**R. delemar** toxin or normal rabbit IgG. The former, but not the latter, bound to ricin and the **R. delemar** toxin in a dose-dependent manner (Fig. 3c). The IgG anti-**R. delemar** toxin also recognized ricin, and a murine monoclonal antibody (8A1 clone) against the ricin B chain**37** recognized the **R. delemar** toxin in a dot blot (Fig. 3d). Furthermore, the IgG anti-**R. delemar** toxin reacted with both the **R. delemar** toxin and ricin in a western blot (Fig. 3e). Importantly, the IgG anti-**R. delemar** toxin protected A549 cells from ricin-induced damage in a manner similar to that of the IgG anti-ricin B chain (8A1 clone) and galactose (the lectin for the ricin B chain; Fig. 3f). Collectively, these data demonstrate the similarities between the two toxins.

**Mucoricin** is a **RIP** that also promotes vascular permeability and induces both necrosis and apoptosis of host cells. After ricin is internalized by cells through its lectin-binding B chain, the A chain exerts its toxic activity by irreversibly inactivating ribosomes via its N-glycosylase activity. This results in the inhibition of protein synthesis**61. The enzyme activity cleaves the N-glycosidic bond between the adenine nucleobase in the α-sarcin–ricin loop and its ribose causing the release of adenosine (depurination)**62. To determine whether the **R. delemar** toxin had similar activity, we compared the ability of the two toxins to inhibit protein synthesis in a cell-free rabbit reticulocyte lysate after adding aniline, albeit at a concentration that was 10^11 higher than that of ricin and after incubation with the ribosomes for a longer period of time (Fig. 4d). Thus, like ricin, the **R. delemar** toxin is a **RIP**.

In addition to its N-glycosylase activity, ricin chain A is known to cause vascular leak both in vitro**23,26** and in vivo**27, mediated by its LDV sequence, a motif that is also present in the **R. delemar** toxin (Fig. 3a). To examine whether the **R. delemar** toxin compromised vascular integrity, we cultured HUVECs on membrane inserts in transwells before treating the confluent monolayers with either **R. delemar** spores or **R. delemar** toxin for 5 h at concentrations that did not kill the HUVECs (Supplementary Fig. 1). The permeability of HUVECs was determined by measuring the amount of fluorescent dextran migrating from the upper to the lower chamber of the transwells after adding the **R. delemar** spores (Fig. 4e) or recombinant **R. delemar** toxin (Fig. 4f). Both **R. delemar** and the toxin induced permeability, which was equivalent to that induced by *Escherichia coli* lipopolysaccharide (LPS), a potent inducer of vascular permeability**63. Furthermore, IgG anti-**R. delemar** toxin blocked this enhanced permeability by 50–60%. Importantly, IgG anti-ricin B chain (8A1 clone) almost completely abrogated the permeability induced by **R. delemar** or its toxin (Fig. 4e,f). These results confirm that **R. delemar** induces permeability in HUVECs through its toxin and that the IgG anti-ricin B chain blocks this **R. delemar**-mediated virulence trait.

Ricin is also known to cause cell damage by inducing both necrosis and apoptosis**66–68. To determine whether the **R. delemar** toxin did the same, we used an Apoptosis/Necrosis detection kit to compare the abilities of **R. delemar** toxin and ricin to damage alveolar epithelial cells. After 2 h, both toxins caused similar levels of apoptosis and necrosis in comparison to the control (Fig. 4g,h). Collectively, these results demonstrate the functional similarities between ricin and the **R. delemar** toxin as RIPs that inhibit protein synthesis via N-glycosylase activity. Both toxins also cause cell death by apoptosis and necrosis. Based on the structural and functional similarity to ricin, we named the **R. delemar** toxin mucoricin and the corresponding gene Ricin-like toxin (RLT1).

**Discussion**

Mucormycosis is a lethal fungal infection often associated with extensive tissue damage. We have now identified a cell-associated/secreted/shed toxin that is widely present in pathogenic Mucorales fungi. We used genetic and biochemical techniques to show that the toxin gene RLT1 and its encoded protein (mucoricin) are required for the full virulence of **R. delemar**. In addition to being produced by pathogenic Mucorales, this toxin seems to be present in other fungi and bacteria. For example, orthologues with 30–40% identity to RLT1 were identified in *Rhizophagus*. *Rhizophagus* lives symbiotically with plants, is recognized as an integral part of the natural ecosystem and was shown to delay plant disease symptoms caused by *Phytophthora infestans***69*. Similarly, RLT1 orthologues were identified in the bacterial genera *Streptomyces* and *Paenibacillus* (approximately 30% identity). Both bacteria are known inhabitants of soil, present in rhizosphere of various plants and used as biological control agents for crops because of their ability to secrete secondary metabolites**70.

Mucoricin has structural and functional similarities to ricin, a prototypic Type II RIP consisting of two polypeptide chains (A and B) that are linked by a disulfide bond**24. The A chain of ricin is an N-glycosidase that is responsible for inactivating ribosomes, and the B chain is a galactose-specific lectin**28 that enables the toxin to bind to target cells**28. In contrast, Type I RIPs are monomeric A-chain-like RIPs**29. Mucoricin seems to have the activities of both ricin A and B chains (and Type I RIPs), and both activities are present in a single 17kDa protein (Fig. 3e). Several other RIPs isolated from plants consist of low-molecular-weight single-chain proteins, including a...
26 kDa TRIP isolated from tobacco leaves and a 7.8 kDa protein isolated from sponge gourd seeds (*Luffa cylindrica*).

The strong sequence and structural similarity between mucoricin and ricin lies in the ricin B chain, although mucoricin inhibits protein synthesis via N-glycosylase activity, leading to depurination, albeit with lower activity than ricin. A possible explanation for the ability of mucoricin to inhibit protein synthesis is probably predicted by its conserved rRNA glycosidase activity. Specifically, the EAARF motif (Fig. 3a, green brackets) in the ricin A chain is known to be responsible for the RIP activity of ricin. It is known to depurinate adenosine 4324 in 28S rRNA with the glutamic acid residue (E) responsible for this activity. Furthermore, the arginine residue (R), which is separated by two amino acids from the glutamic acid residue, is also required for the activity of the bacterial Shiga toxin, a potent ricin-like A chain with a fully conserved EAARF domain of ricin. Mucoricin has the EEGRL motif, in which the glutamic acid and arginine residues are conserved (Fig. 3a, green brackets).

