

1 **Mucoricin is a Ricin-Like Toxin that is Critical for the Pathogenesis of Mucormycosis**

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26 Short title: Mucormycosis involves a ricin-like toxin

27

28 **Abstract**

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

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46 **Keywords:** Mucormycosis, Toxins, Ricin, *Rhizopus*, Virulence, Pathogenesis, Mucoricin

47

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49 Mucormycosis is a lethal fungal infection that usually afflicts immunocompromised hosts
50 such as diabetics in ketoacidosis (DKA), neutropenic patients, patients undergoing hematopoietic
51 cell or solid organ transplant, or patients receiving high-dose corticosteroids¹⁻⁶.
52 Immunocompetent patients with severe trauma are also at risk of contracting mucormycosis by
53 direct inoculation of open wounds^{7,8}. The overall mortality rate of mucormycosis is >40% and it
54 approaches 100% in patients with disseminated disease, persistent neutropenia, or brain
55 infection¹⁻⁶. The two most common forms of the disease are rhino-orbital/cerebral and
56 pulmonary mucormycosis. In both forms of the disease, infection is initiated by the inhalation of
57 spores that germinate in the host to form hyphae, which are capable of invading host tissues
58 while avoiding phagocytic killing^{6,9}.

59 A characteristic feature of mucormycosis is the propensity of Mucorales to invade blood
60 vessels, resulting in thrombosis and subsequent tissue necrosis⁶. The massive tissue necrosis
61 associated with mucormycosis compromises the delivery of antifungal drugs to infected foci,
62 thereby necessitating radical surgical intervention to improve the outcome of therapy. We have
63 previously determined that Mucorales fungi invade human umbilical vein endothelial cells
64 (HUVECs) by expressing the fungal invasin, CotH3, which interacts with the 78kDa host
65 receptor, glucose regulated protein (GRP78). The interaction between CotH3 and GRP78
66 induces the endothelial cells to endocytose the fungi¹⁰⁻¹². However, the mechanisms by which
67 Mucorales damage host cells and cause necrosis are unknown.

68 While studying the capacity of *Rhizopus delemar*, the most common cause of
69 mucormycosis, to damage HUVECs, we observed that killed hyphae of this organism and other
70 Mucorales caused considerable damage to host cells¹³. This experimental finding and the clinical

71 observation of the extensive tissue necrosis observed in patients with mucormycosis led us to
72 speculate that a fungal-derived toxin may be involved in the pathogenesis of this disease.

73 Here, we identify and characterize a hyphal-associated and secreted/shed toxin produced
74 by Mucorales. This toxin damages host cells *in vitro* by inhibiting protein synthesis. The toxin is
75 required for the pathogenesis of mucormycosis in mice, where it induces inflammation,
76 hemorrhage and tissue damage resulting in apoptosis and necrosis. Suppression of toxin
77 production in *R. delemar* by RNAi attenuates virulence in DKA mice, and polyclonal anti-toxin
78 antibodies (IgG anti-toxin) protect mice from mucormycosis by reducing tissue inflammation
79 and damage. Thus, the toxin is a key virulence factor of Mucorales fungi and a promising
80 therapeutic target. Because this toxin shares structural and functional features with ricin
81 produced by the castor bean plant, *Ricinus communis*¹⁴, we named it “mucoricin”.

82

83 **Results**

84 **Mucorales damage host cells by a hyphal-associated toxin**

85 We previously observed that *R. delemar* causes significant damage to HUVECs within 8
86 h of infection¹¹. This organism also damages the A549 alveolar epithelial cell line and primary
87 alveolar epithelial cells, but only after 30 h of incubation (**Extended Data Fig. 1a**). *R. delemar*-
88 mediated damage to both HUVECs and alveolar epithelial cells is associated with the formation
89 of extensive hyphae, suggesting that the hyphal form of this organism produces a factor(s) that
90 damage host cells¹³. To investigate whether viability is required for *R. delemar* hyphae to
91 damage host cells, we compared the extent of damage to A549 cells caused by live and heat-
92 killed hyphae. We found that while heat-killed hyphae caused less damage to these cells than live
93 hyphae, the extent of host cell damage was still significant (**Extended Data Fig. 1b**). These

94 finding suggested that a hyphal-associated heat-stable toxin may be partially responsible for host
95 cell damage. To explore this hypothesis, we compared the ability of aqueous extracts from dead
96 *R. delemar* spores and/or hyphae to damage host cells. Extracts from either hyphae alone or from
97 a mixture of spores and hyphae damaged A549 cells, whereas an extract from spores alone
98 caused no detectable damage (**Extended Data Fig. 1c**). We also found that killed cells and
99 pelleted hyphal debris from four different Mucorales fungi, but not the yeast *Candida albicans*,
100 caused significant damage to HUVECs (**Extended Data Fig. 1d**). Collectively, these results
101 suggest that Mucorales produce a hyphal-associated toxin that damages mammalian cells.

102

103 **Purification and activity of the toxin**

104 To purify the hyphal-associated toxin, *R. delemar* spores were grown in a liquid medium
105 for 4-7 days to generate a hyphal mat. The mat was ground in liquid nitrogen and extracted with
106 sterile water, concentrated and analyzed by size exclusion chromatography¹⁵. When the different
107 fractions were analyzed for their ability to damage A549 cells, activity was found in the fractions
108 with molecular masses of 10-30 kDa (**Extended Data Fig. 2a**). The concentrated water extract
109 was then subjected to three dimensional chromatographic fractionations yielding a fraction that
110 caused significant damage to A549 cells (**Extended Data Fig. 2b-g**). This fraction was further
111 subjected to high-performance liquid chromatography (HPLC), using hydrophobic interaction
112 chromatography (HIC). The purified sample was trypsinized and sequenced using LC-MS/MS,
113 which identified a 17 kDa protein (RO3G_06568).

114 The ORF encoding the 17 kDa protein is widely present in other Mucorales that we and
115 others previously sequenced¹⁶⁻¹⁹ and that are reported to cause disease in humans (*Mucor*,
116 *Cunninghamella*, *Lichtheimia*), animals (*Mortierella*), or plants (*Choanephora cucurbitarum*).

117 Orthologues were also found in the arbuscular mycorrhizal fungus *Rhizophagus* species, and
118 bacterial genera of *Streptomyces* and *Paenibacillus* (**Supplementary Table 1**). Since orthologues
119 were detected in other Mucorales known to cause mucormycosis, we examined the ability of
120 unfractionated hyphal extracts from various Mucorales fungi to damage A549 cells relative to
121 that induced by hyphal extracts from the *R. delemar* 99-880 reference strain. Hyphal extracts
122 from *R. oryzae*, another strain of *R. delemar*, *Lichtheimia corymbifera*, and *Cunninghamella*
123 *bertholletiae* all caused significant damage to A549 cells (**Extended Data Fig. 1e**).

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

131 We also used qRT-PCR to study the expression of this ORF in *R. delemar*. In accordance
132 with data showing a lack of toxin activity in spores (**Extended Data Fig. 1c**), there was minimal
133 expression of this gene during the first 3 h of incubation (prior to germination¹³). Expression of
134 this gene began to increase when the spores germinated at 4 h¹³, peaked by 5 h, and plateaued for
135 at least 16 h of hyphal formation (**Extended Data Fig. 4a**). Protein expression in germlings and
136 hyphae, but not spores, was confirmed by immunostaining using the IgG anti-toxin (**Extended**
137 **Data Fig. 4b**). The expression of the putative toxin gene was high in hyphae growing under
138 aerated conditions, but not in hyphae grown in the absence of aeration (**Extended Data Fig. 4c**).
139 In addition, RNA expression was 5-10-fold higher following 2-5 h of co-culture with A549

140 alveolar epithelial cells as compared to co-culture with HUVECs or human erythrocytes.

141 **(Extended Data Fig. 4d).**

142

143 **The toxin is capable of damaging host cells *in vitro* and *in vivo***

144 We compared the ability of the purified toxin to damage primary lung epithelial cells,
145 A549 alveolar epithelial cells, and HUVECs. After 1 h, the toxin caused significant damage to all
146 the host cells and especially to HUVECs (**Fig. 1a**). After 3 h, there was almost 100% damage to
147 all host cells. We also compared the ability of the purified toxin vs. the recombinant toxin to
148 damage A549 cells. Both caused significant damage (**Fig. 1b**). Therefore, the purified and the
149 recombinant protein act similarly and in a time dependent manner in damaging A549 cells.

150 We next tested the activity of the toxin *in vivo*. Toxin purified from *R. delemar* was
151 injected intravenously into mice every other day for a total of three doses and the mice were
152 monitored for behavioral changes, weight loss, and survival. Within 10-30 minutes after the
153 injection of 0.1 mg/ml (5.9 μ M) purified toxin, we observed behavior highly suggestive of
154 sudden circulatory hypovolemic shock, including rapid and shallow breathing, weakness, and
155 cold skin. The mice lost >25% of their original body weight (**Fig. 1c**); most eventually died.
156 These events were similar to those observed in mice infected with live *R. delemar* spores (**Fig.**
157 **1d**). Finally, histopathology of organs collected from the mice showed pathological changes that
158 included necrosis, hemorrhage, and infiltration of the pulmonary interstitium by macrophages in
159 the lungs. Liver changes included necrosis, clusters of mononuclear cells and the presence of
160 megakaryocytes in the organs, polymorphonuclear cell (PMN) infiltration and tissue calcification
161 indicative of uncontrolled inflammation, hemorrhage and necrosis (**Fig. 1e**). These data suggest

162 that the toxin is sufficient to cause clinical symptoms often associated with disseminated
163 mucormycosis.

164

165 **RNAi knockdown and antibody-mediated neutralization of the toxin reduced the virulence**
166 **of *R. delemar* in vitro and in vivo**

167 To further confirm the role of this toxin in the pathogenesis of mucormycosis, we used
168 RNAi²⁰ to down regulate the gene expression of the toxin. The extent of down regulation of the
169 toxin was measured by qRT-PCR using toxin specific primers and by Western blotting or
170 immunostaining of *R. delemar* with the IgG anti-toxin. This IgG anti-toxin specifically
171 recognized the toxin by ELISA and Western blotting. RNAi knockdown of the toxin caused
172 ~90% inhibition in gene expression (**Extended Data Fig. 5a**). Furthermore, RNAi knockdown
173 resulted in >80% reduction in protein expression (**Extended Data Fig. 5b**) and negligible
174 staining of toxin-RNAi *R. delemar* germlings compared to germlings of a control strain that have
175 been transformed with an empty plasmid (**Extended Data Fig. 5c**). In accord with the lack of an
176 effect by the IgG anti-toxin on the growth and germination of *R. delemar*, the RNAi knockdown
177 of the toxin had no effect on fungal germination or growth (**Extended Data Fig. 6**).

178 We next assessed the effect of downregulation of toxin expression on the ability of *R.*
179 *delemar* to damage A549 alveolar epithelial cells. *R. delemar* with RNAi targeting of the toxin
180 gene induced ~40% reduction in epithelial cell damage relative to either the wild-type strain or
181 *R. delemar* transformed with the empty plasmid (**Fig. 2a**). Similarly, the IgG anti-toxin protected
182 alveolar epithelial cells from wild-type *R. delemar*-induced injury by ~40%, *in vitro* whereas
183 normal rabbit IgG did not (**Fig. 2b**).

184 Finally, we evaluated the effects of RNAi inhibition of toxin production on the virulence
185 of *R. delemar* in our model of pulmonary mucormycosis²¹. DKA mice infected with *R. delemar*
186 harboring the empty plasmid had a median survival time of 6 days and 90% mortality by day 21
187 post-intratracheal infection, whereas mice infected with the toxin-attenuated expression strain
188 had a median survival time of 21 days and mortality of 30% (**Fig. 2c**). Surviving mice had no
189 residual fungal colonies in their lungs when the experiment was terminated on day 21. Inhibition
190 of toxin production appeared to have minimal effects on the early stages of infection because
191 after 4 days of infection, the fungal burden of the lungs and brains (the primary and secondary
192 target organs, respectively²¹) of mice infected with *R. delemar* toxin-attenuated strain and *R.*
193 *delemar* harboring the empty plasmid were similar (**Extended Data Fig. 7a**). Reduced virulence
194 without affecting tissue fungal burden has been reported for non-neutropenic mice infected with
195 an *Aspergillus fumigatus* null mutant that does not produce the Asp f1 ribotoxin²², representing a
196 classical feature of disease tolerance²³. Collectively, our results indicate that while the toxin is
197 dispensable for the initiation of mucormycosis, it plays a central role in the lethality of this
198 disease.

199

200 **IgG anti-toxin protected mice from mucormycosis**

201 To further verify the role of the toxin in the pathogenesis of mucormycosis, we infected
202 DKA mice intratracheally with wild-type *R. delemar* and then 24 h later, treated them with a
203 single 30 µg dose of either the IgG anti-toxin or normal rabbit IgG. While mice treated with
204 normal IgG had a mortality rate of 95%, treatment with the IgG anti-toxin resulted in ~70%
205 long-term survival (**Fig. 2d**). Surviving mice appeared healthy and had no detectable fungal
206 colonies in their lungs when the experiment was terminated on day 21. In accord with data from

207 the fungal burden in tissues of mice infected with the toxin-attenuated strain, antibody treatment
208 had no effect on the fungal burden of lungs or brains when tissues were harvested four days post
209 infection (**Extended Data Fig. 7b**). These data further confirm the role of the toxin in the
210 pathogenesis of mucormycosis and point to the potential of using anti-toxin antibodies to treat
211 the disease.

212 We also performed histopathological examination of the tissues from all groups of mice
213 sampled at the same time of tissue fungal burden studies (day 4) to gain insight into the
214 mechanism of action of the toxin. While, uninfected mice had normal lung architecture with no
215 signs of inflammation or infection (**Fig. 2e**), lungs from mice infected with *R. delemar*
216 transformed with the RNAi empty plasmid (control) showed fungal and granulocyte infiltration
217 (**Fig. 2f left panel**) and angioinvasion with thrombosis (**Fig. 2f right panel**). In contrast, lungs
218 from mice infected with the toxin-attenuated mutant showed only mild signs of inflammation
219 with no angioinvasion (**Fig. 2g**). Importantly, lungs of mice infected with wild-type *R. delemar*
220 and treated with the IgG anti-toxin showed architecture that was similar to the lungs of the
221 uninfected control mice; there were no signs of inflammation or infiltration with *R. delemar* (**Fig.**
222 **2h**).

223

224 **Down regulation of the toxin gene or treatment with IgG anti-toxin attenuated *R. delemar*-** 225 **mediated host cell damage *in vivo***

226 To determine whether the toxin contributed to host cell damage *in vivo*, we used the
227 ApopTag *in situ* apoptosis kit to stain the lung tissues of the infected mice. While extensive lung
228 damage was observed in mice infected with wild-type *R. delemar*, the lungs harvested from mice
229 infected with the toxin-attenuated mutant (**Extended Data Fig. 8a**) or those infected with wild-

230 type *R. delemar* and treated with the IgG anti-toxin (**Extended Data Fig. 8b**) had almost no
231 detectable damage.

232 Finally, we have previously reported on a mucormycosis case in a human with
233 disseminated mucormycosis⁹. Haemotoxylin and Eosin (H&E) staining of lung tissues of this
234 patient showed broad aseptate hyphae that caused necrosis and massive infiltration of tissues
235 compared to thinner septated hyphae present in a patient suffering from invasive pulmonary
236 aspergillosis (**Extended Data Fig. 9a,b**). Subsequent immunohistochemistry of the patient's
237 lungs using the IgG anti-toxin (*vs.* control IgG) showed association of the toxin with fungal
238 hyphae and the surrounding tissues in a mucormycosis patient and lack of staining in tissues of
239 an aspergillosis patient (**Extended Data Fig. 9c,d**). These results show that the toxin is also
240 involved in human mucormycosis, is cell-associated as well as secreted/shed into the
241 surrounding tissues, and confirm the specificity of the antibody used in these studies since the
242 putative toxin does not have an orthologue in *Aspergillus* (**Supplementary Table 1**).

243 To confirm the secretion/shedding of the toxin, we grew *R. delemar* spores in 96-well
244 plate with or without amphotericin B (AmB) and assayed cell-free supernatants for the presence
245 of the toxin using a sandwich ELISA with an IgG₁ anti-toxin monoclonal antibody that we raised
246 in mice as a capture antibody and the rabbit IgG anti-toxin as the detecting antibody. The toxin
247 was detected in cell-free supernatants of *R. delemar* wild-type (26.7 ± 0.87 nM) or *R. delemar*
248 transformed with the empty plasmid (23.0 ± 2.04 nM), but not in *R. delemar* transformed with
249 RNAi targeting the toxin. In accord with secretion/shedding of the toxin by live hyphae,
250 supernatants collected from wild-type *R. delemar* hyphae in which further growth has been
251 hampered by AmB concentrations ≥ 2 μ g/ml showed little to no secretion/shedding of the toxin

252 **(Extended Data Fig. 10)**. These results confirm that the toxin is secreted/shed into the growth
253 medium.

254

255 **The hyphae-associated toxin has structural features of ricin**

256 Given the critical role of the toxin in the pathogenesis of mucormycosis, we did structural
257 and bioinformatics studies to understand its mechanism of action. As reported in
258 **Supplementary Table 1**, many of the toxin orthologues found in other organisms are annotated
259 as ricin domain-containing proteins or ricin B chain-like lectins. Further detailed bioinformatic
260 analysis of the *R. delemar* toxin sequence showed a two domain structure similar to that of ricin
261 (Sequence ID: NP_001310630.1)²⁴ (**Fig. 3a**). Specifically, the *R. delemar* toxin harbored a small
262 region (amino acids 198-289) that resembled a sequence in ricin chain A, known to be involved
263 in inactivating ribosomes (*i.e.* ribosome inactivating protein [RIP]), and two domains (amino
264 acids 304-437 and 438-565) of the lectin-binding ricin chain B. Moreover, the *R. delemar* toxin
265 contained an LDV-motif (**Fig. 3a**) that is present in ricin (**Fig 3a**, red colored amino acids) and
266 that has been reported to cause damage to HUVECs *in vitro*^{25,26}, and *in vivo* in models of
267 vascular leak²⁷ as well as postulated to cause ricin A chain-mediated vascular leak syndrome in
268 humans²⁵. Furthermore, gene ontology studies predicted that *R. delemar* toxin would have
269 functions similar to ricin including sugar binding (GO:0005529, score of 0.64), as well as rRNA
270 glycosylase activity (GO:0030598, score of 0.49) and hydrolase activity (GO:0004553, score of
271 0.35) (**Fig. 3a**, table).