Another functional domain in ricin and Shiga toxin reported to be required for RIP activity is WGRLS (Fig. 3a, cyan underline). This sequence also aligns with the EEGRL motif of mucoricin (Fig. 3a, green brackets and Supplementary Table 3). Furthermore, mucoricin contains the EAANQ motif (Fig. 3a, purple overline), which resembles the ricin sequence EAARF, with the glutamic acid residue conserved and the arginine residue replaced by asparagine, a conserved amino acid with properties that are weakly similar to arginine. The lack of fully conserved functional residues (that is, arginine) between mucoricin and ricin/Shiga toxins probably explains the 800-fold-weaker RIP activity of mucoricin in comparison to ricin (Fig. 4a,b). Finally, mucoricin also has sequence homology with several other RIPs, including saporin of *Saponaria officinalis*, a Type I RIP with a 19% overall identity and 10 of the 17 conserved amino acid residues in the functional domain of EAARF (Supplementary Fig. 2). Notably, the EEGRL and EAANQ motifs are widely present in Mucorales fungi known to cause human disease (Supplementary Table 3). Thus, mucoricin seems to be a RIP that functionally resembles the ricin A chain and Type I RIPs such as saporin. The contribution of the EEGRL and EAANQ motifs to the RIP activity is being further investigated.

Our in vitro studies suggest that *RLT1* is expressed most strongly when the hyphal mat is aerated and in response to alveolar epithelial cells (Extended Data Fig. 4c,d). These results further suggest that mucoricin may be highly active during pulmonary mucormycosis and potentially in rhino-orbital disease, when hyphae are exposed to epithelial cells in the presence of ambient levels of oxygen. It is of interest that mitochondria are believed to play a central role in the ability of RIPs (for example, ricin, Shiga toxin and abrin) to induce cell apoptosis. Although the expression of *RLT1* in *R. delemar* is higher in response to alveolar epithelial cells than to HUVECs (Extended Data Fig. 4d), the latter host cells are damaged much more rapidly by R. *delemar* (that is, considerable R. *delemar*-induced injury to HUVECs occurs at 8 h versus 48 h for alveolar epithelial cells; Extended Data Fig. 1), and by purified mucoricin (Fig. 1a). In agreement with these results, it has been shown that HUVECs are rapidly damaged and their permeability is affected by small peptides containing the LDV motif but lacking the sequences responsible for N-glycosidase activity. In this study we show that R. *delemar* compromises the permeability of HUVEC monolayers in vitro through the direct effect of mucoricin. The LDV and other (x)D(y) motifs (with known vascular leak effector function) are widely present in pathogenic Mucorales (Supplementary Table 3). Thus, it is possible that the LDV motif is responsible for angioinvasion and rapid haematogenous dissemination in mucormycosis by inducing damage to vascular endothelial cells.

The exact mechanism by which mucoricin enters a target cell to exert its lethal effect is not yet known. However, our data strongly indicate that it is cell-associated as well as secreted/shed by Mucorales. The amount of toxin in the medium (27 nM; Extended Data Fig. 10b) from a small-scale culture in a 96-well plate was sufficient to exceed the IC₅₀ in RIP activity of 17 nM (Fig. 4b). Thus, the secreted/shed toxin probably exerts its toxicity by binding to and then entering the host cells in the absence of invading hyphae. Alternatively, invading *R. delemar* hyphae release the toxin once they are phagocyted by immune or barrier cells.

Our in vivo studies clearly demonstrate the contribution of mucoricin to pathogenesis by enhancing angioinvasion, inflammation and tissue destruction. There is also evidence that the lethality of ricin in vivo is related at least in part to its ability to induce a massive inflammatory immune response accompanied by infiltration of PMNs in many settings, such as acute lung injury and gastro-intestinal disease. This is probably due to activation of the innate arm of the immune system by the toxin itself or by toxin–damaged cells. Our histopathological examination of the organs harvested from the mice injected with purified mucoricin detected inflammation and recruitment of PMNs (Fig. 1e). Neutralization of the effect of the toxin by either RNAi or anti-mucoricin antibodies decreased inflammation and host tissue damage (Fig. 2e–h and Extended Data Fig. 8). These results confirm the critical role of mucoricin in the pathogenesis of mucormycosis and suggest that it is involved in mediating inflammation and tissue damage, both of which are clinical features of mucormycosis. Notably, the treatment of patients with mucormycosis with antifungal agents is often hampered by the extensive tissue necrosis that prevents optimal delivery of drugs into the site of infection. Antifungal treatment alone (without surgical intervention) is hence often non-curative. Thus, antibody-mediated neutralization of mucoricin might reduce tissue necrosis, decrease the need for disfiguring surgery and maximize the effect of antifungal therapy.

Based on these results, we propose a model of pathogenesis and the role of mucoricin in this process. We suggest the following events.

1. Infection is initiated when fungal spores are inhaled and in the absence of phagocytes (or the presence of dysfunctional phagocytes, such as in patients with DKA). Fungal spores express CotH and bind to either GRP78 on nasal epithelial cells or to integrin β1 (ref. 39), which activates epidermal growth factor receptor on alveolar epithelial cells to induce invasion.

2. Under aerobic conditions, the calcineurin pathway is activated in the inhaled spores, causing them to germinate, a process that leads to the production of mucoricin.

3. Mucoricin binds to tissue cells through its lectin receptor, inhibits protein synthesis, and causes apoptosis and necrosis. The toxin can also compromise vascular permeability, resulting in rapid haematogenous dissemination and tissue oedema often seen in patients with mucormycosis.

4. While tissue damage is occurring—and because the toxin and debris from necrotic cells are recognized by the immune system—an inflammatory immune response leads to the recruitment of PMNs and other tissue-resident phagocytes.

5. Although the recruited phagocytes damage some of the invading hyphae, both the dead and live hyphae release mucoricin, resulting in more host cell death and more inflammation.

6. In the necrotic tissue, the fungus can proliferate, protected from both phagocytes and antifungal drugs.

Our finding that mucoricin remains active even in dead organisms offers an explanation for why antifungal therapy alone has limited efficacy in patients with mucormycosis and why the fun-gal lesions must frequently be surgically excised. Importantly, other toxins/mechanisms of host cell damage probably exist in Mucorales, given that antibody-blocking studies and downregulation of toxin gene expression do not fully abrogate the ability of *R. delemar* to damage host tissues.
In summary, we have identified a ricin-like toxin (mucoricin) that is widely present in Mucorales fungi, where it plays a central role in the pathogenesis of mucormycosis. We postulate that strategies to neutralize mucoricin will have important therapeutic benefits.

Methods

Organisms, culture conditions and reagents. R. delemar 99–880 and R. oryzae 99–892 were isolated from the brain and lungs of patients with mucormycosis and obtained from the Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio, which had the genomes sequenced18. C. herbthellettiae 182 is a clinical isolate and was a gift from T. Walsh (Cornell University). L. corymbifera is also a clinical isolate obtained from the DEFEAT Mucor clinical study24. R. delemar M16 is a pyrF null mutant derived from R. delemar 99–880 and was used for transformation to attenuate mucoricin expression23. The organisms were cultured on potato dextrose agar (PDA) or potato dextrose plates for 5–7 d at 37 °C. For R. delemar M16, the PDA was supplemented with 100 mg ml⁻¹ uracil. The spore suspensions were collected in endotoxin-free Dulbecco’s PBS containing 0.01% Tween 80, washed with PBS and counted with a haemocytometer to prepare the final inoculum. To form grommels, spores were incubated in yeast extract peptone dextrose (YPD; Becton Dickinson) medium at 37 °C with shaking for different time periods. Finally, for the growth studies, 1 × 10⁸ spores of the R. delemar wild-type or the mutant strains were plated in the middle of PDA agar plates. The plates were incubated at 37 °C and the diameter of the colony was calculated every day for 6 d. The monoclonal anti-ricin B chain antibody (clone 8A1)27,30 and affinity-purified rabbit anti-ricin antibodies were prepared and characterized in the Vitetta and Mantis laboratories. Galactose was obtained from Fisher Scientific (cat. no. BP656500) and used to block the damaging effect of ricin holotoxin.

Host cells. Human alveolar epithelial cells (A549 cells) were obtained from a 58-year-old male Caucasian patient with carcinoma and procured from the American Type Culture Collection (ATCC). The cells were propagated in F12K Medium developed for lung A549 epithelial cells. Primary alveolar epithelial cells were obtained from ScienCell (cat. no. 3200), propagated in Alveolar Epithelial Cell medium (cat. no. 3201) and passaged once.

HUVECs were collected using the method of Jaffe and colleagues8. The cells were harvested using collagenase and cultured in M-199 medium (Gibco) for 5–7 d at 37 °C. The reagents were tested for endotoxin using a chromogenic Limulus amoebocyte lysate assay (BioWhittaker, Inc.), and the endotoxin concentrations were less than 0.01 IU ml⁻¹.

Fresh red blood cells were isolated from blood samples collected from healthy volunteers after obtaining a signed informed consent form and processed as previously described17. The endothelial and red-blood-cell collection was approved by the Institutional Review Board at The Lundquist Institute at Harbor-UCLA Medical Center.

Purification and characterization of ricin. Two sources of ricin were used. One was purified from a large stock of pulverized castor beans in the Vitetta Medical Center. Limulus amebocyte lysates (LAL) were obtained from Fisher Scientific and used to determine the endotoxin levels. The endotoxin levels were measured using a Limulus amebocyte lysate kit (Charles River) and determined to be <0.8 endotoxin units (EU) ml⁻¹, which is below the 5 EU kg⁻¹ body weight set for intraperitoneal injection17.

Mouse monoclonal antibody clone 8A1 was raised against the ricin B chain27, which has 33% sequence identity to mucoricin. Clone 8A1 recognizes an epitope mapped to ricin B chain (2γ; amino acids 221–262).

Cell damage assay. The damage to epithelial cells (A549 and primary (ScienCell, cat. no. 3200)) and HUVECs was quantified using a 14C release assay. Briefly, confluent cells cultured in 24-well tissue culture plates were incubated with 1 μCi Na14CO3 (ICN) per well in either F12K (epithelial cells) or M-199 (HUVECs) medium for 16 h. On the day of the experiment, the medium was aspirated and the cells were washed twice with pre-warmed Hank’s balanced salt solution (ScienCell). The cells were treated with toxin suspended in either 1 ml F12K (epithelial cells) or RPMI 1640 (endothelial cells) medium supplemented with glutamine and incubated at 37 °C in 5% CO2 incubator. The medium was changed every 2 d. At different time points, and after the data were corrected for variations in the amount of tracer incorporated in each well, the percentage of specific cell release of 14C was calculated as follows: (experimental release) – (spontaneous release) / (1 – (spontaneous release)) × 100. Each experimental
condition was tested at least in triplicate and the experiment was repeated at least once.

In some experiments, the effect of mucoricin gene silencing on HUVESCs or A549 cell damage was measured by incubating the cells with *R. delemar* spores (1.0 × 10^10 or 2.5 × 10^10 spores ml^-1) for 6 or 48 h, respectively. In other experiments, the protective effect of IgG anti-mucoricin was measured by incubating the fungal cells with either 50 μg ml^-1 IgG anti-mucoricin or normal rabbit IgG (R&D Systems, cat. no. AB-105-C) for 1 h on ice before adding the mixture to A549 cells radiolabelled with 35S-Cl. The assay was carried out for 48 h. The cell damage was quantified as described earlier.

To study the effect of fungal cell viability on host cell damage, fungal spores (1 × 10^10 ml^-1) were cultured in F12K medium and left to grow overnight at 37°C. The fungal hyphae were collected by filtration, dried by passing with a sterile filter paper, weighed and then aseptically cut into four equal small pieces with a wet needle. The fungal hyphal mass was resuspended in 2 ml 1×F12K, heated to 60°C in a water bath for 4 h and then cooled down. A loop full of the hyphal mass was plated on PDA plates to check the viability of the hyphal mass. The other two groups of fungal hyphae were suspended in preheated and cooled F12K culture medium. F12K culture medium, heated to 60°C and then cooled, was prepared to preserve the fungal growth control. The fungal samples were incubated with Cr-labelled A549 cells previously seeded into 24-well plates as described earlier, the damage assay was carried out for 24 h at 37°C and the amount of Cr released in the supernatant was measured as above.

To determine whether IgG anti-mucoricin protected cells against ricin-damaged cells, mucoricin (1 μg ml^-1) (about 77 nM) ricin was incubated with 10 μg ml^-1 of the monoclonal IgG anti-rinic B chain (clone 8A1)68, IgG anti-mucoricin, normal rabbit IgG (R&D Systems, cat. no. AB-105-C) or 10 mM galactose on ice for 1 h before being added to Cr-labelled confluent A549 cells in a 24-well plate. The damage assay was conducted as described earlier for 24 h.

Western blotting. Hyphal expression of mucoricin was determined in *R. delemar* wild-type or RNAi mutants from a hyphal matt cultured overnight at 37°C in YNB medium without uracil. Briefly, mycelia were collected by filtration, washed briefly with PBS and then thoroughly ground in liquid nitrogen for 3 min using a mortar and pestle. The ground powder was immediately transferred to a microtube containing 500 μl extraction buffer, which consisted of 50 mM Tris–HCl, pH 7.5, 150 mM NaCl and 10 mM MgCl2. The extraction buffer was supplemented with 1×Halt protease inhibitor cocktail (Thermo Scientific) and 1 mM phenylmethylsulfonyl fluoride. The sample was vortexed vigorously for 1 min and then centrifuged at 21,000 × g for 5 min at 4°C. The supernatant was filtered using FCS syringe filters (Bioland Scientific, cat. no. SF01-02), transferred to a new tube and the protein concentration was determined using the Bradford method.