272 We produced a 3-D structural model of the *R. delemar* toxin by searching templates
273 within the SWISS-Model template library (SMTL). The greatest resemblance was with sugar-
274 binding proteins, especially galactose, the known lectin for ricin²⁸. Other proteins with predicted

275 resemblance to mucoricin included those with cell adhesion, toxin and hydrolase (glycosylase)
276 activities (**Supplementary Table 2**). The rRNA glycosylase activity is a feature of ricin and is
277 required for inactivating ribosomes²⁹. Finally, the structures of the *R. delemar* toxin and ricin B
278 chain were superimposable with a highly significant Tm-align score of 0.81 and a score of 0.78
279 for ricin B domain I (amino acids 304-437) and domain II (438-565 amino acid), respectively
280 (**Fig. 3b**). However, the 17 kDa *R. delemar* toxin is much smaller than either the A or B chains of
281 ricin (32 kDa each). Nevertheless, the fungal toxin appears to share structural homology with
282 portions of ricin that are responsible for inactivating ribosomes, inducing vascular leak and
283 binding to galactose.

284

285 ***R. delemar* toxin is immunologically cross-reactive with ricin**

286 To further explore the similarities between *R. delemar* toxin and ricin, we used the IgG
287 anti-*R. delemar* toxin in an ELISA to determine whether the toxin and ricin were
288 immunologically cross-reactive. Plates were coated with ricin or *R. delemar* toxin, and then
289 incubated with the IgG anti *R. delemar* -toxin, or normal rabbit IgG. The former but not the latter
290 bound to ricin or *R. delemar* toxin in a dose dependent manner (**Fig. 3c**). The IgG anti-*R.*
291 *delemar* toxin also recognized ricin, and a murine monoclonal antibody (8A1) against the ricin B
292 chain³⁰ recognized the *R. delemar* toxin in a dot blot (**Fig. 3d**). Furthermore, the IgG anti-*R.*
293 *delemar* toxin reacted with both the *R. delemar* toxin and ricin in a Western blot (**Fig. 3e**).
294 Importantly, the IgG anti-*R. delemar* toxin protected A549 alveolar epithelial cells from ricin-
295 induced damage in a manner similar to that of the IgG anti-ricin B chain (8A1 clone) or
296 galactose [the lectin for the ricin chain B (**Fig. 3f**)]. Collectively, these data demonstrate the
297 similarities between the two toxins.

298

299 **Mucoricin is a RIP that also promotes vascular permeability and induces both necrosis and**
300 **apoptosis of host cells**

301 After ricin is internalized by cells *via* its lectin-binding B chain, it's A chain exerts its
302 toxic activity by irreversibly inactivating ribosomes *via* its N-glycosylase activity. This result in
303 the inhibition of protein synthesis³¹. The enzyme activity cleaves the N-glycosidic bond between
304 the adenine nucleobase in the α -sarcin-ricin loop and its ribose causing the release of adenine
305 (depurination)³². To determine whether the *R. delemar* toxin had similar activity, we compared
306 the ability of the two toxins to inhibit protein synthesis in a cell-free rabbit reticulocyte assay³³.
307 As reported previously³⁴, ricin concentration that caused 50% inhibition in protein synthesis
308 (IC₅₀) was ~ at 2.2×10^{-11} M (**Fig. 4a**). The recombinant toxin of *R. delemar* also inhibited
309 protein synthesis, albeit with ~ 800-fold weaker activity than ricin (*i.e.* an IC₅₀ of 1.7×10^{-8} M)
310 (**Fig. 4b**). To determine if the protein inhibition is due to depurination, we used HPLC to
311 measure the amount of adenine released from template RNA extracted from A549 alveolar
312 epithelial cells³². In contrast to RNA exposed to the buffer control, RNA incubated with
313 mucoricin released detectable amounts of adenine (**Fig. 4c**). The depurination is due to rRNA N-
314 glycosylase activity since in contrast to the negative control, ovalbumin (OVA) which did not
315 cleave 28S rRNA, the *R. delemar* toxin cleaved the rRNA in rabbit reticulocyte lysates after
316 adding aniline, albeit at a concentration of 10^4 higher than ricin and after incubation with the
317 ribosomes for a longer period of time (**Fig. 4d**). Thus, like ricin, the *R. delemar* toxin is a RIP.

318 In addition to its N-glycosylase activity, ricin chain A is known to cause vascular leak *in*
319 *vitro*^{25,26} and *in vivo*²⁷ mediated by its LDV sequence, a motif that is also present in the *R.*
320 *delemar* toxin (**Fig. 3a**). To examine whether the *R. delemar* toxin compromised vascular
321 integrity, we grew HUVECs on membrane inserts in transwells prior to treating the confluent

322 monolayers with either *R. delemar* spores or *R. delemar* toxin for 5 h at concentrations that did
323 not kill the HUVECs (**Supplementary Fig. 1**). The permeability of HUVECs was determined by
324 measuring the amount of fluorescent dextran migrating from the upper chamber to the lower
325 chamber of the transwells after adding *R. delemar* spores (**Fig. 4e**) or recombinant *R. delemar*
326 toxin (**Fig. 4f**). Both *R. delemar* and the toxin induced permeability, which was equivalent to
327 that induced by *E. coli* lipopolysaccharide (LPS), a potent inducer of vascular permeability³⁵.
328 Furthermore, IgG anti-*R. delemar* toxin blocked this enhanced permeability by 50-60%.
329 Importantly, IgG anti-ricin B chain (8A1 clone) almost completely abrogated the permeability
330 induced by *R. delemar* or its toxin (**Fig. 4e,f**). These results confirm that *R. delemar* induces
331 permeability in HUVECs *via* its toxin and that IgG anti-ricin B chain blocks this *R. delemar*-
332 mediated virulence trait.

333 Ricin is also known to cause cell damage by inducing both necrosis and apoptosis^{36,37}. To
334 determine whether the *R. delemar* toxin did the same, we used an Apoptosis/Necrosis detection
335 kit to compare the abilities of *R. delemar* toxin and ricin to damage alveolar epithelial cells. As
336 compared to the control, after 2 h both toxins caused comparable apoptosis and necrosis (**Fig.**
337 **4g,h**). Collectively, these results demonstrate the functional similarities between ricin and *R.*
338 *delemar* toxin as RIPs that inhibits protein synthesis *via* N-glycosylase activity. Both toxins also
339 cause cell death by apoptosis and necrosis. Based on the structural and functional similarity to
340 ricin, we named the *R. delemar* toxin **mucoricin** and the corresponding gene **Ricin-Like Toxin**
341 (***RLTI***).

342

343 **Discussion**

344 Mucormycosis is a lethal fungal infection often associated with extensive tissue damage. We
345 have now identified a cell-associated/secreted/shed toxin that is widely present in pathogenic
346 Mucorales fungi. We used genetic and biochemical techniques to show that the toxin gene, *RLT1*
347 and its encoded protein (mucoricin) are required for the full virulence of *R. delemar*. In addition
348 to being produced by pathogenic Mucorales, this toxin appears to be present in other fungi and
349 bacteria. For example, orthologues in *Rhizophagus* were identified to have 30-40% identity with
350 *RLT1*. *Rhizophagus* lives symbiotically with plants and is recognized as an integral part of the
351 natural ecosystem and was shown to delay plant disease symptoms caused by *Phytophthora*
352 *infestans*³⁸. Similarly, *RLT1* orthologues were identified in the bacterial genera of *Streptomyces*
353 and *Paenibacillus* (~30% identity). Both bacteria are known inhabitants of soil, present in
354 rhizosphere of various plants, and are used as biological control agents for crops because of their
355 ability to secrete secondary metabolites³⁹.

356 Mucoricin has structural and functional similarities to ricin, a prototypic Type 2 RIP
357 consisting of two polypeptide chains (A and B) that are linked by a disulfide bond²⁴. The A-
358 chain of ricin is an *N*-glycosidase that is responsible for inactivating ribosomes, and the B chain
359 is a galactose-specific lectin²⁹ that enables the toxin to bind to target cells²⁸. In contrast, Type I
360 RIPs are monomeric A-chain-like RIPs²⁹. Mucoricin appears to have the activities of both ricin
361 A and B chains (and Type I RIPS) and both activities are present in a single 17 kDa protein (**Fig.**
362 **3e**). Several other RIPs isolated from plants consist of low molecular weight single chain
363 proteins including a 26 kDa TRIP isolated from tobacco leaves⁴⁰ and a 7.8 kDa protein isolated
364 from sponge gourd seeds (*Luffa cylindrica*)⁴¹.

365 The strong sequence and structural similarity between mucoricin and ricin lies in the ricin
366 B-chain, although mucoricin inhibits protein synthesis *via* N-glycosylase activity leading to

367 depurination, albeit with lower activity than ricin. A possible explanation for the ability of
368 mucoricin to inhibit protein synthesis is likely predicted by its conserved rRNA glycosidase
369 activity. Specifically, the EAARF motif in ricin A chain is known to be responsible for the RIP
370 activity of ricin. It is known to depurinate adenosine 4324 in 28S rRNA with the glutamic acid
371 residue (E, shaded) responsible for this activity⁴². Furthermore, the arginine residue (R, shaded)
372 separated by two amino acids from the glutamic acid residue (**Fig. 3a**, green brackets), is also
373 required for the activity of the bacterial Shiga toxin, a potent ricin-like A chain with a fully
374 conserved EAARF domain of ricin⁴³. Mucoricin has the EEGRL, in which the glutamic acid and
375 arginine residues are conserved (**Fig. 3a**, green brackets).

376 Another functional domain in ricin and Shiga toxin reported to be required for RIP
377 activity is the WGRSL⁴⁴ (**Fig. 3a**, cyan underline). This sequence also aligns with the EEGRL
378 motif of mucoricin (**Fig. 3a**, green brackets; and **Supplementary Table 3**). Furthermore,
379 mucoricin contains the EAANQ motif (**Fig. 3a**, purple overline) which resembles the ricin
380 sequence of EAARF, with the glutamic acid residue conserved and the arginine residue replaced
381 by asparagine, a conserved amino acid with properties that are weakly similar to arginine. The
382 lack of fully conserved functional residues (*i.e.* arginine) between mucoricin and ricin/Shiga
383 toxins likely explains the 800-fold weaker RIP activity of mucoricin as compared to ricin (**Fig.**
384 **4a,b**). Finally, mucoricin also has sequence homology with several other RIPs including saporin
385 of *Saponaria officinalis*, a Type 1 RIP with 19% overall identity and 10 out of the 17 conserved
386 amino acid residues in the functional domain of EAARF (**Supplementary Fig. 2**). Notably, the
387 EEGRL and EAANQ are widely present in Mucorales fungi known to cause human disease
388 (**Supplementary Table 3**). Thus, mucoricin appears to be a RIP that functionally resembles ricin

389 A chain and Type-1 RIPs such as saporin⁴⁵. The contribution of the EEGRL and EAANQ motifs
390 to the RIP activity is being further investigated.

391 Our *in vitro* studies suggest that *RLT1* is expressed most strongly when the hyphal matt is
392 aeriated and in response to alveolar epithelial cells (**Extended Data Fig. 4c,d**). These results
393 further suggest that mucoricin may be highly active during pulmonary mucormycosis and
394 potentially in rhinoorbital disease, when hyphae are exposed to epithelial cells in the presence of
395 ambient levels of oxygen. It is of interest that, mitochondria are believed to play a central role in
396 RIPs' (e.g. ricin, Shiga toxin and abrin) ability to induce host cell apoptosis⁴⁶. Although the
397 expression of *RLT1* in *R. delemar* is higher in response to alveolar epithelial cells than to
398 HUVECs (**Extended Data Fig. 4d**), the latter host cells are damaged much more rapidly by *R.*
399 *delemar* (*i.e.* significant *R. delemar*-induced HUVEC injury occurs at 8 h vs. 48 h for *R.*
400 *delemar*-induced alveolar epithelial cells [**Extended Data Fig. 1**])¹³, and by purified mucoricin
401 (**Fig. 1a**). In accord with these results, it has been shown that HUVECs are rapidly damaged and
402 their permeability affected by small peptides containing the LDV-motif but lacking the
403 sequences responsible for N-glycosidase activity²⁶. In this study we show that *R. delemar*
404 compromises the permeability of HUVEC monolayers *in vitro* by the direct effect of mucoricin.
405 The LDV and other (x)D(y) motifs (with known vascular leak effector function) are widely
406 present in pathogenic Mucorales (**Supplementary Table 3**)²⁵. Thus, it is possible that the LDV-
407 motif is responsible for angioinvasion and rapid hematogenous dissemination in mucormycosis⁶
408 by inducing damage to vascular endothelial cells.

409 The exact mechanism by which mucoricin enters a target cell to exert its lethal effect is
410 not yet known. However, our data strongly indicate that it is cell-associated as well as
411 secreted/shed by Mucorales. The amount of toxin in the medium (27 nM, **Extended Data Fig.**

412 **10b)** from a small-scale growth in a 96-well plate was sufficient to exceed the IC₅₀ in RIP
413 activity of 17 nM (**Fig. 4b**). Thus, secreted/shed toxin likely exerts its toxicity by binding to and
414 then entering the host cells in the absence of invading hyphae. Alternatively, invading *R.*
415 *delemar* hyphae release the toxin once they are phagocytosed by immune⁹ cells or barrier
416 cells^{13,47,48}.

417 Our *in vivo* studies clearly demonstrate the contribution of mucoricin to pathogenesis by
418 enhancing angiogenesis, inflammation and tissue destruction. There is also evidence that the
419 lethality of ricin *in vivo* is related at least in part to its ability to induce a massive inflammatory
420 immune response accompanied by infiltration of PMN⁴⁹ in many settings such as acute lung
421 injury^{49,50} and gastrointestinal disease⁵¹. This is likely due to activation of the innate arm of the
422 immune system by the toxin itself or by toxin-damaged cells. Indeed, our histopathological
423 examination of organs harvested from mice injected with purified mucoricin shows inflammation
424 and recruitment of PMNs (**Fig. 1e**). Neutralizing the effect of the toxin either by RNAi or by
425 anti-mucoricin antibodies decreased inflammation and host tissue damage (**Fig. 2e-h** and
426 **Extended Data Fig. 8**). These results confirm the critical role of mucoricin in the pathogenesis
427 of mucormycosis and suggest that it is involved in mediating inflammation and tissue damage,
428 both of which are clinical features of mucormycosis. Of note, treatment of mucormycosis
429 patients with antifungal agents is often hampered by the extensive tissue necrosis that prevents
430 optimal delivery of drugs into the site of infection. Hence, antifungal treatment alone (without
431 surgical intervention) is often non-curative⁶. Thus, antibody-mediated neutralization of
432 mucoricin might reduce tissue necrosis, decrease the need for disfiguring surgery, and maximize
433 the effect of antifungal therapy.

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

- 436 i. Infection is initiated when fungal spores are inhaled, and in the absence of phagocytes
437 (or presence of dysfunctional phagocytes such as in patients with DKA). Fungal
438 spores express CotH¹⁰⁻¹² and bind to either GRP78 on nasal epithelial cells⁴⁸, or to
439 integrin β 1⁴⁸ which activates epidermal growth factor receptor (EGFR)⁴⁷ on alveolar
440 epithelial cells to induce invasion.
- 441 ii. Under aerobic conditions, the calcineurin pathway is activated in the inhaled spores,
442 causing them to germinate^{52,53}, a process that leads to the production of mucoricin.
- 443 iii. Mucoricin binds to tissue cells by its lectin receptor, inhibits protein synthesis, and
444 causes apoptosis and necrosis. The toxin can also compromise vascular permeability
445 resulting in rapid hematogenous dissemination and tissue edema often seen in patients
446 with mucormycosis.
- 447 iv. While tissue damage is occurring, and because the toxin and debris from necrotic cells
448 are recognized by the immune system, an inflammatory immune response leads to the
449 recruitment of PMNs and other tissue-resident phagocytes.
- 450 v. Although the recruited phagocytes damage some of the invading hyphae, both the
451 dead and live hyphae release mucoricin, resulting in more host cell death and more
452 inflammation.
- 453 vi. In the necrotic tissue, the fungus can proliferate, protected from both phagocytes and
454 anti-fungal drugs.

455 Our finding that mucoricin remains active even in dead organisms offers an explanation
456 for why antifungal therapy alone has limited efficacy in patients with mucormycosis, and why

457 the fungal lesions must frequently be surgically excised. Importantly, other toxins/mechanisms
458 of host cell damage likely exist in Mucorales, since antibody blocking studies and
459 downregulation of toxin gene expression do not fully abrogate the ability of *R. delemar* to
460 damage host tissues.

461 In summary, we have identified a ricin-like toxin (mucoricin) that is widely present in
462 Mucorales fungi, where it plays a central role in the pathogenesis of mucormycosis. We postulate
463 that strategies to neutralize mucoricin will have significant therapeutic benefits.

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466

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476 Slyke for their work on the 8A1 monoclonal antibody and Dr. Robert Munford, NIH for his
477 insightful suggestions concerning the nature of the toxin.