For western blotting, 10 μg of each sample was used to separate proteins using SDS–PAGE. The separated proteins were transferred to PVDF membranes (GE Water & Process Technologies) and treated with Western blocking reagent (Roche). Western blotting was performed using 0.1% gelatin, washed with Tris–HCl 1×, and the membranes were blocked for 1 h in 1×PBS, pH 7.5. The membranes were incubated with the primary antibody for 1 h and then washed with 1×PBS. The membranes were incubated with HRP–IgG anti-rabbit IgG (Jackson ImmunoResearch, cat. no. 715-035-150) or HRP–IgG anti-mouse (Invitrogen, cat. no. 31450; when murine 8A1 antibody was used) horseradish peroxidase (HRP)–IgG anti-rabbit IgG (Jackson ImmunoResearch, cat. no. 715-035-150) or HRP–IgG antirabbit IgG (Jackson ImmunoResearch, cat. no. 715-035-150). The membranes were washed with 1×PBS, pH 7.5, and then visualized by the chemiluminescence detection Kit (GE Healthcare Life Technologies). The bands were visualized using the ImageJ software.

In vitro apoptosis/necrosis assay. A549 cells were cultured to confluence on fibronectin-coated circular glass coverslips in 24-well tissue culture plates and then incubated with 50 μg ml^-1 (2.9 μM) mucoricin or 5 μg ml^-1 (77 nM) ricin (concentrations found to cause in vitro damage to alveolar epithelial cells) for 2 h, after which the cells were washed and stained with 1×Apoxin green indicator and 1×7-AAD (Apoptosis/necrosis detection kit, Abcam) for 45 min. The cells were fixed and mounted in ProLong Gold antifade containing 4,6-diamidino-2-phenylindole (Life Technologies) and visualized with the confocal microscope. The apoptotic and necrotic cells were determined by their green and red fluorescence, respectively. The number of apoptotic and necrotic events per HPF was determined; ten HPFs were counted per coverslip. The experiment was performed three times in triplicate.

In vitro protein translation assay. The ability of the two toxins to inhibit protein synthesis was measured using a previously described method. Briefly, a rabbit reticulocyte lysate (Promega, cat. no. L4151) was thawed at 37°C immediately before use and supplemented with 40 μl of 1 mM haemin stock solution, 10 μl of 1 mM creatine phosphate (Sigma-Aldrich, cat. no. 27920) and 10 μl of 5 mM γ-creatinine phosphokinase (Sigma-Aldrich, cat. no. C7886) before the lysate had fully thawed. The reaction mixture was prepared in 96-well plates as follows: 1 μl of 1 mM amino acid mixture minus methionine (Promega, cat. no. L9691), 35 μl rabbit reticulocyte lysate and 1 μl of sevenfold diluted ricin, control OVA or cycloheximide (Fisher Scientific, cat. no. AC575420100). Diluted distillate water was added to a final volume of 48 μl. Two replicates were employed in all experiments and the experiments were repeated at least three times. After a pre-incubation period of 30 min at 37°C, 2 μl 5′-S methionine (1,200 Cimol⋅μl^-1, PerkinElmer) was added to a final volume of 50 μl. The 96-well plate was incubated at 30°C for 60 min; 0.2 μl from each well was added per well of a 24-well plate containing 98 μl of 0.5 M H2O. The proteins were precipitated with 900 μl of 25% trichloroacetic acid before harvesting the precipitates on Whatman filter strips (Sigma-Aldrich, cat. no. WHA1828053). The filter paper discs were placed in Biofluor scintillation fluid (PerkinElmer, cat. no. 6013329) and the 5′S methionine incorporation was quantitated by scintillation counting. Background (a small amount determined from a well containing all of the reagents except rabbit reticulocyte lysate) was subtracted from all counts per minute.

Assay for the depurination activity of mucoricin. The depurination activity of mucoricin was measured by the release of adenine when mammalian RNA was treated with mucoricin for 24 h at 37°C (ref. 1). Mammalian RNA extracted from A549 cells, using QIAGEN RNaseasy mini kits according to manufacturer’s instructions, was treated with 20 μg ml^-1 mucoricin in 0.1 M HEPES and 10 mM ammonium acetate buffer containing 1 mg ml^-1 BSA for 24 h at 37°C. The solution was then filtered through a 10-kDa size-exclusion column and 40 μl was injected for HPLC using a Phenomenex Luna C18 reverse phase column (10×250 mm) attached to a Varian ProStar HPLC 212 system (Varian). Solvent A was 20 mM ammonium acetate and solvent B was 100% acetonitrile. The column gradient was as follows: 97 to 60% solvent A in 10 min at a flow rate of 1 ml min^-1. The column effluent was monitored at 260 nm.

Glycosylase activity assay. The N-glycosylase activity of the toxins was determined using rabbit reticulocyte lysate. Briefly, 40 μl lysate was incubated with ricin (1 nM), mucoricin (10 μM) or control OVA (1 nM or 10 μM) in the presence of 10 mM MgCl2, at 30°C for 1 or 4 h. After the treatment, the ribosomal protein was denatured using 50 mM Tris and 0.5% SDS to release RNA. The RNA was purified through phenol–Tris extraction, followed by ethanol precipitation. Half of each purification sample was incubated with 20 μg ml^-1 of glycosylase 2 (ref. 4) for 10 min on ice, and the other half was incubated without aniline treatment. The RNA were further extracted using water-saturated ether, followed by ethanol precipitation. The RNA samples (3 μg each) were resolved through 7 M urea polyacrylamide gel electrophoresis and the RNA fragment bands were visualized by staining with ethidium bromide.
Transwell permeability assay. HUVECs were seeded on a 24-transwell plate (Corning) with permeable polyester inserts (0.4 μm; Fisher) coated with fibronectin (15 μg/ml in PBS, Fisher). The HUVECs were cultured to confluency in M-199 medium with phenol red. R. delamar spores (1 × 10⁵) in M-199 (without phenol red) were added to the upper chamber and the plate was incubated for 5 h at 37 °C. As a positive control for the permeability of HUVECs, 2 μg/ml E. coli LPS (Sigma-Aldrich) was added to uninfected HUVECs. Following incubation, 3 μl of 50 mg/ml FITC-dextran-10K (Sigma) was added to the upper chamber of the transwell and the migration of the dextran through the HUVEC monolayer to the lower transwell was determined 1 h later by quantifying the concentration of the dye in the bottom chamber using a fluorescence microplate reader at 490 nm (ref. 19). To determine the direct effect of mucorin on the permeability of the HUVEC monolayer, 30 pg/ml (2.9 μM) mucorin or control OVA were added to the HUVEC-seeded wells instead of R. delamar. To determine the effect of antibiotics on mucorin-induced cell death by R. delamar, normal rabbit IgG (R&D Systems, cat. no. AB-105-C) or IgG anti-mucoricin, or 10 μg/ml IgG anti-toxin ricin chain B (clone 8QA1) were incubated for 30 min on ice with R. delamar spores or mucorin before their addition to the upper chamber of the transwell.