478

479 **Author contributions**

480 S.S.M.S. conceived, designed and performed studies to purify and identify the toxin, and screen
481 its activity *in vitro* and *in vivo* and wrote the manuscript. C.B. generated mucoricin mutants and
482 characterized their virulence *in vitro* and *in vivo* and conducted the antibody efficacy studies.
483 Y.G. helped in animal studies, conducted confocal microscopy, cross reactivity studies, and RIP
484 activity studies. S.S. designed and performed homology modeling, cross reactivity studies, and
485 toxin secretion studies. T.G. helped in the animal studies. M.S. performed the necrosis/apoptosis
486 assay and the mouse immunohistochemistry studies. A.A. performed permeability studies,

487 E.G.Y. performed sequence alignment and gene ontology studies. S.A. purified recombinant
488 toxin and polyclonal antibodies. A.P. and G.C. provided and performed the human
489 immunohistochemistry studies. C.P. and V.V. performed and interpreted the mouse histology
490 studies. AR carried out studies on cross-reactivity of mucoricin and ricin. V.M.B. and J.D.H.
491 performed phylogenetic studies and blast search of mucoricin in Mucorales. N.J.M. generated
492 and characterized the 8A1 monoclonal antibody. J.E.E. and S.G.F. provided intellectual advice,
493 designed studies, and edited the manuscript. E.S.V. conceived, designed and carried out studies
494 of cross reactivity, provided reagents and expertise on ricin and helped write the manuscript.
495 A.S.I. conceived, designed, coordinated and supervised the studies, performed experiments,
496 analyzed data, and wrote the manuscript along with comments from co-authors.

497

498 **Materials and Correspondence:**

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500

501 **Competing interests**

502 A.S.I. owns shares in Vitalex Biosciences, a start-up company that is developing
503 immunotherapies and diagnostics for mucormycosis. The remaining authors declare no
504 competing interests.

505 The Lundquist Institute has filed intellectual property rights concerning mucoricin. Vitalex
506 Biosciences has an option to license the technology from The Lundquist Institute for Biomedical
507 Innovation.

508 Other authors declare no conflict of interest.

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510 **Methods**

511 **Organisms, culture conditions and reagents.** *R. delemar* 99-880 and *R. oryzae* 99-892 were
512 isolated from the brain and lungs of patients with mucromycosis and obtained from the Fungus
513 Testing Laboratory, University of Texas Health Science Center at San Antonio which had its
514 genome sequenced¹⁹. *Cunninghamella bertholletiae* 182 is a clinical isolate and is a kind gift
515 from Thomas Walsh (Cornell University). *Lichtheimia corymbifera* is also a clinical isolate
516 obtained from the DEFEAT Mucor clinical study⁵⁴. *R. delemar* M16 is a *pyrf* null mutant derived
517 from *R. delemar* 99-880 and was used for transformation to attenuate mucoricin expression⁵⁵.
518 The organisms were grown on potato dextrose agar (PDA, Becton Dickinson) plates for 5-7 days
519 at 37°C. For *R. delemar* M16, PDA was supplemented with 100 mg/ml uracil. The
520 sporangiospores were collected in endotoxin free Dulbecco's phosphate buffered saline (PBS)
521 containing 0.01% Tween 80, washed with PBS, and counted with a hemocytometer to prepare
522 the final inoculum. To form germlings, spores were incubated in YPD (Becton Dickinson)
523 medium at 37°C with shaking for different time periods. Finally, for growth studies 10⁵ spores of
524 *R. delemar* wild-type, or mutant strains were plated in the middle of PDA agar plates. The plates
525 were incubated at 37°C and the diameter of the colony was calculated every day for 6 days. The
526 monoclonal anti-ricin B chain antibody (clone 8A1)³⁰ and affinity purified rabbit anti-ricin
527 antibodies were prepared and characterized in the Vitetta and Mantis laboratories. Galactose was
528 obtained from Fisher Scientific (Cat # BP656500) and used for blocking the damaging effect of
529 ricin holotoxin.

530

531 **Host cells.** Human alveolar epithelial cells (A549 cells) were obtained from a 58-year-old male
532 Caucasian patient with carcinoma and procured from American Type Culture Collection

533 (ATCC). The cells were propagated in F-12K Medium developed for lung A549 epithelial cells.
534 Primary alveolar epithelial cells were obtained from ScienCell (Cat # 3200) and propagated in
535 Alveolar Epithelial Cell Medium (Cat#3201) and passaged once.

536 HUVECs were collected by the method of Jaffe *et al.*⁵⁶. The cells were harvested by
537 using collagenase and were grown in M-199 (Gibco BRL) enriched with 10% fetal bovine
538 serum, 10% defined bovine calf serum, L-glutamine, penicillin, and streptomycin (all from
539 Gemini Bio-Products, CA). Second-passage cells were grown to confluency in 24- or 96-well
540 tissue culture plates (Costar, Van Nuys, CA) on fibronectin (BD Biosciences). All incubations
541 were in 5% CO₂ at 37°C. The reagents were tested for endotoxin using a chromogenic limulus
542 amoebocyte lysate assay (BioWhittaker, Inc.), and the endotoxin concentrations were less than
543 0.01 IU/ml.

544 Fresh red blood cells were isolated from blood samples collected from healthy volunteers
545 after obtaining a signed informed consent form and processed as previously described⁵⁷.
546 Endothelial cell and red blood cell collection was approved by Institutional Review Board at The
547 Lundquist Institute at Harbor-UCLA Medical Center.

548

549 **Purification and characterization of ricin.** Two sources of ricin were used. One was purified
550 from a large stock of pulverized castor beans in the Vitetta laboratory⁵⁸. Its IC₅₀ and toxicity were
551 tested on Daudi lymphoma cells (ATCC® CCL-213™), HUVECs, and in cell free reticulocyte
552 assays^{25,34,59}. The other source was purchased from Vector Laboratories (Burlingame, CA; Cat
553 No. L-1090). Both sources were similar in purity and activity.

554

555 **Purification and identification of mucoricin.** To purify mucoricin, *Rhizopus* fungal spores
556 (10^3 /ml) were grown for 5 days at 37°C in YPD culture medium. The supernatant was separated
557 from the fungal mat by filtration and the fungal mycelia was ground in liquid nitrogen and
558 extracted with sterile water, concentrated and analyzed through size exclusion columns¹⁵. Host
559 cell damage assay showed that substances >10 kDa and < 30kDa is responsible for injuring the
560 host cells (**Extended Data Fig. 1a**). The concentrated water extract was then subjected to 3D
561 chromatographic separations (**Extended Data Fig. 1**). For the first dimension, the concentrated
562 extract run on native polyacrylamide gel under electrophoretic force (**Extended Data Fig. 1b**).
563 The gel was cut into 6 pieces and then eluted separately in PBS buffer prior to testing for their
564 damaging activity. Only fraction #6 corresponding to 15-20 kDa showed damaging effect to host
565 cells (**Extended Data Fig. 1c**). Next, this fraction was concentrated and subjected to separation
566 using methanol:water (4:1) as a solvent on cellulose plates. Fractions were scraped from the
567 cellulose plate and dissolved in irrigation water, followed by incubation with host cell after filter
568 sterilization. A third dimensional fractionation was applied to the fraction that caused damage
569 using cellulose plates and with the solvent system as above. This round of fractionation resulted
570 in one fraction causing damage to host cells.

571
572 **Structural modeling of mucoricin.** For amino acid sequence comparisons, mucoricin and ricin
573 (Sequence ID: NP_001310630.1) protein sequences were aligned using MUSCLE/CLUSTAL-
574 W. We searched the SWISS-MODEL template library (SMTL) (<https://swissmodel.expasy.org/>)
575 to find templates for building 3-D structural model of mucoricin. Briefly, a BLAST search of the
576 SMTL against the primary amino acid sequence identified the target sequence. To build the
577 model, we performed target-template alignment using ProMod3, and templates with the highest

578 quality were selected for model building. Insertions and deletions were re-modeled using a
579 fragment library, and the side chains were rebuilt. Finally, the geometry of the resulting model is
580 regularized by using a force field. In case loop modeling with ProMod3 fails, an alternative
581 model is built with PROMOD-II. The models showing high accuracy values were finalized for
582 similarity comparisons. Ricin 3-D structure models were also built using ricin chain B amino
583 acids 313-435 and 440-565⁶⁰⁻⁶². Ricin and mucoricin 3-D protein models were aligned using
584 MacPyMOL software and Tm align score was calculated by webtool Tm Align
585 (<https://zhanglab.ccmb.med.umich.edu/TM-align>)⁶³. Using gene ontology term prediction,
586 mucoricin was predicted to have carbohydrate-binding, hydrolase activity and negative
587 regulation of translation functions.

588

589 **Expression and purification of mucoricin.** Heterologous expression of mucoricin gene in *S.*
590 *cerevisiae* was performed to ensure the production of a functional toxin since we used this yeast
591 to generate functional *R. delemar* proteins before^{11,64}. The heterologous expression was
592 conducted as follows; total RNA was isolated from *R. delemar* hyphae grown on YPD broth and
593 reverse transcribed into cDNA. The entire ORF of mucoricin was PCR amplified from cDNA
594 using Phusion High-Fidelity PCR Kit (New England Biolabs) using the primers 5'-
595 GATAAGACTAGTATGTATTTCGAAGAAGGC-3' and 5'-
596 GGTGATGCACGTGTCCTTCAAATGGCACTA-3'. The amplified PCR product was verified
597 by sequencing and then cloned into modified XW55 yeast dual expression vector⁶⁵ in the
598 highlighted *SpeI* and *PmlI* sites downstream of the ADH2 promoter by yeast recombinase
599 technology [protocol Yeastmaker™ Yeast Transformation System 2 (Clontech)] and according to
600 the manufacturer's instructions. The generated yeast expression vector was transformed into *S.*

601 *cerevisiae* strain BJ5464 using protocol Yeastmaker™ Yeast Transformation System 2
602 (Clontech). The transformants were screened on yeast nitrogen base (YNB) medium lacking
603 uracil. *S. cerevisiae* transformed with empty plasmid was served as negative control.
604 Transformants were grown on YNB without uracil for 1-3 days then transformed into YPD
605 medium for 3 days at 30°C with shaking. The expression of mucoricin was induced once the
606 glucose was exhausted from the medium and yielded a recombinant mucoricin that was both 6x
607 His- and Flag-tagged. Purification of the recombinant mucoricin was performed by Ni-NTA
608 matrix affinity purification according to the manufacturers' instructions (Sigma-Aldrich). The
609 purity of the protein was confirmed by SDS-PAGE and quantified by a modified Lowry protein
610 assay (Pierce).

611
612 **Anti-mucoricin and anti-ricin antibodies.** Rabbit and mouse monoclonal antibodies against
613 recombinant mucoricin coupled to KLH were raised by ProMab Biotechnologies Inc.¹¹. The IgG
614 fraction was purified from the antisera using protein A/G spin column (Thermo Fisher Scientific)
615 according to the manufacturer's instructions. Normal IgG was purified from the sera of non-
616 immunized rabbits and used as a control. Rabbit and monoclonal antibodies against ricin and
617 ricin B chain were prepared as described previously^{27,30}. Hybridoma cells producing anti-
618 muroricin antibodies were propagated in WHEATON CELLLine bioreactor 350 using protein-free
619 hybridoma medium 1× (Gibco) for 5 to 7 days at 37°C in 5% CO₂. The antibody-containing
620 supernatant was collected and purified using protein G spin columns (Thermo Fisher Scientific).
621 Eluted purified IgG were dialyzed in PBS using a dialysis cassette (Thermo Fisher Scientific),
622 and the purity of the antibodies was confirmed by SDS-PAGE prior to determining the
623 concentration using the Bradford protein assay (Bio rad, Hercules, CA). Endotoxin levels were

624 measured by the Limulus Amebocyte Lysate (LAL) kit (Charles River) and determined to be
625 <0.8 EU/ml which is below the 5 EU/kg body weight set for intraperitoneal injection⁶⁶.
626 Mouse monoclonal antibody clone 8A1 was raised against the ricin B chain³⁰, which has 33%
627 sequence identify with mucoricin. Clone 8A1 recognizes an epitope mapped to ricin B chain (2γ,
628 amino acids 221-262).
629
630 **Cell damage assay.** The damage of epithelial cell [A549 and Primary (ScienCell, Cat # 3200)]
631 and HUVECs was quantified using a ⁵¹Cr-release assay⁶⁷. Briefly, confluent cells grown in 24-
632 well tissue culture plates were incubated with 1 μCi/well Na₂⁵¹CrO₄ (ICN) in F12K-medium (for
633 epithelial cells) or M-199 medium (HUVECs) for 16 h. On the day of the experiment, the media
634 was aspirated, and cells were washed twice with pre-warmed Hanks' balanced salt solution
635 (HBSS, ScienCell). Cells were treated with toxin suspended in either 1 ml of F12K-medium (for
636 epithelial cells) or RPMI 1640 medium (for endothelial cells) supplemented with glutamine and
637 incubated at 37°C in a 5% CO₂ incubator. Spontaneous ⁵¹Cr release was determined by
638 incubating the untreated cells in the same volume of the culture medium supplemented with
639 glutamine. At different time points, and after data were corrected for variations in the amount of
640 tracer incorporated in each well, the percentage of specific cell release of ⁵¹Cr was calculated as
641 follows: [(experimental release) – (spontaneous release)]/[1 – (spontaneous release)]⁶⁸. Each
642 experimental condition was tested at least in triplicate, and the experiment was repeated at least
643 once.

644 In some experiments, the effect of mucoricin gene silencing on damage to HUVECs or
645 A549 cells was measured by incubating 1.0 x 10⁶/ml or 2.5 x 10⁵/ml spores of *R. delemar* and
646 incubated for 6 or 48 h, respectively. In other experiments the protective effect of IgG anti-

647 mucoricin was measured by incubating the fungal cells with either 50 µg/ml of IgG anti-
648 mucoricin or normal rabbit IgG (R & D Systems, Cat # AB-105-C) for 1 h on ice prior to adding
649 the mixture to A549 cells radiolabeled with ⁵¹Cr. The assay was carried out for 48 h. The amount
650 of damage was quantified as above.

651 To study the effect of fungal cell viability on host cell damage, fungal spores (10⁶/ml)
652 were cultured in F12K media and left to grow overnight at 37°C. The fungal hyphae were
653 collected by filtration, dried by padding with a sterile filter paper, weighed and then aseptically
654 cut into four equal small pieces of 0.1 mg wet weight. The fungal hyphal matt was suspended in
655 1 ml F12K and heated at 60°C in a water bath for 4 h and then cooled down. To check the
656 viability of the hyphal matt, a loop full of the hyphae was plated on PDA plates. The other two
657 groups of fungal hyphae were suspended in preheated and cooled in F12K culture media.
658 Another group of F12K culture media was prepared by heating at 60°C and then cooled to
659 represent spontaneous control. The fungal samples were incubated with ⁵¹Cr-labelled A549
660 alveolar epithelial cells previously seeded into 24-well plates as above and the damage assay was
661 carried out for 24 h at 37°C and the amount of ⁵¹Cr released in the supernatant was measured as
662 above.

663 To determine whether IgG anti-mucoricin protected cells against ricin-induced damage, 5
664 µg/ml (~77 nM) of ricin was incubated with either 10 µg/ml of the monoclonal IgG anti-ricin B
665 chain (clone 8A1)⁶⁹, 10 µg/ml of IgG anti-mucoricin, or normal rabbit IgG (R & D Systems, Cat
666 # AB-105-C) or 10 mM of galactose on ice for 1 h prior to adding to ⁵¹Cr-labelled confluent
667 A549 alveolar epithelial cells in 24-well plate. The damage assay was conducted as above for 24
668 h.

669

670 **Western blotting.** Hyphal expression of mucoricin was determined in *R. delemar* wild-type, or
671 RNAi mutants from hyphal mat grown for overnight at 37°C in YNB without uracil medium⁷⁰.
672 Briefly, mycelia were collected by filtration, washed briefly with PBS, and then ground
673 thoroughly in liquid nitrogen using mortar and pestle for 3 min. The ground powder was
674 immediately transferred to microfuge tube containing 500 µl extraction buffer which consisted of
675 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl₂. The extraction buffer was
676 supplemented with 1X Halt Protease Inhibitor Cocktails (Thermo Scientific) and 1 mM
677 phenylmethylsulfonyl fluoride (PMSF). The sample was vortexed vigorously for 1 min, then
678 centrifuged for 5 min at 21000 g at 4°C. The supernatant was filtered with PES syringe filters
679 (Bioland Scientific, Cat# SF01-02) and transferred to a new tube and the protein concentration
680 determined using Bradford method.

681 For Western blotting, 10 µg of each sample was used to separate proteins on an SDS-
682 PAGE. Separated proteins were transferred to PVDF membranes (GE Water & Process
683 Technologies) and treated with Western blocking reagent (Roche) for overnight at 4°C. The IgG
684 anti-mucoricin (2 µg/ml) and the murine 8A1 monoclonal anti-ricin B chain were used as
685 primary antibodies. After 1 h, 0.5 µg/ml of HRP- IgG anti-rabbit IgG (Jackson ImmunoResearch,
686 Product number 111-035-144) (when rabbit IgG anti-mucoricin was used as a primary) or HRP-
687 IgG anti-mouse (Invitrogen, Cat #31450) (when murine 8A1 antibody anti-ricin B chain used as
688 a primary) secondary antibodies (Jackson Immuno Research) were added for another 1 h at room
689 temperature. Mucoricin bands were visualized by adding the HRP substrate (SuperSignal West
690 Dura Extended Duration Substrate, Thermo Scientific), and the chemiluminescent signal was
691 detected using an In-gel Azure Imager c400 fluorescence system (Azure Biosystems). The
692 intensity of the bands was quantified by ImageJ software.

693 To examine the cross reactivity of ricin with IgG anti-mucorin, 5 μ g of ricin (77 pmol) or
694 recombinant mucorin (294 pmol) were submitted to SDS-PAGE under reducing and denaturing
695 conditions. Western blotting was conducted as above using IgG anti-mucorin as a primary
696 followed by HRP- IgG anti-rabbit IgG as secondary (Jackson ImmunoResearch, Product number
697 111-035-144).

698
699 **Gene expression analysis.** Expression of the mucorin gene was studied in *R. delemar* as
700 spores (10^3 /ml) germinated into hyphae in YPD medium for 16 h at 37°C. At selected times, cells
701 or mycelia were collected by centrifugation, followed by filtration using 0.22 μ m membrane
702 units. The cells were washed once with PBS, and ground in liquid nitrogen using mortar and
703 pestle. RNA was extracted using RNeasy Plant Mini kit (Qiagen). To quantify the expression
704 of the mucorin gene in response to host cells, fungal spores (10^6 /ml) were incubated with either
705 epithelial, HUVECs or human erythrocytes in 24-well plate using F12K, RPMI, or PBS,
706 respectively. The fungal cells were collected at different time intervals including zero, 2 and 5 h
707 and directly ground with liquid nitrogen followed by RNA extraction using RNeasy Plant Mini
708 Kit. Contaminating genomic DNA was removed from RNA samples by treatment with 1 μ l of
709 Turbo-DNase (Ambion) for 30 min at room temperature. DNase was then removed using an
710 RNA Clean-Up kit (Zymo Research). First-strand cDNA synthesis was performed using the
711 Retroscript first-strand synthesis kit (Ambion). Toxin specific primers were designed with the
712 assistance of online primer design software (Genscript). Mucorin gene primers include G7F1;
713 5'-CTGGCGTTACGAAAATGGTT-3' and G7R1; 5'-TAAATCAGGACGGGCTTCAC-3'. The
714 amplification efficiency was determined by serial dilution experiments, and the resulting
715 efficiency coefficient was used for the quantification of the products ⁷¹. Gene expression was

716 analyzed by an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using the
717 QuantiTect Sybr Green PCR kit (Qiagen). PCR conditions were 10 min at 90°C and 40 cycles of
718 15 s at 95°C and 1 min at 60°C. Single PCR products were confirmed with the heat dissociation
719 protocol at the end of the PCR cycles. The amount of gene expression was normalized to
720 actin [ACT1-RT5'; 5'-TGAACAAGAAATGCAAAGTGC-3' and ACT1-RT3'; 5'-
721 CAGTAATGACTTGACCATCAGGA-3'] and then quantified using the $2(-\Delta\Delta C(T))$ method ⁷².
722 All reactions were performed in triplicate, and the mixture included a negative no-reverse
723 transcription (RT) control in which reverse transcriptase was omitted.

724
725 ***In vitro* apoptosis/necrosis assay.** A549 lung epithelial cells were grown to confluency on
726 fibronectin-coated circular glass coverslips in 24-well tissue culture plates and then incubated
727 with 50 µg/ml (2.9 µM) mucoricin or 5 µg/ml (77 nM) ricin (concentrations shown to cause *in*
728 *vitro* damage to alveolar epithelial cells [**Fig. 3f**]) for 2 hours after which the cells were washed
729 and stained with 1x Apoxin Green Indicator and 1x 7-AAD (Apoptosis/Necrosis detection kit,
730 Abcam) for 45 min. The cells were fixed and mounted in ProLong Gold antifade containing
731 DAPI (Life Technologies) to visualize cells. Microscopic z-stack pictures were taken using a
732 Leica SP8confocal laser scanning platform. Apoptotic cells *vs.* necrotic cells were identified by
733 their green and red fluorescence, respectively. The number of apoptotic and necrotic events per
734 high-power field (HPF) was determined, counting 10 HPF per coverslip. The experiment was
735 performed three times in triplicate.

736
737 ***In vitro* protein translation assay.** The ability of the two toxins to inhibit protein synthesis was
738 measured by using a modification of a previously described method³³. Briefly, a rabbit

739 reticulocyte lysate (Promega, Cat: L4151) was thawed at 37°C immediately before use and
740 supplemented with 40 µl of 1 mM hemin stock solution and 10 µl of 1 M creatine phosphate
741 (Sigma-Aldrich, Cat: 27920) and 10 µl of 5 mg/ml creatine phosphokinase (Sigma-Aldrich,
742 Cat:C7886) before the lysate had fully thawed. The reaction mixture was prepared into 96-well
743 plates as follows: 1 µl of 1 mM amino acid mixture minus methionine (Promega, Cat: L9961),
744 35 µl of rabbit reticulocyte lysate, 1 µl of 7-fold dilutions of ricin, mucoricin, control OVA, or
745 cycloheximide (Fisher Scientific, Cat: AC357420010). Diluted distilled water was added to a
746 final volume of 48 µl. Two replicates were employed in all experiments and the experiments
747 were repeated at least three times. After a pre-incubation period of 30 min at 37°C, 2 µl ³⁵S
748 Methionine (1,200 Ci/mmol) (PerkinElmer) was added to a final volume of 50 µl. The 96-well
749 plate was incubated at 30°C for 60 min. Two µl from each well was added per well of a 24-well
750 plate containing 98 µl of 0.5 M H₂O₂. Proteins were precipitated with 900 µl of 25%
751 trichloroacetic acid (TCA) before harvesting precipitates on Whatman filter strips (Sigma-
752 Aldrich, Cat: WHA1823035). Filter paper disks were placed in Biofluor scintillation fluid
753 (Perkin Elmer, Cat: 6013329), and [³⁵S] Methionine incorporation was quantitated by
754 scintillation counting. Background counts determined from well containing all reagents without
755 rabbit reticulocyte lysate were subtracted from all CPMs.

756
757 **The depurination activity assay of mucoricin.** The depurination activity of mucoricin was
758 measured by the release of adenine when mammalian RNA was treated with mucoricin for 24 h at
759 37°C³². Mammalian RNA extracted from A549 alveolar epithelial cells by Qiagen RNasy mini
760 kits according to manufacturer's instruction were treated with 20 µg/ml of mucoricin in 0.01M
761 HEPES/10 mM ammonium acetate buffer containing 1 mg/ml BSA for 24 h at 37°C. The solution

762 was then filtered through a 10 kDa size exclusion column and 40 μ l was injected into HPLC using
763 Phenomenex Luna C18 reverse phase column (10 x 250 mm) attached to a Varian ProStar HPLC
764 218 system (Varian, Walnut Creek, CA). Solvent A was 20 mM ammonium acetate, and solvent
765 B was 100% acetonitrile. The column gradient was as follows: 97% to 60% solvent A in 10 min
766 at flow rate of 1 ml/min. The column effluent was monitored at 260 nm.

767
768 **Glycosylase activity assay.** The N-glycosylase activity of toxins were determined by using rabbit
769 reticulocyte lysate^{73,74}. Briefly, 40 μ l of lysate was incubated with ricin (1 nM), mucorin (10 μ M)
770 or control OVA (1 nM or 10 μ M) in the presence of 10 mM $MgCl_2$ at 30°C for 1 or 4 hours. After
771 the treatment, ribosomal protein was denatured by 50 mM Tris/0.5% SDS to release RNA. The
772 RNA was purified by phenol-Tris extraction followed by ethanol precipitation. Half of each
773 purified RNA sample was subjected to 2 M aniline acetate (pH 4.5) treatment for 10 minutes on
774 ice, while the other half of RNA was incubated without aniline treatment. rRNA were further
775 extracted using water saturated ether followed by ethanol precipitation. Three micrograms of each
776 rRNA sample were resolved on 7 M Urea Polyacrylamide gel electrophoresis and RNA fragment
777 bands were visualized by staining with ethidium bromide.

778
779 **Transwell permeability assay**⁷⁵. HUVECs were seeded on 24-Corning transwell plate with
780 permeable polyester inserts (0.4 μ m, Fisher) coated with fibronectin (15 μ g/ml in PBS, Fisher).
781 HUVECs were grown to confluency in M-199 medium with phenol red. *R. delemar* spores (10^5)
782 in M-199 (without phenol red) were added to the upper chamber, and the plate was incubated for
783 5 h at 37°C. As a positive control for the permeability of HUVECs, *E. coli* LPS (Sigma-Aldrich)
784 was added at 2 μ g/ml to uninfected HUVECs. Following incubation, 3 μ l of 50 mg/ml FITC-

785 dextran-10K (Sigma) was added to the upper chamber of the trans-well and the migration of the
786 dextran through the HUVEC monolayer to the lower trans-well was determined 1 h later by
787 quantifying the concentration of the dye in the bottom chamber using fluorescence microplate
788 reader at 490 nm⁷⁵. To determine the direct effect of mucoricin on the permeability of the
789 HUVEC monolayer, 50 µg/ml (2.9 µM) mucoricin or control OVA were added to the HUVEC-
790 seeded wells instead of *R. delemar*. To determine the effect of antibodies on permeability
791 induced by *R. delemar* or mucoricin, 50 µg/ml of normal rabbit IgG (R & D Systems, Cat # AB-
792 105-C), IgG anti-mucoricin, or 10 µg/ml of IgG anti-ricin toxin chain B (clone 8A1) were
793 incubated for 30 min on ice with *R. delemar* spores or mucoricin prior to their addition to the
794 upper chamber of the trans-well.

795

796 ***In vivo* effects induced by mucoricin.** To test the effect of the purified toxin *in vivo*, male (ICR
797 mice, ~27-32g) were immunosuppressed by intraperitoneal injection of 200 mg/kg of
798 cyclophosphamide and subcutaneous injection of 250 mg/kg cortisone acetate on day -2 and +3,
799 relative to toxin injection. This regimen results in approximately 10 days of leucopenia with
800 reduction in neutrophils, lymphocytes and monocytes as described previously⁷⁶. Mouse gender
801 has no effect on the pathogenesis of mucormycosis, or antifungal treatment as determined by an
802 NIH Contract No. HHSN272201000038I/Task Order HHSN27200008, unpublished data. Mice
803 were given irradiated food and sterile water containing 50 µg/ml baytril (Bayer) ad libitum. 100
804 µl of purified mucoricin (0.1 mg/ml) was then injected into the tail vein on days 0, +2, and +4.
805 The differences in survival between normal mice receiving vehicle (*i.e.* PBS) and those received
806 toxin were compared by the Log Rank test. The primary efficacy endpoint was time to
807 morbidity.

808 Mouse tissues were fixed in 10% ZnCl₂ formalin solution prior to histopathological
809 examination. The fixed organs were dehydrated in graded alcohol solutions, embedded in
810 paraffin, and 5- μ m sections were cut and stained with H&E⁷⁷. Cumulative histopathological
811 scores of hemorrhages, neutrophil infiltration (inflammation), and edema were used to determine
812 the effects of toxin by observing 5 fields per slide. The observer was not told the origin of the
813 samples.

814
815 **Mucoridin RNAi knockdown.** RNAi knockdown of mucoridin was employed using our
816 previously described RNAi method²⁰. Briefly, a 330-bp mucoridin transcript was PCR amplified
817 using 5'-AAATTTAAAGCATGCACACACAAAAGTATGAAGATTGCT-3' and 5'-
818 CTGCTTACCATGGCGCGCCCAAATGGCACTAATTCCCAGC-3' primers and cloned into
819 the *Sph*I and *Asc*I sites of pRNAi-pdc⁷⁸. The inverted repeat fragment was PCR amplified by 5'-
820 TTAAGCGATCGCTAGCACACACAAAAGTATGAAGATTGCT-3' and 5'-
821 TTATTCTTATAGCCCGCGGCAAATGGCACTAATTCCCAGC-3' at cloned downstream the
822 intro fragment at the *Nhe*I and *Sac*II sites. The developed construct was transformed into *R.*
823 *delemar* pyrF mutant (strain M16)⁵⁵ using the biolistic delivery system (BioRad), and the
824 homogenous transformants were selected on minimal medium lacking uracil¹¹. The down
825 regulation of mucoridin expression was confirmed by qRT-PCR using primers 5'-
826 CTTGGATATCCGTGGAGGTGA-3' and 5'-GGCAGCTTCTTCGACCATCT-3' as described
827 before¹² and by confocal microscopy using immunostaining (see below)¹¹.

828
829 **Secretion/shedding of mucoridin into the culture supernatant.** Wild-type *R. delemar* spores
830 (2×10^4 /100 μ l/well), *R. delemar* transformed with the empty plasmid or those transformed with
831 mucoridin RNAi were grown in 96-well plates for 24 h at 37°C followed by additional 24 h of

832 incubation in the presence or absence of 2-fold serially diluted amphotericin B (0.06-32 $\mu\text{g/ml}$).
833 100 μl of culture supernatant samples from each well were collected and stored at -20°C until
834 used for toxin detection by ELISA. To determine corresponding fungal growth, 100 $\mu\text{l/well}$ XTT
835 substrate (0.20 mg/ml activated with 6.25 μM menadione) was added to the remaining *R.*
836 *delemar* culture plate.⁷⁹ After a 2 h incubation at 37°C , absorbance at 450 nm was measured for
837 metabolized XTT. Sandwich ELISAs were used to detect and quantify mucoricin in the cell-free
838 supernatants. Briefly, 96-well plates were coated with 2 $\mu\text{g/ml}$ mouse anti-*R. delemar* toxin
839 monoclonal antibodies at 4°C overnight. After washing the plate with 1X PBST (PBS+ 0.05%
840 Tween-20) 5 times, diluted recombinant mucoricin or undiluted culture supernatant samples
841 were added to the ELISA plate. Bound mucoricin was detected by the IgG anti-toxin antibodies
842 (2 $\mu\text{g/ml}$), and subsequently by HRP- IgG anti-rabbit IgG (Jackson ImmunoResearch, product
843 number 111-035-144) and a TMB substrate detection system (Invitrogen). A standard curve was
844 generated using linear regression of OD_{450} of known recombinant mucoricin concentrations and
845 the concentrations of toxin in the medium were extrapolated from the standard curve.

846
847 **Confocal microscopy.** IgG anti-toxin was used to localize the toxin in the *Rhizopus* fungus¹⁰.
848 Fungal spores ($10^5/\text{ml}$) were pre-germinated in YPD media at 1, 4, or 12 h. Each fungal stage
849 was fixed in 4% paraformaldehyde followed by permeabilization for 10 min in 0.1% Triton X-
850 100. The permeabilized fungal growth stages were incubated with the IgG anti-toxin for 2 h at
851 room temperature. The fungal stages were then washed 3 times with Tris-buffered saline (TBS,
852 0.01 M Tris HCl [pH 7.4], 0.15 M NaCl) containing 0.05% Tween 20 and counterstained with
853 anti-rabbit IgG Alexa Fluor 488 (Life Technologies, Cat # A-11034). The stained fungi were
854 imaged with Leica confocal microscope at excitation wavelength of 488 nm. The final confocal

855 images were produced by combining optical sections taken through the z axis.

856

857 ***In vivo* virulence studies and immunohistochemistry.** Male ICR mice (≥ 20 g) were rendered
858 DKA with a single intraperitoneal injection of 210 mg/kg streptozotocin in 0.2 ml citrate buffer
859 10 days prior to fungal challenge. On days - 2 and +3 relative to infection, mice were given a
860 dose of cortisone acetate (250 mg/kg). Diabetic ketoacidotic (DKA) mice were given irradiated
861 food and sterile water containing 50 $\mu\text{g/ml}$ Baytril (Bayer) ad libitum. DKA mice were infected
862 intratracheally with fungal spores with a target inoculum of 2.5×10^5 spores of RNAi-empty
863 plasmid (Control strain) or RNAi-mucoricin (targeting mucoricin gene expression) in 25 μl . To
864 confirm the fidelity of the inoculum, three mice were sacrificed immediately after inoculation,
865 their lungs were homogenized in PBS and quantitatively cultured on PDA plates containing 0.1%
866 triton, and colonies were counted after a 24-hour incubation period at 37°C. Average inhaled
867 inoculum for RNAi-empty plasmid and RNAi-mucoricin were 8.6×10^3 and 3.3×10^3 spores
868 from two experiments, respectively. Primary endpoint was time to moribundity analyzed by
869 Kaplan Meier plots. In another experiment, DKA mice were infected as above and then
870 sacrificed on Day +4 relative to infection, when their lungs and brains (primary and secondary
871 target organs) were collected and processed for determination of tissue fungal burden by qPCR²¹.
872 The ability of the IgG anti-toxin to protect against *Rhizopus* infection was also evaluated in the
873 DKA mouse model. Briefly, DKA mice were infected with *R. delemar* 99-880 as above (average
874 inhaled inoculum of 5.6×10^3 spores from two experiments) and 24 h later were injected
875 intraperitoneally with either a 30 μg of IgG anti-toxin or normal rabbit IgG (R & D Systems, Cat
876 # AB-105-C). The survival of mouse and tissue fungal burden of target organs collected on Day
877 +4 post infection served as endpoints as above. Furthermore, histopathological examination was

878 carried out on sections of the organs harvested on Day +4 post infection. These organs were
879 fixed in 10% zinc formalin and processed as above for histological examination with H&E, PAS
880 or Grocott staining.

881 Apoptotic cells in the lung were detected by immunohistochemistry using the ApopTag
882 *in situ* apoptosis detection kit (EMD Millipore) following the manufacturer's directions. Briefly,
883 paraffin-embedded sections were rehydrated in Histo-Clear II (National Diagnostics) and
884 alcohols followed by washing with phosphate-buffered saline (PBS). The sections were pre-
885 treated with 20 µg/ml Proteinase K (Ambion) in PBS for 15 min at room temperature.
886 Endogenous peroxidases were blocked by incubation of the slides for 15 min in 3% hydrogen
887 peroxide. Sections were incubated with equilibration buffer (EMD Millipore) for 30 sec at RT,
888 followed by terminal deoxynucleotidyl transferase (TdT; EMD Millipore) at 37°C for 1 h.
889 Sections were further exposed to anti-Digoxigenin for 30 min at RT, and the positive reaction
890 was visualized with DAB 3, 3-diaminobenzidine (DAB) substrate (Thermo Scientific). After
891 counterstaining the specimens with 0.5% methyl green (Sigma), they were imaged by bright field
892 microscopy. For quantification, apoptotic areas were quantified using PROGRES GRYPHAX®
893 software (Jenoptik).

894

895 **Immunofluorescence staining for mucorin in human tissue samples.** Paraffin-embedded
896 human lung tissue from a patient diagnosed with disseminated mucormycosis⁹ or a patient with
897 proven invasive pulmonary aspergillosis were cut into 5 µm sections that were then mounted
898 onto glass slides. Organ sections on slides were deparaffinized and rehydrated with an ethanol
899 gradient (100%-70%) followed by incubation of the slides in water and heat-induced antigen
900 retrieval in sodium citrate buffer (10 mM, pH 6). Sections were blocked with 3% bovine serum

901 albumin (BSA) in PBS (BSA-PBS), incubated for 1 h with 1:50 dilution of the IgG anti-
902 mucoricin in PBS, washed twice in PBS, stained with 1:500 dilution of the appropriate goat anti-
903 rabbit IgG Alexa Fluor[®] 488 (Life Technologies, Cat #A-11034) in 1x PBS, followed by DNA
904 staining with 1 μ M TOPRO-3 iodide (642/661; Invitrogen) and staining of the fungal hyphae
905 with 100 μ g/ml Fluorescent Brightener 28 (Sigma-Aldrich, Cat #475300). After washing with 1x
906 PBS, slides were mounted in Prolong Gold antifade media (Molecular Probes). Images were
907 acquired using a laser-scanning spectral confocal microscope (TCS SP8; Leica), LCS Lite
908 software (Leica), and a 40 \times Apochromat 1.25 NA oil objective using identical gain settings. A
909 low fluorescence immersion oil (11513859; Leica) was used, and imaging was performed at
910 room temperature. Serial confocal sections at 0.5 μ m steps within a z-stack spanning a total
911 thickness of 10 to 12 μ m of tissue, and 3D images were generated using the LCS Lite software.
912 Corresponding tissue sections from the same area were also stained with hematoxylin and eosin.