In vivo effects induced by mucorin. To test the effect of the purified toxin in vivo, male (ICR mice, approximately 27–32 g) were immunosuppressed by injection of 250 mg/kg cortisone acetate on days −2 and +3 relative to the toxin injection. This regimen results in approximately 10 d of leukopenia with a reduction in neutrophils, lymphocytes and monocytes as described previously75. The mouse gender has no effect on the pathogenesis of mucormycosis or antifungal treatment, as determined by NIH contract no. HHSN272201000388/Task order HHSN22720008 (unpublished data). The mice were given irradiated food and sterile water containing 50 μg/ml Baytril (Bayer) ad libitum. Purified mucorin (100 μl) was injected into mice intraperitoneally. R. delemar 2 × 10⁶ spores or mucoricin before their addition to the upper chamber of the transwell. To determine the effect of the purified toxin to protect against Rhizopus infection was also evaluated in the DKA mouse model. Briefly, DKA mice were infected with R. delamar 99–880 as above (average inhaled inoculum of 5.6 × 10⁷ spores from two experiments) and injected intraperitoneally 24 h later with 30 μg IgG anti-toxin or normal rabbit IgG (R&D Systems, cat. no. AB-105-C). The survival of the mice and fungal burden of the target organs collected on day +4 post infection served as the endpoints as above. Furthermore, histopathological examination was carried out on sections of the organs harvested on day +4 post infection. These organs were fixed in 10% zinc formalin and processed as above for histological examination with H&E, Periodic acid–Schiff or Grocott staining.

Histological analysis of the lung tissue was performed using light microscopy and histopathological examination. The fixed organs were dehydrated in graded alcohol (100–70%), followed by incubation of the slides in water and heat-induced antigen retrieval in sodium citrate buffer (10 mM; pH 6). The sections were blocked with 3% BSA in PBS, incubated for 1 h with a 1:50 dilution of the IgG anti-mucoricin, or 10 μg/ml IgG anti-toxin ricin chain B (clone 8QA1) and stained with 100 μg/ml H&E76. Cumulative histopathology scores of haemorrhages, neutrophil infiltration (inflammation) and oedema were used to determine the effects of toxin by optical sections taken through the z axis.

In vivo virulence studies and immunohistochemistry. A single intraperitoneal injection of 210 mg/kg streptozotocin in 0.2 ml citrate buffer was used to induce DKA in male ICR mice (≥20 g) 10 d before fungal challenge. On days −2 and +3 relative to infection, the mice were given a dose of cortisone acetate (250 μg/ml). The DKA mice were given irradiated food and sterile water containing 50 μg/ml Baytril (Bayer) ad libitum. The DKA mice were infected intratracheally with fungal spores with a target inoculum of 2.5 × 10⁷ spores of RNAi empty plasmid (RNAi-empty plasmid) or RNAi-mucoricin. To confirm the fidelity of the inoculum, three mice were killed immediately after inoculation, their lungs were homogenized in PBS and quantitatively cultured on PDA plates containing 0.1% Triton X-100, and colonies were counted after a 24-h incubation at 37 °C. The average inhaled inoculum for the RNAi-empty plasmid group and RNAi-mucoricin was 6.8 × 10⁵ and 2.9 × 10⁵ spores respectively, 2 × 10⁶ spores in two experiments, respectively. The primary endpoint was the time to morbidity, analysed using Kaplan–Meier plots. In another experiment, DKA mice were infected as above and then killed on day +4 relative to infection, when their lungs and brains (primary and secondary target organs) were collected and processed for the determination of the fungal burden in tissue using quantitative PCR. The ability of the IgG anti-toxin to protect against Rhizopus infection was also evaluated in the DKA mouse model. Briefly, DKA mice were infected with R. delamar 99–880 as above (average inhaled inoculum of 5.6 × 10⁷ spores from two experiments) and injected intraperitoneally 24 h later with 30 μg IgG anti-toxin or normal rabbit IgG (R&D Systems, cat. no. AB-105-C). The survival of the mice and fungal burden of the target organs collected on day +4 post infection served as the endpoints as above. Furthermore, histopathological examination was carried out on sections of the organs harvested on day +4 post infection. These organs were fixed in 10% zinc formalin and processed as above for histological examination with H&E, Periodic acid–Schiff or Grocott staining.

Confocal microscopy. IgG anti-toxin was used to localize the toxin in the Rhizopus fungus10. Fungal spores (1 × 10⁶ spores/ml) were germinated in PPDY medium at 1, 4 or 12 h. Each fungal stage was fixed in 4% paraformaldehyde, followed by permeabilization with Triton X-100 in 0.1% Triton X-100. The permeabilized fungal growth stages were incubated with the IgG anti-toxin for 2 h at room temperature. The fungal stages were then washed three times with Tris-buffered saline (0.1 M Tris–HCl, pH 7.4 and 0.15 M NaCl) containing 0.05% Tween 20 and counterstained with anti-rabbit IgG Alexa Fluor 488 (Life Technologies, cat. no. A-11034). The stained fungal spores were imaged using the Leica confocal microscope at an excitation wavelength of 488 nm. The final confocal images were produced by combining the optical sections taken through the z axis.

Immunofluorescence staining for mucorin in human tissue samples. Paraffin-embedded human lung tissue from a patient diagnosed with disseminated mucormycosis10 and a patient with proven invasive pulmonary aspergillosis were cut into 5-μm sections, which were then mounted on glass slides. The organ sections on the slides were deparaffinized and rehydrated with an ethanol gradient (100–70%), followed by incubation of the slides in water and heat-treated antigen retrieval in sodium citrate buffer (10 mM pH 6). The sections were blocked with 3% BSA and incubated with a 1:50 dilution of the appropriate goat anti-rabbit IgG Alexa Fluor 488 (Life Technologies, cat. no. A-11034) in 1× PBS, followed by DNA staining with 1 μM TOPRO-3 iodide (642/661; Invitrogen) and staining of the fungal hyphae with 100 μg/ml 4′,6-diamidino-2-phenylindole (Molecular Probes). After incubation with a laser scanning spectral confocal microscope (TCS SP8; Leica), LCS Light software (Leica) and a ×40 Apochromat 1.25 numerical aperture objective using identical gain settings. A low fluorescence immersion oil (Leica, 11513859) was used and imaging was performed at room temperature. Serial confocal sections at 0.5-μm steps with a z-stack spanning a total thickness of 10–12μm of tissue and 3D images were generated using the LCS Light software. Corresponding tissue sections from the same area were also stained with H&E.