913

914 **Statistical analysis.** The data was collected and graphed and statistically analyzed using
915 Microsoft Office 360 and Graph Pad 8.0 for Windows or Mac (GraphPad Software, La Jolla,
916 CA, USA). Cell damage and gene expression were analyzed using one-way analysis of variance
917 (ANOVA) using Dunnett's Multiple Comparison Test. The non-parametric log-rank test was
918 used to determine differences in mouse survival times. Differences in tissue fungal burdens were
919 compared by the non-parametric Wilcoxon rank sum test for multiple comparisons. $P < 0.05$ was
920 considered as significant. All *in vitro* experiments were performed at least in triplicate and
921 replicated at least once.

922

923 **Study approval.** All procedures involving mice were approved by the IACUC of The Lundquist
924 Institute for Biomedical Innovations at Harbor-UCLA Medical Center, according to the NIH
925 guidelines for animal housing and care. Human endothelial cell collection was approved by the
926 IRB of The Lundquist Institute for Biomedical Innovations at Harbor-UCLA Medical Center.
927 Because umbilical cords are collected without donor identifiers, the IRB considers them medical
928 waste not subject to informed consent. The purification and testing of ricin were approved by the
929 IRB at UT Southwestern and carried out under BSL3 guidelines. Approval for the collection of
930 tissue samples from the patients with mucormycosis and invasive pulmonary aspergillosis was
931 obtained and the Ethics Committee of the University Hospital of Heraklion, Crete, Greece
932 (5159/2014). The patients provided written informed consent in accordance with the Declaration
933 of Helsinki.

934

935 **Data availability.** Source data are provided with this paper.

936

937

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1160 **Figure Legends**

1161 **Figure 1. *R. delemar* toxin is sufficient to cause damage *in vitro* and *in vivo*.** (a) The effect of
1162 toxin on different cell lines (n=7 wells /time point pooled from three independent experiments).
1163 Data are median \pm interquartile range. Statistical analysis was performed by using the non-
1164 parametric Mann-Whitney (two-tailed) test comparing HUVECs vs. primary alveolar epithelial
1165 cells or A549 alveolar cells. (b) Damage of extracted or recombinant toxin (~ 500 μ g/ml or 29.4
1166 μ M) on epithelial cells at different time points (n=3 wells/time point). Data are representative of
1167 three independent experiments and presented as median \pm interquartile range. (c) Mouse (n=3
1168 mice/group) weight loss (data are median \pm interquartile range) and (d) percent survival (n=3
1169 mice/group) following intravenous injection with 0.1 mg/ml (5.9 μ M) toxin QOD x 3. (e) Mouse
1170 organ H&E histomicrographs showing the effects of the toxin. Livers showed necrosis (white
1171 arrow), infiltration and calcification of PMNs (black arrow) due to inflammation and a cluster of
1172 mononuclear cells (cyan arrow). Lungs showed megakaryocytes (black arrow) and hemorrhage
1173 (yellow arrow). Data in each group are representative of 2 mice. Scale bar 50 μ m for first liver
1174 micrograph and 100 μ m for all other. For lung micrographs scale bar 50 μ m.

1175 **Figure 2. Inhibition of *R. delemar* toxin attenuates virulence of *R. delemar*.** (a) RNAi toxin
1176 shows reduced damage to A549 cells compared to wild type or empty plasmid *R. delemar* (n=6
1177 wells/group pooled from three independent experiments). Data are median \pm interquartile range.
1178 Statistical comparisons are by the non-parametric Mann-Whitney (two-tailed) test. (b) IgG anti-
1179 toxin antibodies reduced *R. delemar*-induced injury of A549 cells compared to *R. delemar* without
1180 IgG or normal rabbit IgG (n = 13 wells/group pooled from three independent experiments). Data
1181 are median \pm interquartile range. Statistical comparisons are by the non-parametric Mann-Whitney
1182 (two-tailed) test. (c) RNAi toxin inhibition prolonged survival of mice (n= 18 mice) compared to

1183 *R. delamar* with empty plasmid (n= 17 mice). Data were pooled from two independent
1184 experiments. Survival data were analyzed by Log-rank (Mantel-Cox) test. **(d)** IgG anti-toxin
1185 prolonged survival of mice compared to normal rabbit IgG (n=20 mice/group). Data were pooled
1186 from two independent experiments. Survival data were analyzed by Log-rank (Mantel-Cox) test.
1187 **(e)** Histopathological sections of lungs from uninfected mice, **(f)** mice infected with the RNAi
1188 empty plasmid *R. delamar* strain showed hyphae and granulocyte infiltration (left panel, arrows)
1189 and angioinvasion (right panel, arrow), vs. **(g)** mild signs of inflammation and no angioinvasion
1190 for mice infected with RNAi toxin. **(h)** IgG anti-toxin group had normal lung tissue architecture.
1191 Data in **e-h** are representative of 3 mice and scale bar is 20 μ m.

1192 **Figure 3. *R. delamar* toxin and ricin share structural features.** **(a)** *R. delamar* toxin has 29%
1193 amino acid sequence identity with ricin. Both toxins share similar motifs and molecular functions.
1194 **(b)** 3-D structure model of *R. delamar* toxin shows similarities to ricin B chain. Protein 3D
1195 structure models of *R. delamar* toxin and ricin chain B (amino acids 304-437, and 338-565) were
1196 aligned residue-to-residue based on structural similarity using heuristic dynamic programming
1197 iterations and sequence independent TM-align score (0-1) were calculated based on structural
1198 similarity. TM-align score >0.5 considered significant similarity. **(c)** IgG anti-*R. delamar* toxin
1199 binds to ELISA plates coated with either *R. delamar* toxin or ricin. **(d)** Ricin is recognized on a
1200 dot blot by IgG anti-*R. delamar* toxin. **(e)** Western blot of *R. delamar* toxin and ricin using IgG
1201 anti-*R. delamar* toxin IgG. **(f)** IgG anti-*R. delamar* toxin, IgG anti-ricin (8A1 clone) (10 μ g/ml
1202 each) or galactose (10 mM) inhibit ricin (77 nM)-mediated A549 cell damage (n=9 wells for
1203 normal rabbit IgG and Anti-ricin toxin B chain (8A1) group, n=8 for IgG anti-*R. delamar* toxin
1204 and galactose group pooled from three independent experiments). Data are median \pm interquartile

1205 range. Statistical comparisons were made by using the non-parametric Mann-Whitney (two-tailed)
1206 test.

1207 **Figure 4. *R. delemar* toxin and ricin have functional similarities. (a,b)** Cell-free rabbit
1208 reticulocyte assay showing protein synthesis inhibition by ricin (IC₅₀ of 2.2 x 10⁻¹¹ M) (**a**) and *R.*
1209 *delemar* toxin (IC₅₀ of 1.7 x 10⁻⁸ M) (**b**). Data (n=7 wells/concentrations for ricin; and n=6
1210 wells/concentration for *R. delemar* toxin, pooled from three experiments) are presented as median
1211 ± interquartile range. (**c**) Representative HPLC chromatograms demonstrating the depurination
1212 activity of *R. delemar* toxin of A549 RNA at 3.6 min similar to adenine standard. (**d**) A
1213 representative gel (from three experiments) showing rRNA glycosidase activity of *R. delemar*
1214 toxin (1 μM) compared to ricin (1 nM) and control OVA (1 nM or 1 μM). Ribosomes were treated
1215 with ricin for 1 h or *R. delemar* toxin for 4 h. Extracted RNA were treated with (+) or without (-)
1216 aniline prior to running the gel. Arrows point to endo fragment at ~500 bp. (**e, f**) *R. delemar* induces
1217 HUVEC permeability via its toxin. *R. delemar* (**e**) or recombinant toxin (2.9 μM) (**f**) were
1218 incubated with HUVEC for 5 h with or without 50 μg/ml of IgG isotype-matched or anti-*R.*
1219 *delemar* toxin or 10 μg/ml of IgG anti-ricin chain B (clone 8A1). LPS or OVA were added as a
1220 positive and negative controls, respectively. For **e**, n= 13 wells except for IgG anti-ricin 8A1 which
1221 n=12 wells pooled from three independent experiments. For **f**, n= 6 wells for Ova, n=10 wells for
1222 *R. delamar* toxin alone and *R. delamar* toxin + IgG anti-*R. delamar* toxin, n=11 wells for *R. delamar*
1223 toxin + Isotype IgG, n=12 wells for *R. delamar* toxin +IgG anti-ricin (8A1), and n=13 wells for
1224 HUVECs and LPS. Data in **e** and **f** were pooled from three independent experiments and presented
1225 as median ± interquartile range. (**g**) Detection of apoptosis/necrosis of A549 cells incubated for 2
1226 h with 50 μg/ml (2.9 μM) of *R. delemar* toxin or 5 μg/ml (77 nM) ricin. Apoptotic cells (closed
1227 triangle) were identified by green fluorescence while necrotic cells (open triangle) are shown in

1228 red. Scale bar is 50 μm . **(h)** The number of apoptotic and necrotic events per high-power field
1229 (HPF) was determined, counting 10 HPF per coverslip. The data is combined from 3 independent
1230 experiments with each group in triplicate (total n=9 wells) and presented as median \pm interquartile
1231 range. Kruskal-wallis test was used to compare control vs. *R. delamar* toxin or ricin.

Extended Data Figures Legends

Extended Data Figure 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 \pm 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 \pm 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 \pm 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 \pm 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 \pm interquartile range. (f) IgG anti-*R. delemar* toxin but not normal rabbit IgG (50 μ g/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 \pm 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 Figure 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 \pm 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 \pm interquartile range. (d) Cellulose plate separation of fraction # 6 purified from the polyacrylamide gel and (e) its corresponding A 549 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 \pm 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 \pm interquartile range.

Extended Data Figure 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 μ g/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 Figure 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 \pm 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 μ m. (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 \pm 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 \pm interquartile range. Statistical analysis was performed by using unpaired t-test (two-tailed).

Extended Data Figure 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 \pm 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 \pm 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 μ m.

Extended Data Figure 6. Down regulation 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 μ m. (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 \pm interquartile range.

Extended Data Figure 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 1.4×10^4 for empty plasmid [n=22 mice] vs. 1.3×10^4 for RNAi toxin mutants [n=20 mice]). Data are pooled from two independent experiments and presented as median \pm 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 \pm 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 Figure 8. Histology of organs showing involvement of the toxin in tissue damage. (a) Damaged lung tissues (brown color) 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 μ m. (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 μ m.

Extended Data Figure 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 μ m. Box magnification 1400 X. Staining of a mucormycosis (c) or aspergillosis (d) patient lungs using IgG anti-toxin (green color). 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 μ m in all micrographs.

Extended Data Figure 10. Secretion/shedding of *R. delemar* toxin in culture supernatant of growth media. (a) Cell-free culture supernatants were collected from *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 *R. delemar* (left axis, blue bar, n=8 wells/amphotericin B concentration), while toxin release assayed by sandwich ELISA using anti-*R. delemar* mouse monoclonal IgG1 as the capture antibody and rabbit anti-*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 \pm SD. (b) The released toxin concentration from *R. delemar* wild-type, *R. delemar* transformed with empty plasmid RNAi or *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 \pm SD.

Supplementary Figure Legends

Supplementary Figure 1. Incubation of lower inoculum of *R. delemar* with HUVECs induces minimal to no host cell injury. Data are presented as % ^{51}Cr -released from HUVECs challenged with 1×10^5 spores of *R. delemar* for 5 hours after subtracting the amount of ^{51}Cr -released from HUVECs without *R. delemar* challenge. (n= 10 data pooled from three independent experiments). Data presented as median + interquartile range.

Supplementary Figure 2. CLUSTAL multiple sequence alignment by MUSCLE (3.8) between mucoricin and saporin from *Saponaria officinalis*. The predicted Type 1 RIP domain in saporin (shown in yellow) aligned with sequence from mucoricin with 10 out of 17 amino acid residues conserved.

Supplementary Table 1: Results of BLAST search of a ricin-like toxin gene from *R. delemar* 99-880.

Supplementary Table 2. Ten proteins that are structurally similar to mucoricin. The 3-D model of mucoricin was used to identify structurally similar proteins in the protein data bank (PDB) by Tm align.

Supplementary Table 3: Ricin orthologues in different Mucorales and the presence of vascular leak and RIP motifs.

Figure 1

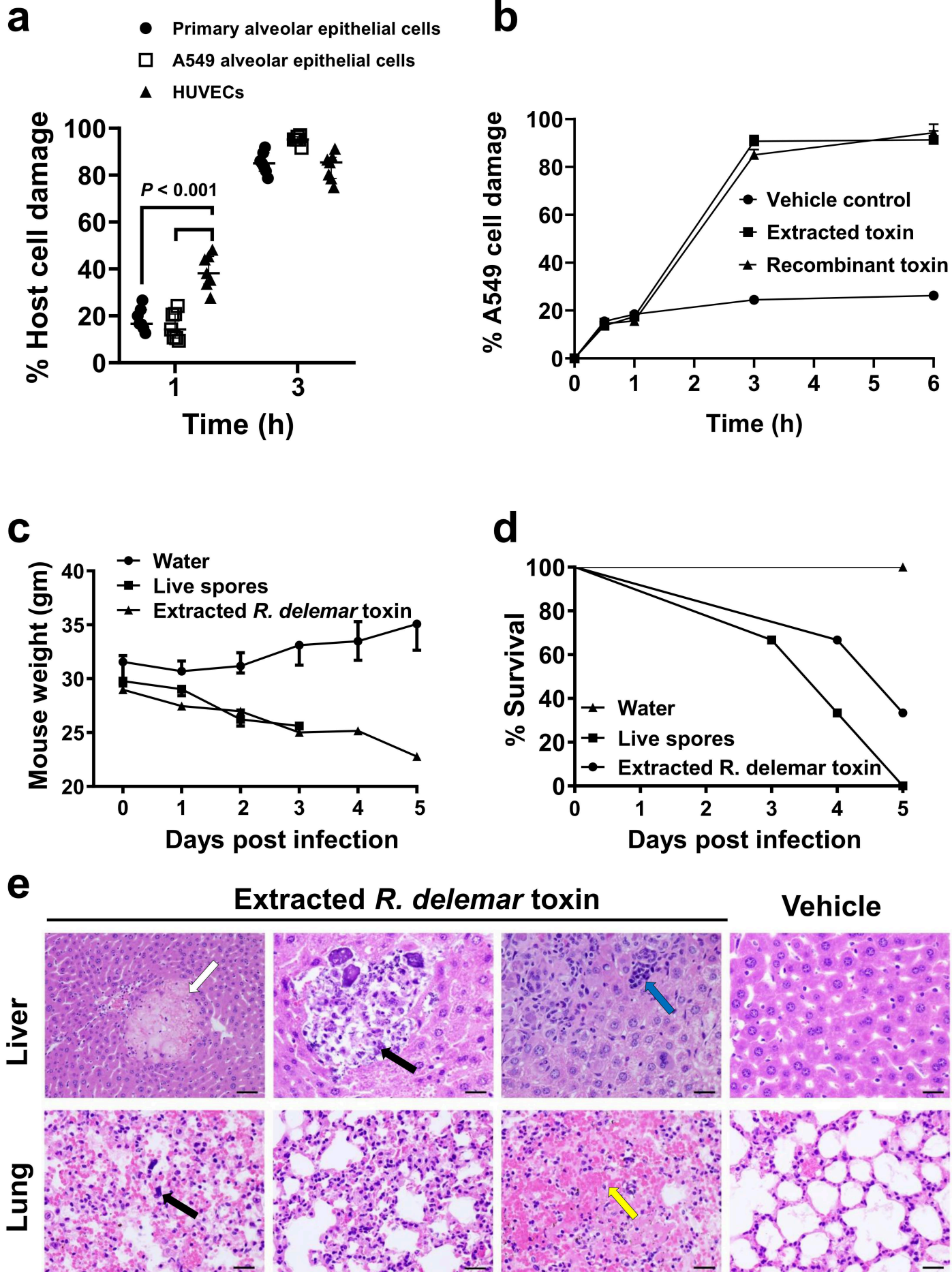
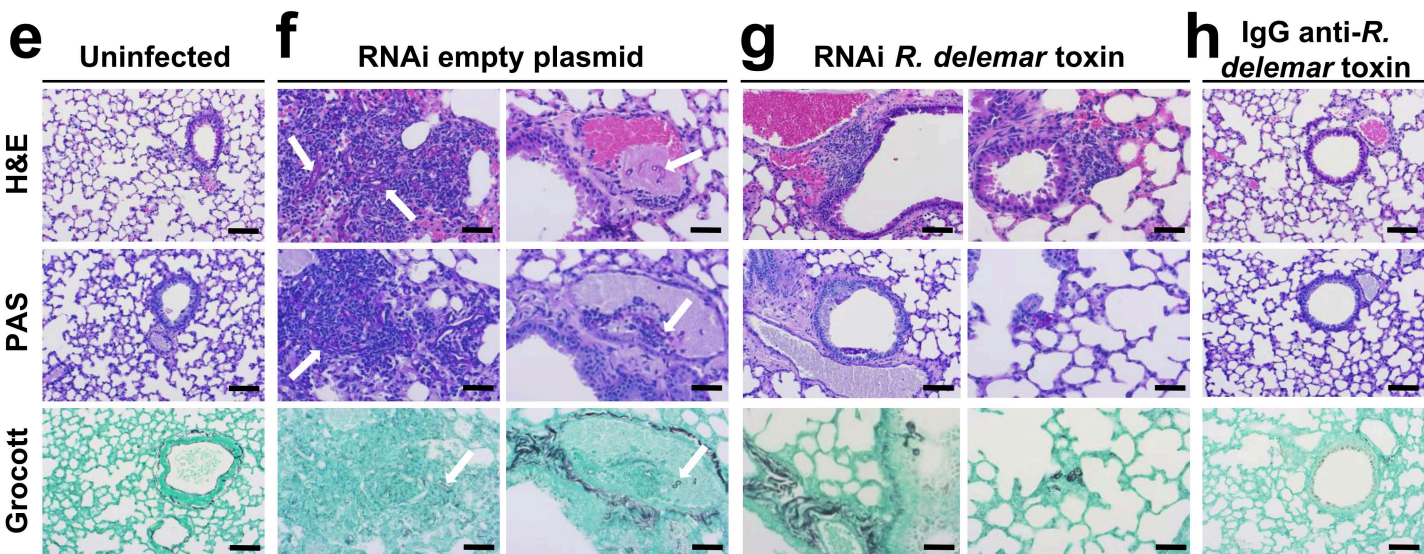
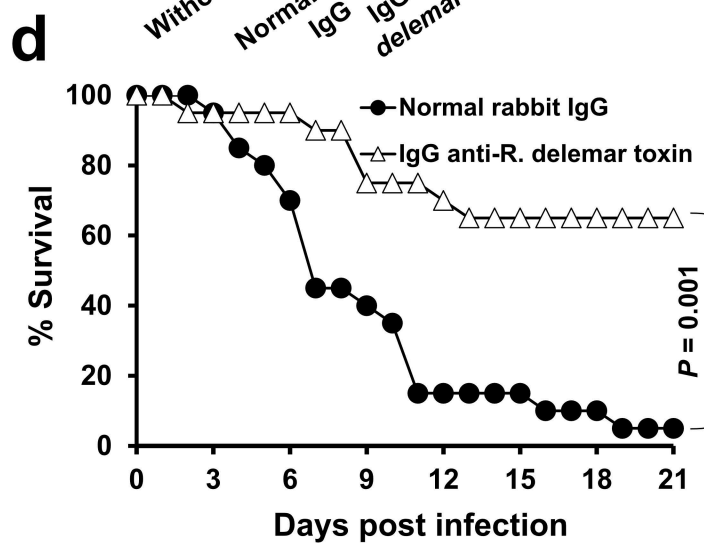
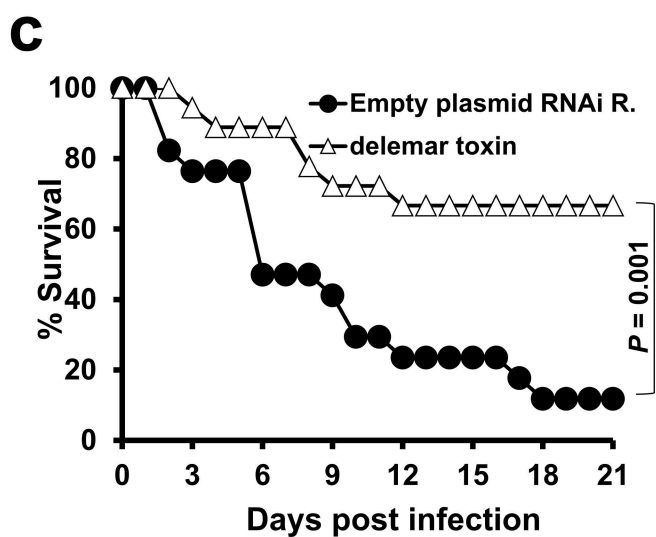
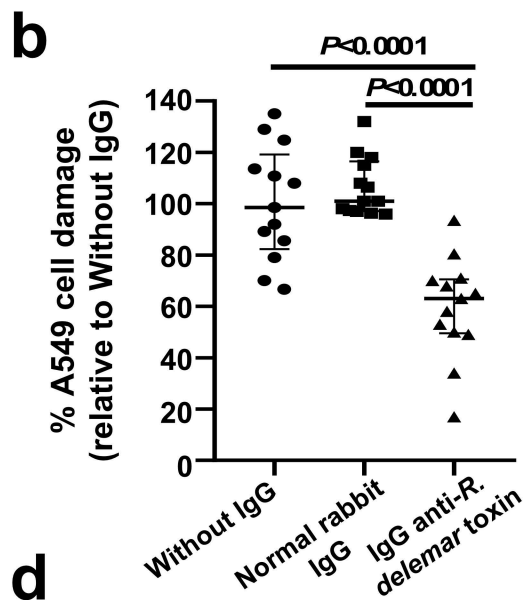
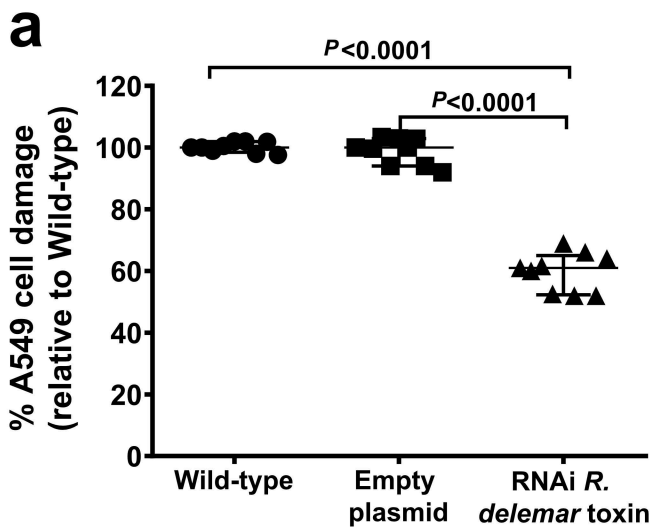
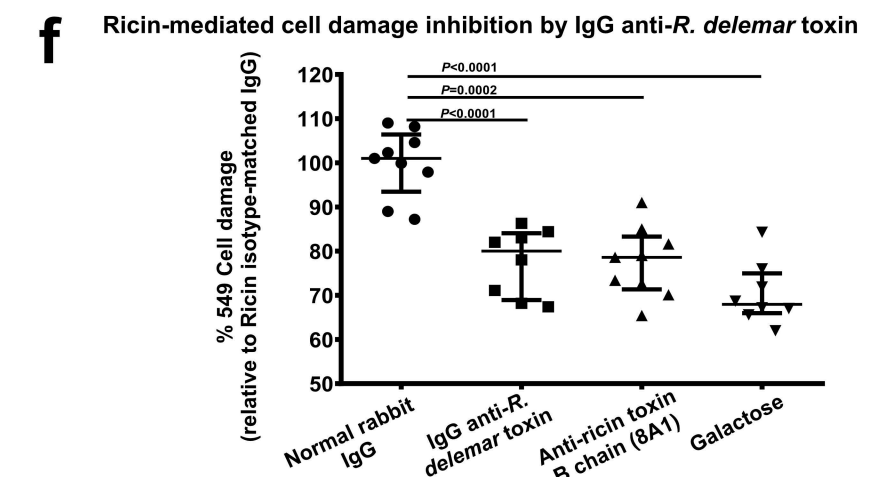
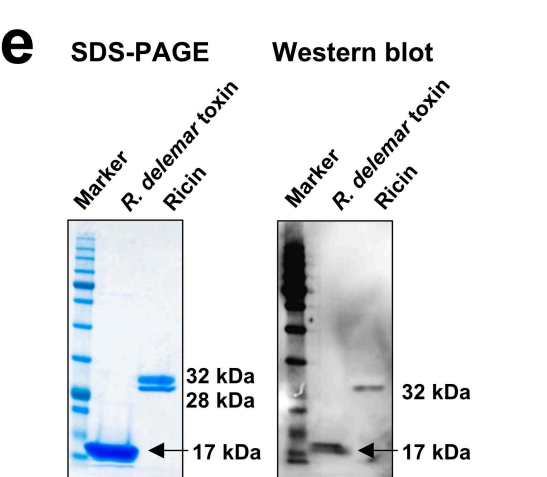
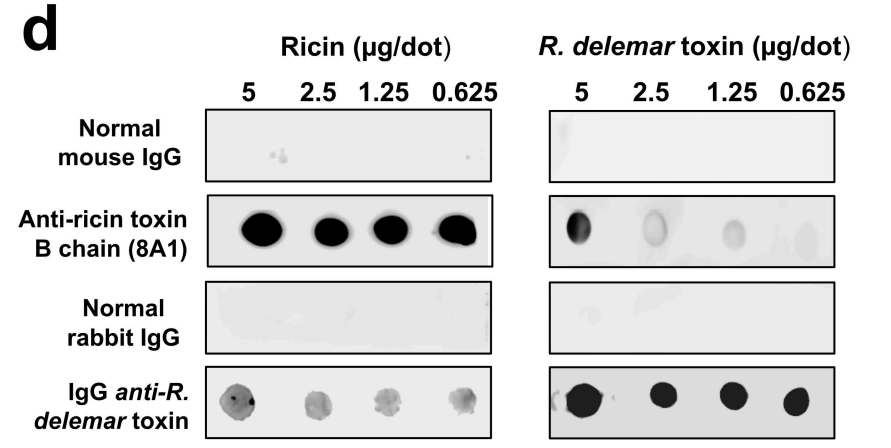
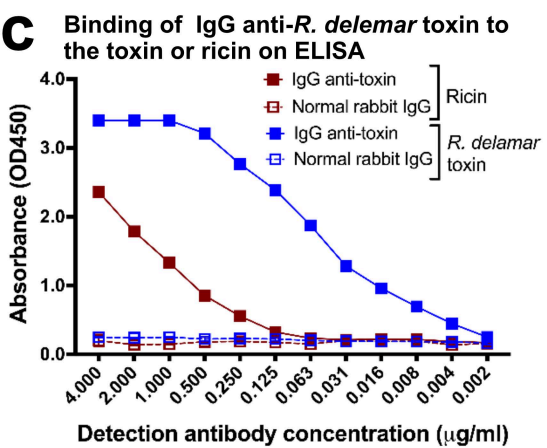
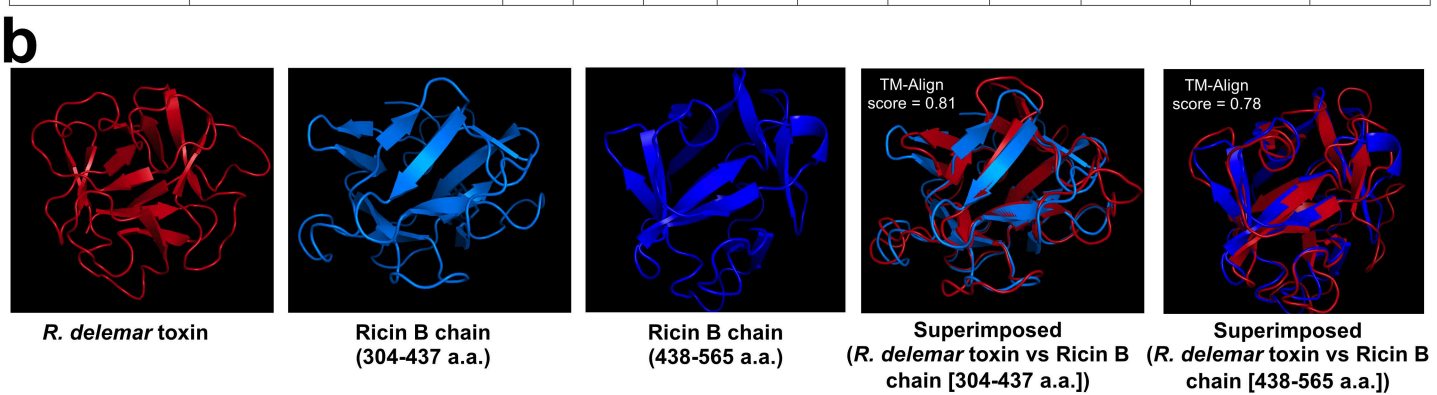


Figure 2





NCBI Sequence ID	Name	Protein sequence homology					Vascular leak motif (LDV/VDV)	Predicted molecular functions			
		Length	Max Score	Total Score	Query Cover	Identity		Sugar Binding	Lectin Receptor Binding	rRNA N-glycosylase activity	Hydrolase activity
NP_001310630.1	Ricin precursor [<i>R. communis</i>]	565	26.6	105	87%	29%	Yes	Yes	Yes	Yes	Yes
EIE81863.1	<i>R. deleamar</i> toxin	147					Yes	Yes	Yes	Yes	Yes