 Statistical analysis. The data were collected, graphed and statistically analysed using Microsoft Office 360 and GraphPad Prism 8.0 for Windows or Mac.
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Author contributions
S.S.M.S. conceived, designed and performed studies to purify and identify the toxin, screen its activity both in vitro and in vivo, and wrote the manuscript. C.B. generated mucoricin mutants and characterized their virulence in vitro and in vivo, and conducted the antibody efficacy studies. Y.G.S. assisted with the animal studies, conducted confocal microscopy, crossreactivity studies and RIP activity studies. S.S. designed and performed the homology modelling, crossreactivity studies and toxin secretion studies. T.G. helped in the animal studies. M.S. performed the necrosis/apoptosis assay and the mouse immunohistochemistry studies. A.A. performed the permeability studies, E.G.Y. performed the sequence alignment and gene ontology studies. S.A. purified recombinant toxin and polyclonal antibodies. A.F. and G.C. provided and performed the human immunohistochemistry studies. C.P. and V.V. performed and interpreted the mouse histology studies. A.R. carried out studies on the crossreactivity of mucoricin and ricin. V.M.B. and J.D.H. performed the phylogenetic studies and BLAST search of mucoricin in Mucorales. N.J.M. generated and characterized the 8A1 monoclonal antibody. J.E.E. Jr and S.G.F. provided intellectual advice, designed studies and edited the manuscript. V.M.B. and J.D.H. provided reagents and expertise on ricin, and helped write the manuscript. A.S.I. conceived, designed, coordinated and supervised the studies, performed experiments, analysed data and wrote the manuscript with comments from the co-authors.

Competing interests
A.S.I. owns shares in Vitalex Biosciences, a start-up company that is developing immunotheapies and diagnostics for mucormycosis. The Lundquist Institute has filed intellectual property rights concerning mucoricin (US patent application no. 16/462,511). Vitalex Biosciences has an option to license the technology from The Lundquist Institute for Biomedical Innovation. The remaining authors declare no competing interests.

Additional information
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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | A heat stable and hyphae-associated Mucorales extract damages mammalian host cells in vitro. a, R. delemar caused time dependent alveolar epithelial cell damage (n = 9 wells/time point, pooled from three independent experiments). Data are median ± interquartile range. b, Heat-killed R. delemar hyphae showed ~50% damage to mammalian cells compared to ~100% damage caused by living hyphae (n = 6 wells/group, pooled from three independent experiments). Data are median ± interquartile range. Statistical analysis was performed by using Mann-Whitney non-parametric (two-tailed) test comparing live vs killed hyphae. c, Extracts from comparable wet weight of R. delemar hyphae/spores, or hyphae, but not spores, damaged alveolar epithelial cells (n = 6 wells/group, pooled from three independent experiments). Data are median ± interquartile range. Statistical analysis was performed by using Mann-Whitney non-parametric (two-tailed) test comparing spores vs spore/hyphae or hyphae. d, Disrupted pellet from Mucorales germlings containing the cell-associated fraction was compared to live or heat-killed cells in causing injury to HUVECs (n = 3 wells/group, pooled from three independent experiments). Data are median ± interquartile range. e, Fungal hyphae from representative clinical Mucorales isolates ground in liquid nitrogen and extracted with mammalian cell culture caused significant A549 alveolar epithelial cell damage (n = 3 wells/Mucorales, pooled from three independent experiments). Data are median ± interquartile range. f, IgG anti-R. delemar toxin but not normal rabbit IgG (50 μg/ml) blocked host cell damage caused by heat-killed hyphae from different Mucorales (n = 8 or 9 replicates/treatment/Mucorales, pooled from three independent experiments). Data presented as median ± interquartile range. Statistical analysis was performed by Mann-Whitney non-parametric (two-tailed) test comparing IgG anti-toxin vs. without IgG or normal rabbit IgG.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Fractionation and purification of *R. delemar* toxin. **a**, Size exclusion of hyphae extracts indicating a 10–30 kDa fraction causing A549 cell damage (*n* = 6 wells/fraction, pooled from three independent experiments). Data are median ± interquartile range. **b**, Native polyacrylamide fractionation of hyphae extract and **c**, its corresponding A549 cell damage, showing fraction #6 causing injury. (*n* = 6 wells/fraction, pooled from three independent experiments). Data are median ± interquartile range. **d**, Cellulose plate separation of fraction #6 purified from the polyacrylamide gel and **e**, its corresponding A549 cell damage, showing a high polar fraction #6 causing injury. Data are *n* = 6 wells/fraction, and pooled from three independent experiments. Data are median ± interquartile range. **f**, Third dimension fractionation of the previous fraction #6 on cellulose plates and **g**, its corresponding A549 cell injury (*n* = 6 wells/fraction, pooled from three independent experiments). Data are median ± interquartile range.
Extended Data Fig. 3 | IgG anti-toxin had no effect on growth or germination of *R. delemar*. **a**, Fungal spores (10^4/ml) were inoculated in 96-well plates with or without 50 μg/ml IgG anti-toxin or normal rabbit IgG for 6 h prior to measuring absorbance at 450 nm. (n = 12 wells, data pooled from three independent experiments) Data presented as median + interquartile range. Statistical analysis was performed by Mann-Whitney non-parametric (two-tailed). **b**, *R. delemar* spores (10^4/ml) were germinated at 37 °C for 6 h prior to measuring the germ tube length using light microscopy equipped with a micrometer lens. Each data point represents 20–50 germ tubes/HPF. (n = 12 wells, data pooled from three independent experiments) Data presented as median + interquartile range from three experiments. Statistical analysis was performed by Mann-Whitney non-parametric (two-tailed).
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Putative toxin gene expression is cell-, time- and oxygen-dependent. **a**, Toxin gene expression in *R. delemar* germinating cells in YPD medium. Data (n=3 wells/timepoint, pooled from three independent experiments) are presented as median ± interquartile range. Statistical analysis was performed by using unpaired t-test (two-tailed). **b**, Confocal imaging of Alexa Flour 488-labelled IgG anti-toxin (green) during the growth of *R. delemar* from spores to hyphae. Scale bar is 50 µm. **c**, Toxin gene expression from *R. delemar* hyphae grown in YPD culture in sufficient versus limited oxygen (n=6 wells, data pooled from three independent experiments). Data presented as median ± interquartile range. Statistical analysis was performed by using unpaired t-test (two-tailed). **d**, Toxin gene expression analysis of fungal germlings on different cell types showed a time dependent expression on alveolar epithelial cells compared to HUVECs and erythrocytes (n=3 wells/group, pooled from three independent experiments). Data presented as median ± interquartile range. Statistical analysis was performed by using unpaired t-test (two-tailed).
Extended Data Fig. 5 | RNAi targeting the putative *R. delemar* toxin inhibits its expression. **a**, *R. delemar* spores were transformed with RNAi plasmids targeting the putative toxin (RNAi-toxin) or empty plasmid (Empty-plasmid) using biolistic delivery system. Cells were grown in minimal medium without uracil for 24 h prior to extracting RNA (n = 6/group, pooled from three independent experiments). Data presented as median ± interquartile range. Statistical analysis was performed by using Mann-Whitney non-parametric (two-tailed) test comparing RNAi- *R. delemar* toxin vs wild-type or empty plasmid **b**, Representative western blot and densitometry analyses of the wild-type, empty plasmid, or RNAi toxin strains (n = 4 pictures data pooled from four independent experiments) Data presented as median ± interquartile range. Statistical analysis was performed by using Mann-Whitney non-parametric (two-tailed) test comparing RNAi- *R. delemar* toxin vs wild-type or empty plasmid. **c**, confocal images showing reduced expression of the toxin in the RNAi toxin mutant. Scale bar is 50 µm.
Extended Data Fig. 6 | Downregulation of *R. delemar* toxin by RNAi did not affect germination or the growth of the fungus. a, *Wild-type R. delemar*, RNAi empty plasmid, or RNAi toxin strains were germinated in minimal medium without uracil at 37 °C with shaking. At times, samples were taken from the medium and examined by light microscopy. Scale bar is 5 µm. b, 10^5 spores of wild-type *R. delemar*, RNAi empty plasmid, or RNAi toxin strains were plated in the middle of the minimal medium without uracil agar plates for several days at 37 °C and the colony diameter measured (n = 6 plates/group, pooled from three independent experiments). Data are presented as median ± interquartile range.
Extended Data Fig. 7 | Effect of blocking the expression or the function of *R. delemar* toxin on fungal burdens in mice. 

**a**, Inhibition of the toxin by RNAi did not affect the fungal burden in the lungs or brain of mice harvested on Day +4 post infection (average inoculum from two experiments of $1.4 \times 10^4$ for empty plasmid [n = 22 mice] vs. $1.3 \times 10^4$ for RNAi toxin mutants [n = 20 mice]). Data are pooled from two independent experiments and presented as median ± interquartile range. Statistical analysis was performed by using Mann-Whitney non-parametric (two-tailed) test comparing RNAi-*R. delemar* toxin vs. empty plasmid.

**b**, The IgG anti-*R. delemar* toxin had no effect on the fungal burden of lungs or brains of DKA mice harvested on Day +4 post intratracheal infection with wild-type *R. delemar* (average inhaled inoculum of $5.6 \times 10^3$ spores from two experiments [n = 20 mice]). Data are pooled from two independent experiments and presented as median ± interquartile range. Statistical analysis was performed by using Mann-Whitney non-parametric (two-tailed) test comparing IgG anti-*R. delemar* toxin vs. normal rabbit IgG.
Extended Data Fig. 8 | Histology of organs showing involvement of the toxin in tissue damage. **a.** Damaged lung tissues (brown colour) of mice infected with *R. delemar* transformed with RNAi empty plasmid (n = 31 field counts) or RNAi toxin. Statistical analysis was performed by using Mann-Whitney non-parametric (two-tailed) test. Scale bar is 200 µm. **b.** Damaged lung tissues from mice infected with wild-type *R. delemar* and treated with either normal rabbit IgG (n = 18 field counts) or IgG anti-toxin (n = 18 field counts) were quantified by ApopTag kit. Data were pooled from two independent experiments, are presented as median + interquartile range. Statistical analysis was performed by using Mann-Whitney non-parametric (two-tailed) test. Scale bar is 200 µm.
Extended Data Fig. 9 | *R. delemar* toxin is expressed in lung tissue collected from a mucormycosis patient but not in lung samples from an aspergillosis patient. H&E staining of lung tissues from mucormycosis a, or aspergillosis b, patients showing broad aseptate hyphae with angioinvasion (Mucorales) and thinner septated hyphae of *Aspergillus*. Scale bar is 10 μm. Box magnification 1400 X. Staining of a mucormycosis c, or aspergillosis d, patient lungs using IgG anti-toxin (green colour). Mucorales or *Aspergillus* hyphae are shown in yellow (stained with calcofluor white) and nuclei are shown in magenta. *R. delemar* toxin staining is shown in association with hyphae (grey arrow) and released in the tissue (white arrow). Scale bar is 10 μm in all micrographs.
Extended Data Fig. 10 | Secretion/shedding of \textit{R. delemar} toxin in culture supernatant of growth media. \textbf{a}, Cell-free culture supernatants were collected from \textit{R. delemar} hyphae grown in the presence or absence of 2-fold dilutions of amphotericin B. The XTT assay was used to determine growth of \textit{R. delemar} (left axis, blue bar, n = 8 wells/amphotericin B concentration), while toxin release assayed by sandwich ELISA using anti-\textit{R. delemar} mouse monoclonal IgG1 as the capture antibody and rabbit anti-\textit{R. delemar} toxin IgG as the detector antibody (right axis, red bar, n = 2 wells/amphotericin B concentration). Data in are representative of three independent experiments and presented as mean ± SD. \textbf{b}, The released toxin concentration from \textit{R. delemar} wild-type, \textit{R. delemar} transformed with empty plasmid RNAi or \textit{R. delemar} with RNAi-toxin was extrapolated from a standard curve using recombinant toxin in the same ELISA assay. Toxin concentrations (n = 3 samples from three independent experiments tested in duplicate in ELISA for each strain) are presented as mean ± SD.
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### Software and code

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Microsoft office 360, Graph Pad 8.0, MacPyMOL: PyMOL v1.7 4.5, NCBI BLAST, RCSB Protein Data Bank, Tm align by Zhang lab (https://zhanglab.ccb.med.umich.edu/TM-align/), Protein 3D model were generated by SWISS Prot (https://swissmodel.expasy.org/), Protein sequences were aligned using MUSCLE in CLUSTAL format, Protein motif search using FIMO (Find Individual Motif Occurrences): Version 5.1.1 tool (http://meme-suite.org), MUSCLE (3.8).

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Data supporting the findings of this study are available within the article and its Supplementary Figures. The source data underlying Fig. 1-4, Extended Data Figures 1-10, Supplementary Information 1-2, Supplementary Tables 1-3 are provided as a Source Data file. Figure: 3b: 3D models were generated using SWISS PROT Template library, 3D models were aligned by using Tm Align tool by Zhang lab (https://zhanglab.ccb.med.umich.edu/TM-align). The data that support the findings of this study are available from the corresponding author upon request.
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Sample size

For survival studies and from our 25+ years experience with animal models, 10 mice/group would provide at least 80% power to test the hazard ratio of 0.2 or more with a level of significance p=0.025 using Log Rank test and Cox proportional model (one-sided test) assuming 100% and 50% mortality in the control and tested group, respectively. For the tissue fungal burden, 16 mice/group would provide at least 90% power to detect the effect size of 3 or 3SD difference in CFU equivalent/g of tissue by Wilcoxon Rank Sum test with Bonferroni correction for post-hoc analysis (α of 0.05), assuming the standard deviation of the test is twice of the one for the control group.