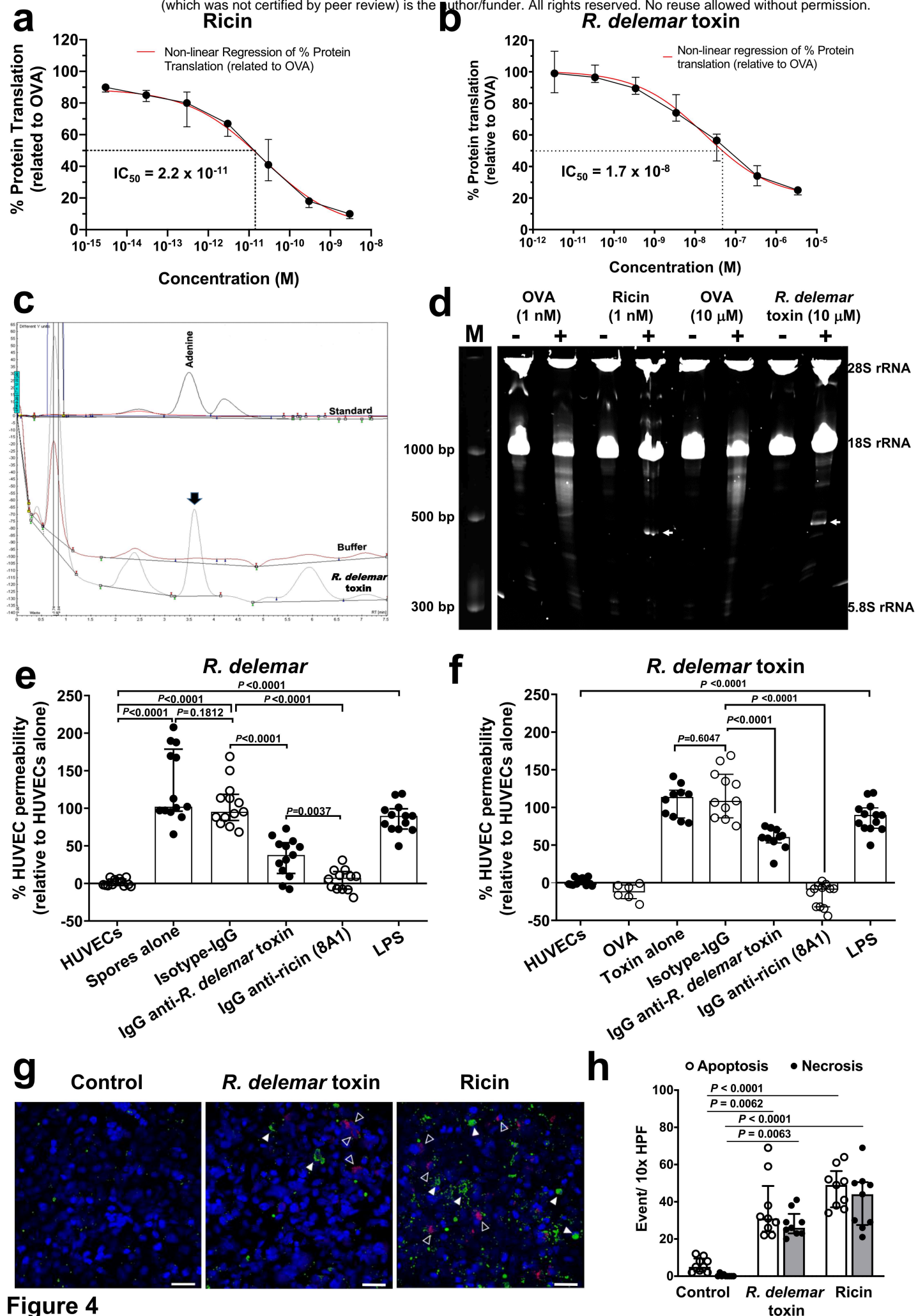
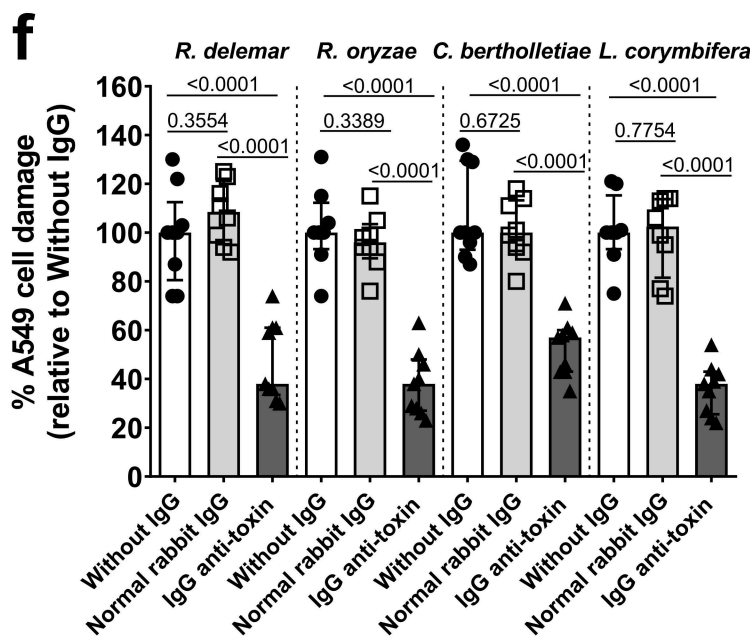
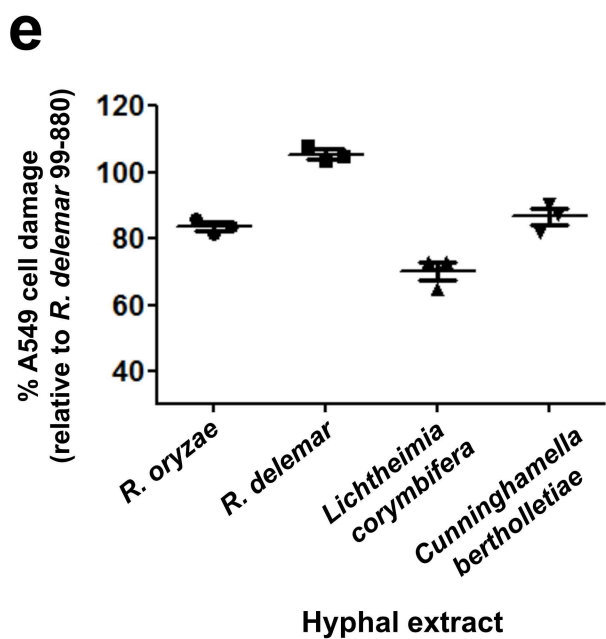
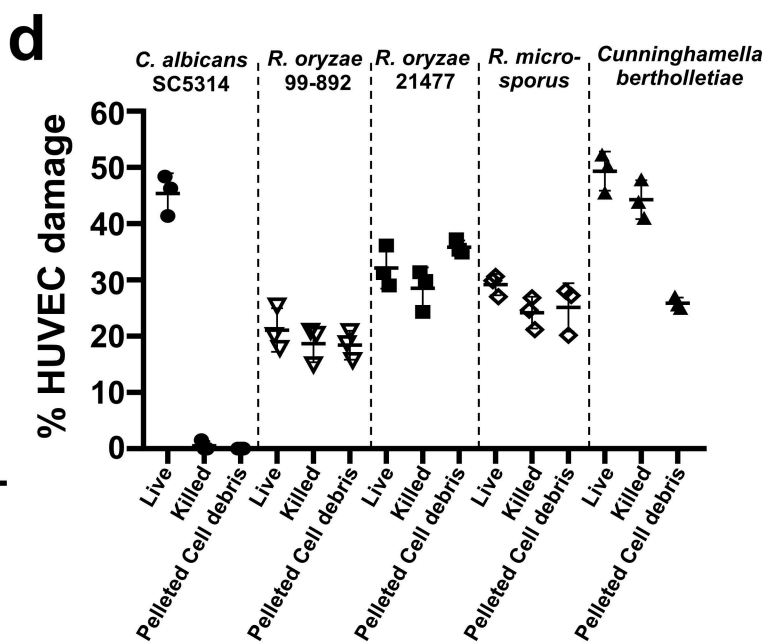
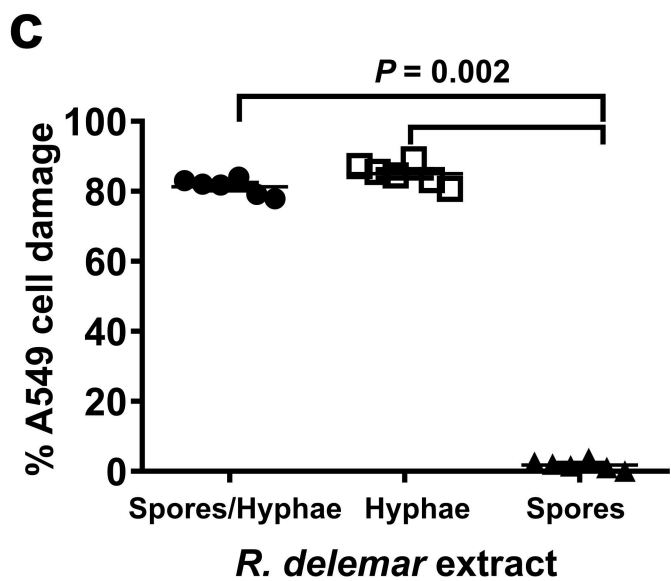
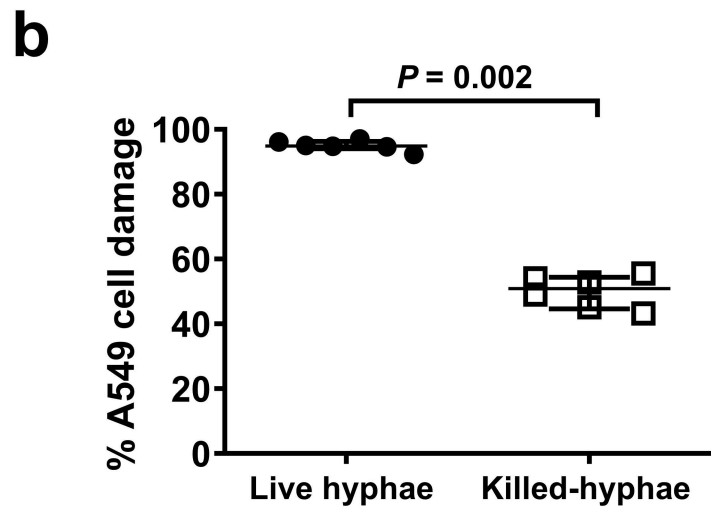
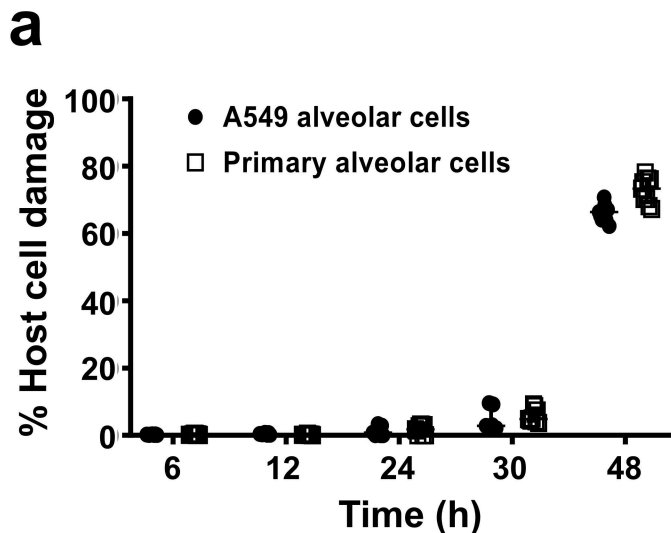
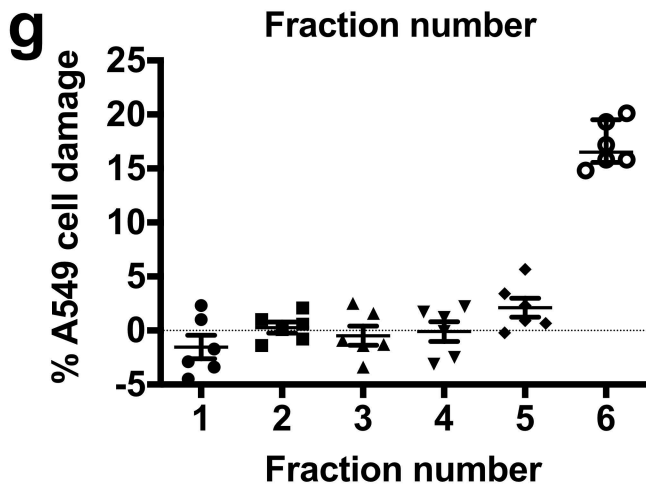
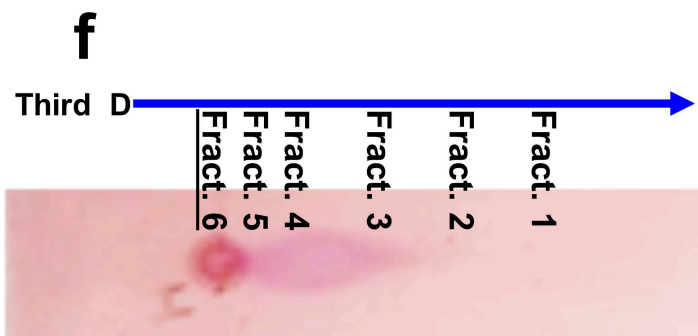
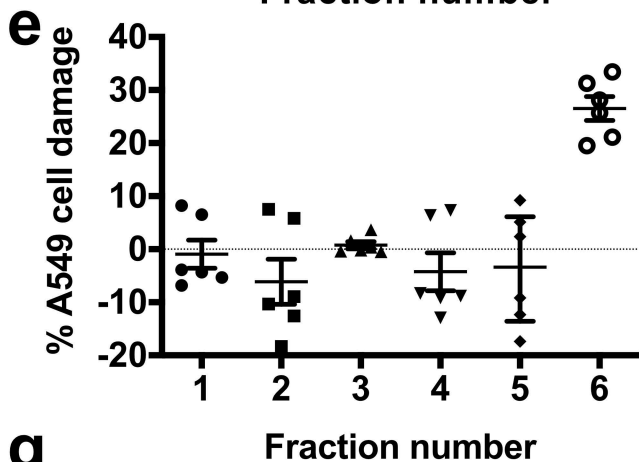
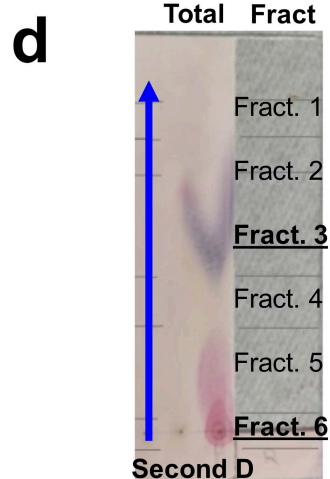
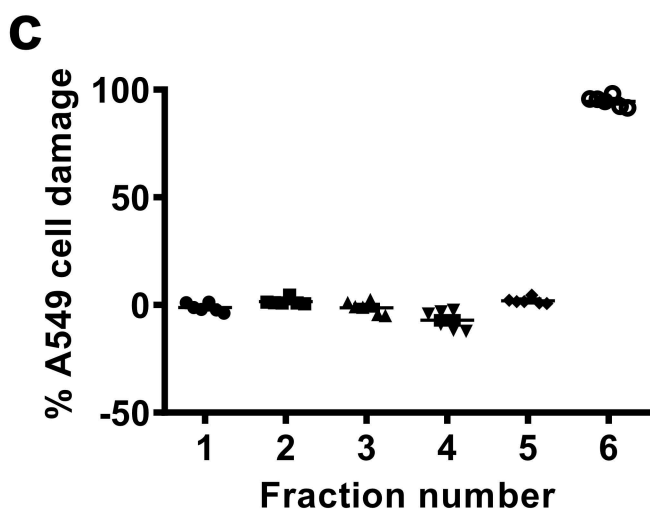
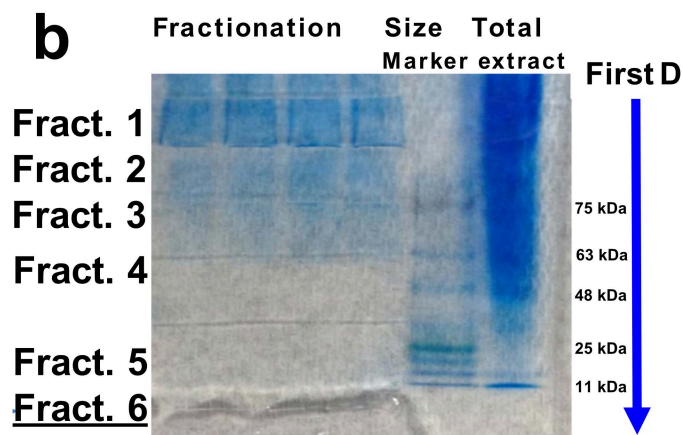
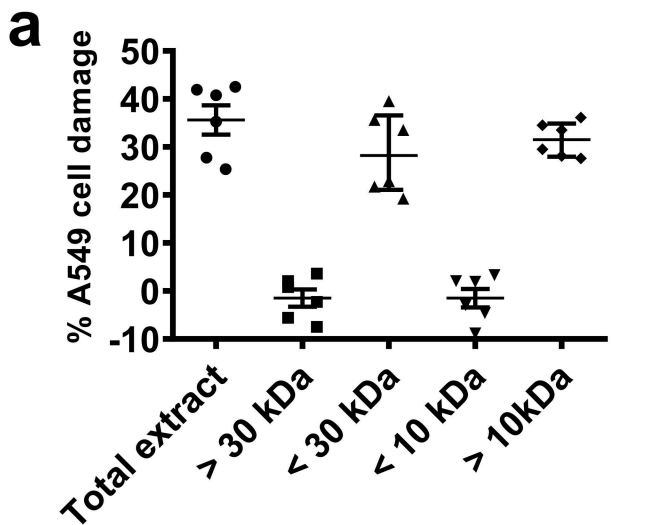


Figure 4

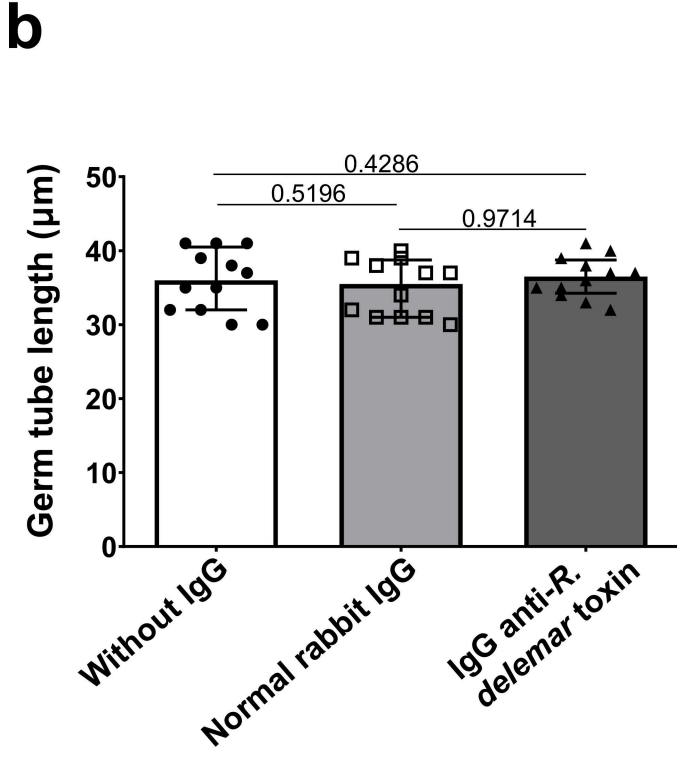
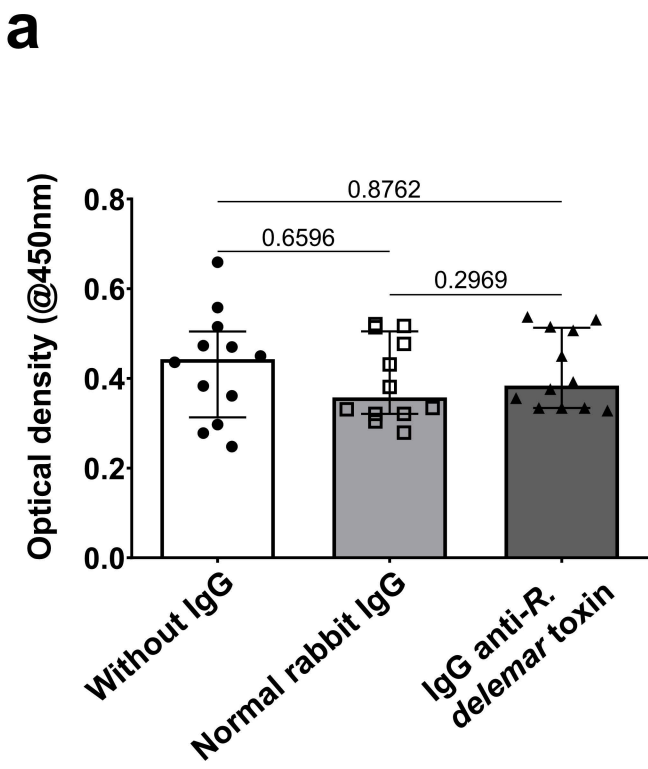
Extended Data Figure 1



Extended Data Figure 2

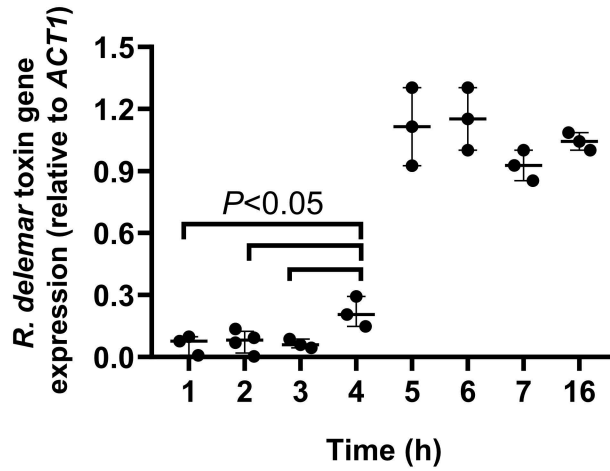


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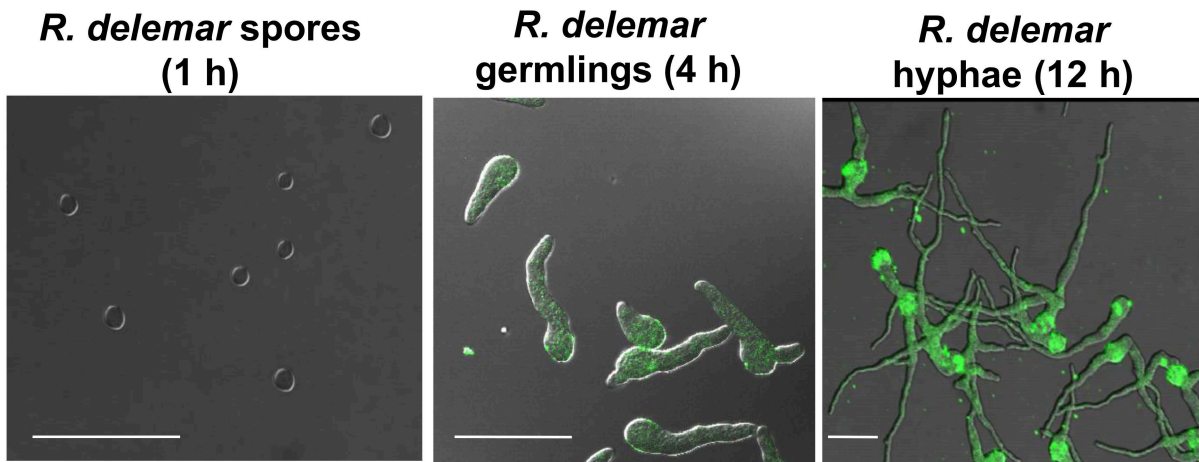