Data exclusions

No data was excluded from the analysis

Replication

All in vitro experiments were performed at least in duplicate and replicated at least twice. All in vivo experiments used 7-10 mice per group and repeated once (for a total of 17-20 mice/group). Screening for the activity of the toxin used only three mice and the experiment was not repeated (Fig 2c-e) because the function of the toxin in vivo was further confirmed in Fig 3, Extended Data Figure 4, Extended Data Figure 5 and Extended Data figure 6. Repeat studies confirmed initial findings.

Randomization

All in vivo experiments were randomized prior to infection or treatment. Up to 20 mice/cage were combined in a cage prior to infection or treatment. After infection or treatment mice were housed 5/cage. Once treated, mice no longer can be randomized. There are no covariates in these experiments since mice are pathogen-free and infected with only Rhizopus delemar.

Blinding

Animal studies were conducted by blinded observers. Some of the in vitro data collection was collected by blinded observes and some were not. For example the isolation of the toxin was not blinded because we did not know what we are looking for. Also, findings in these experiments were verified several times by repeated extraction from other Mucorales or from R. delemar.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a                              |         |
| Antibodies                       | n/a     |
| Eukaryotic cell lines            | ChIP-seq|
| Palaeontology                    | Flow cytometry |
| Animals and other organisms      | MRI-based neuroimaging |
| Human research participants      |         |
| Clinical data                    |         |

Antibodies

| Antibodies used | Validation |
|-----------------|------------|
| HRP-Conjugated anti-rabbit IgG (Jackson ImmunoResearch), Product number (111-035-144); Rabbit IgG (R&D systems) Cat # AB-105-C; Anti-rabbit IgG Alexa Fluor 488 (Life Technologies) Cat # A-11034. HRP-IgG anti-mouse (Invitrogen, Cat #31450). Anti-ricin B chain antibodies (clone 8A1) and affinity purified rabbit anti-ricin antibodies were obtained from the laboratories of Ellen Vitetta and Nicolas Mantis. Affinity purified polyclonal anti-mucoricin IgG and monoclonal anti-mucoricin IgG1 were produced by Promab Biotechnologies. The anti-ricin and the antimucoricin antibodies were verified by its reactivity to ricin or mucoricin by ELISA and Western blots, respectively, as mentioned in the Methods. All antibodies and their sources are described in the Methods. |

1. HRP-Conjugated anti-rabbit IgG (Jackson ImmunoResearch) : Based on immunoelectrophoresis and/or ELISA, the antibody reacts with whole molecule rabbit IgG as per manufacture provided data sheet.
2. Rabbit IgG (R&D systems) Cat # AB-105-C : Serum was obtained from naive (non-immunized) rabbits and purified for use as normal rabbit IgG as per manufacture provided data sheet.
3. Anti-rabbit IgG Alexa Fluor 488 (Life Technologies) Cat # A-11034: The secondary antibody solution is passed through a column matrix containing immobilized serum proteins from potentially cross-reactive species. Only the nonspecific-binding
secondary antibodies are captured in the column, and the highly specific secondaries flow through. Further passages through additional columns result in 'highly cross-adsorbed' preparations of secondary antibody as per manufacture provided data sheet.

4. HRP-IgG anti-mouse (Invitrogen, Cat #31450) : Antibody Specificity: This antibody reacts with the heavy chains of mouse IgG and with the light chains common to most mouse as per manufacture provided data sheet. Immunoglobulins. No antibody was detected against non-immunoglobulin serum proteins. The polyclonal antibodies raised against mucorcin and the anti-ricin B clone BA1, were verified by ELISA, dot blot, and Western blots as detailed in the Methods section.

Eukaryotic cell lines

Policy information about cell lines

| Cell line source(s)          | AS49 cells CCL-185™, and Daudi lymphoma cells CCL-213™ were bought from the ATCC. |
|------------------------------|-----------------------------------------------------------------------------------|
| Authentication               | Both cells were verified by appearance after they were purchased from ATCC        |
| Mycoplasma contamination     | All cell lines including AS49 cells, Daudi cells, and HUVECs were routinely tested for Mycoplasma contamination with negative results. |
| Commonly misidentified lines | None.                                                                             |

Animals and other organisms

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| Laboratory animals          | Male ICR mice (≥20 g). All mice were housed in our animal facility according to IACUC guidelines with ambient room temperature (20–26 °C), humidity (50-70%) and dark / light cycle (12 hours). |
| Wild animals                | No wild animals were used in this study.                                           |
| Field-collected samples     | The study did not involve samples collected from the field                          |
| Ethics oversight            | All procedures involving mice were approved by the IACUC of The Lundquist 860 Institute for Biomedical Innovations at Harbor-UCLA Medical Center, according to the NIH guidelines for animal housing and care. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

| Population characteristics | Human endothelial cell collection was approved by the IRB of The Lundquist Institute for Biomedical Innovations at Harbor-UCLA Medical Center. Because umbilical cords are collected without donor identifiers, the IRB considers them medical waste not subject to informed consent. These umbilical cords were collected from healthy pregnant women after delivery regardless of age, and ethnicity. The population contains the normal patients population seen at Harbor-UCLA Medical Center including White, African American, Latino and Pacific Islander women. The IRB of the Lundquist Institutes consider these umbilical cords are human waste. Thus, no consent form is needed. Additionally, collection of the umbilical cords are done without recording of any human data such as name, ethnicity or age. |
| Recruitment                 | Cords were collected after delivery from all women irrespective of their race or ethnicity and without recording personal information. There is no bias of collection since cords are collected from all delivering women. The only exclusion criteria is for short umbilical cords since they do not produce good endothelial cells. |
| Ethics oversight            | Human endothelial cell collection was approved by the IRB of The Lundquist Institute for Biomedical Innovations at Harbor-UCLA Medical Center. Because umbilical cords are collected without donor identifiers, the IRB considers them medical waste not subject to informed consent. The purification and testing of ricin were approved by the IRB at UT Southwestern and carried out under BSL3 guidelines. Approval for the collection of tissue samples from the patients with mucormycosis and invasive pulmonary aspergillosis was obtained and the Ethics Committee of the University Hospital of Heraklion, Crete, Greece (5159/2014). The patients provided written informed consent in accordance with the Declaration of Helsinki. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.