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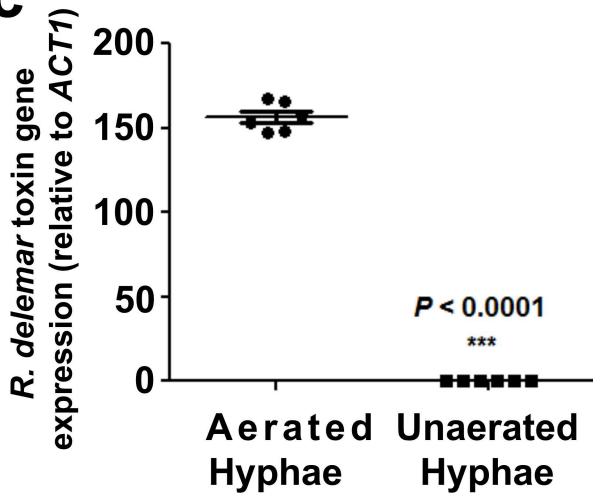
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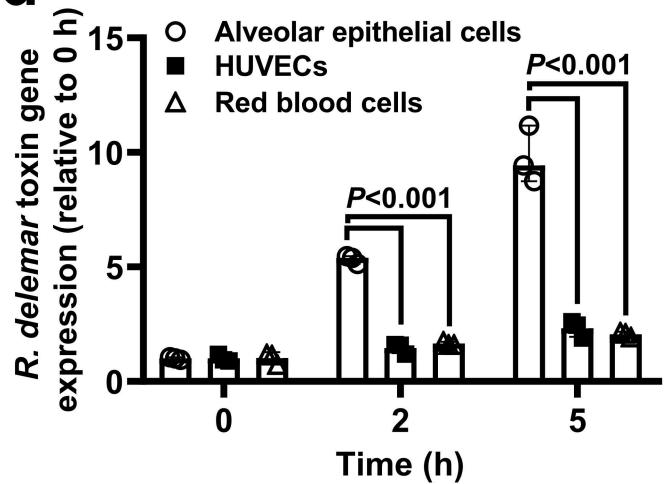
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c

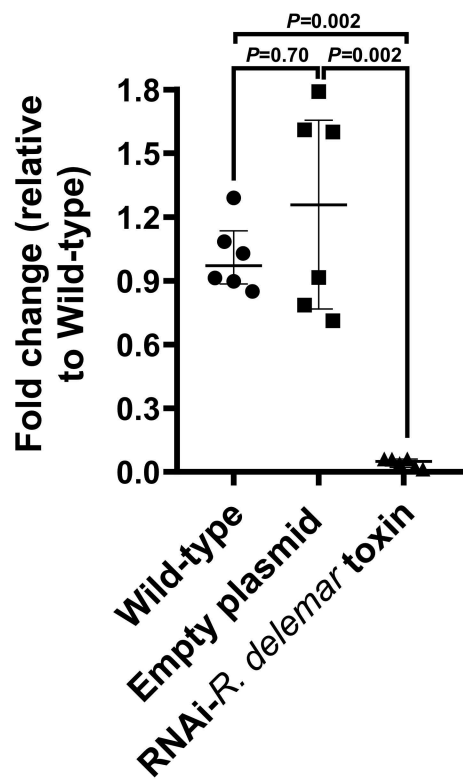


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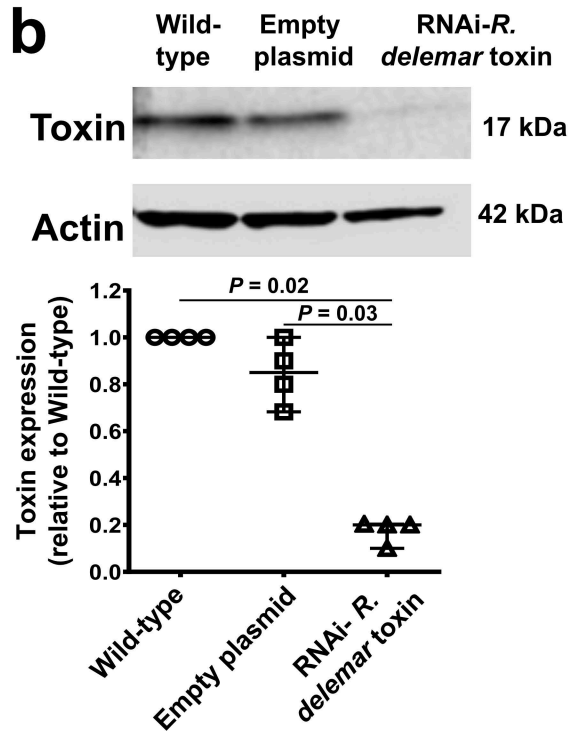


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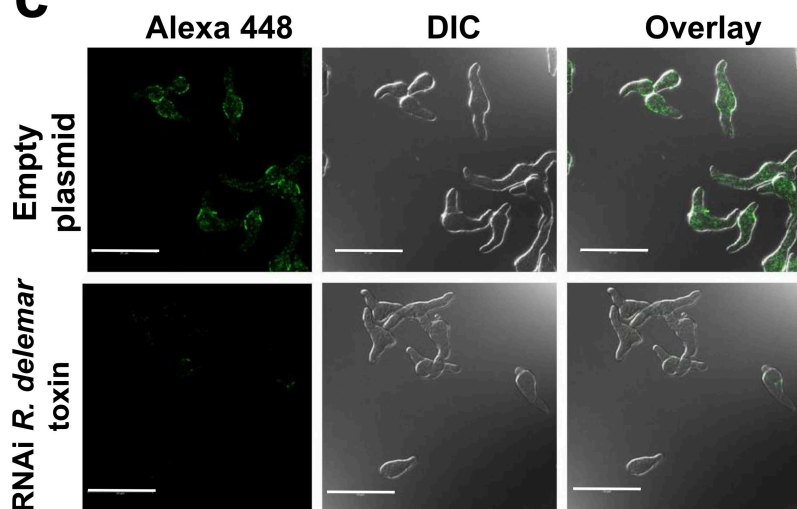
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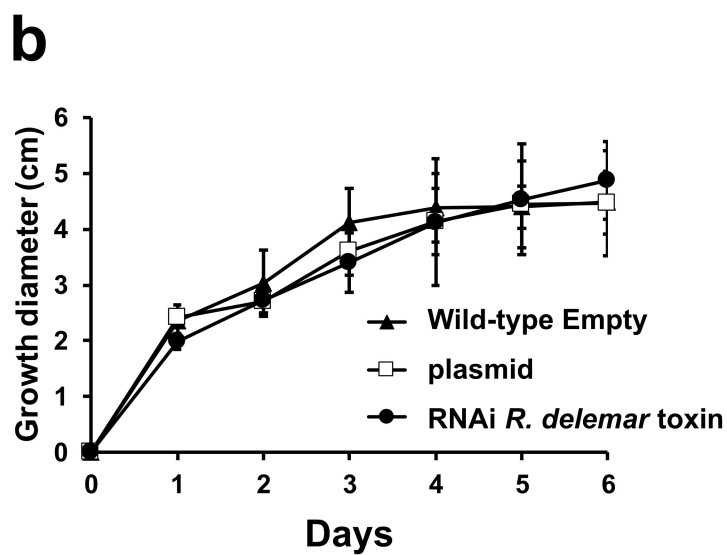
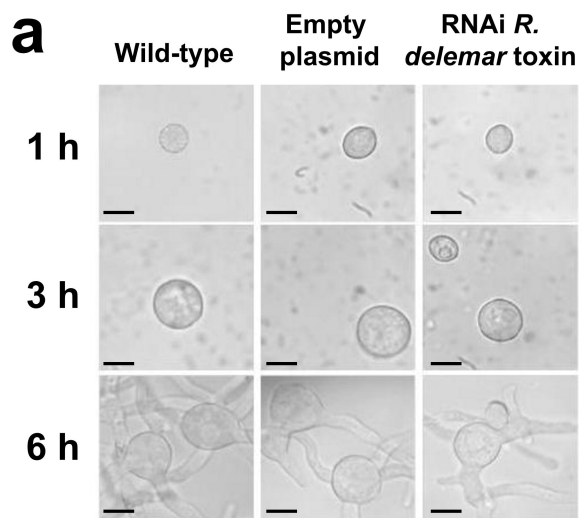
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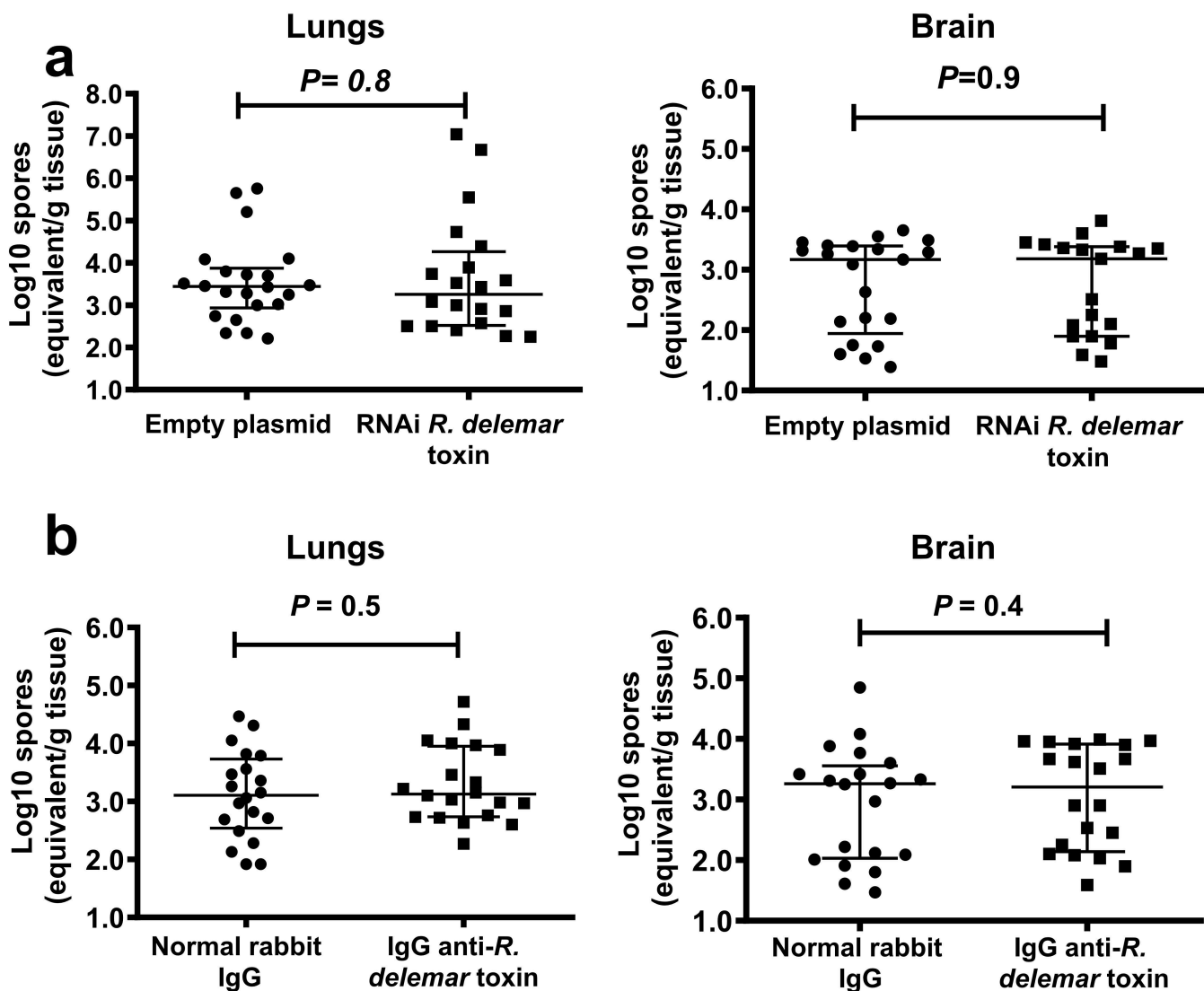
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Extended Data Figure 6.



Extended Data Figure 7.

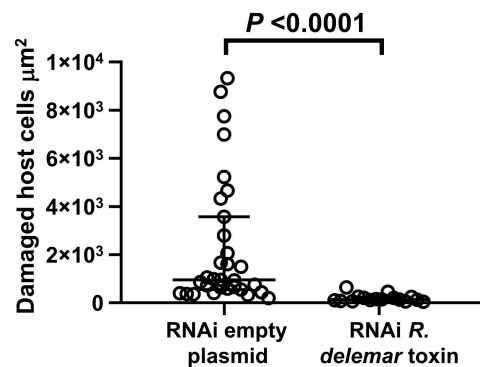
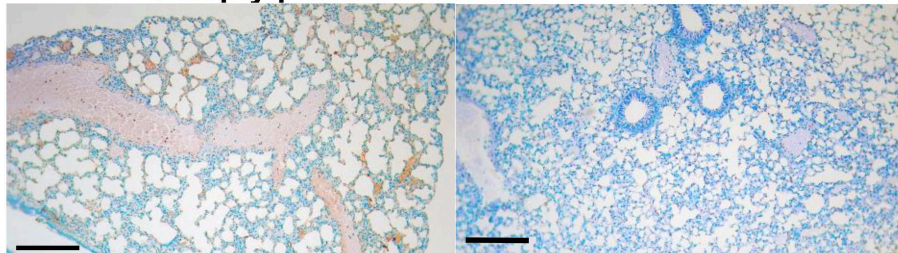


Extended Data Figure 8

a

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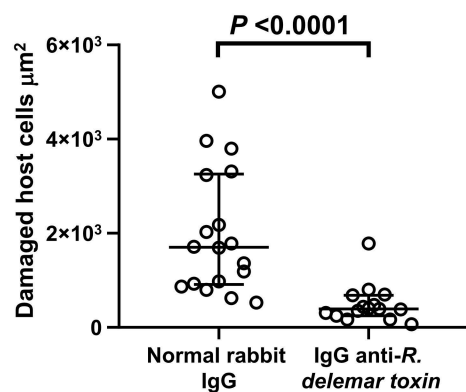
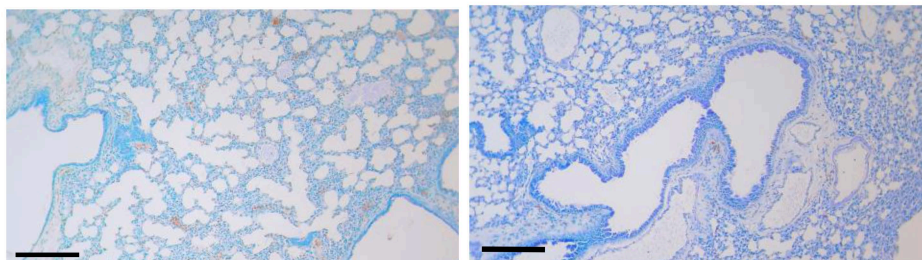
RNAi *R. delemar* toxin



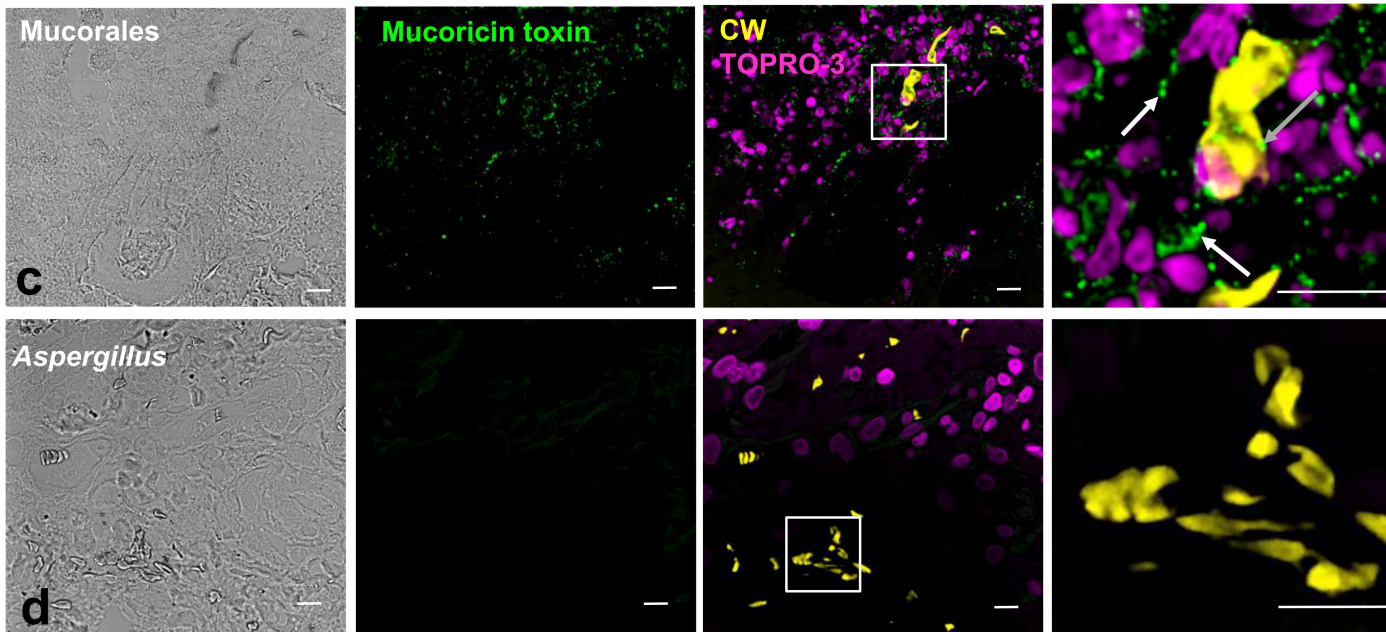
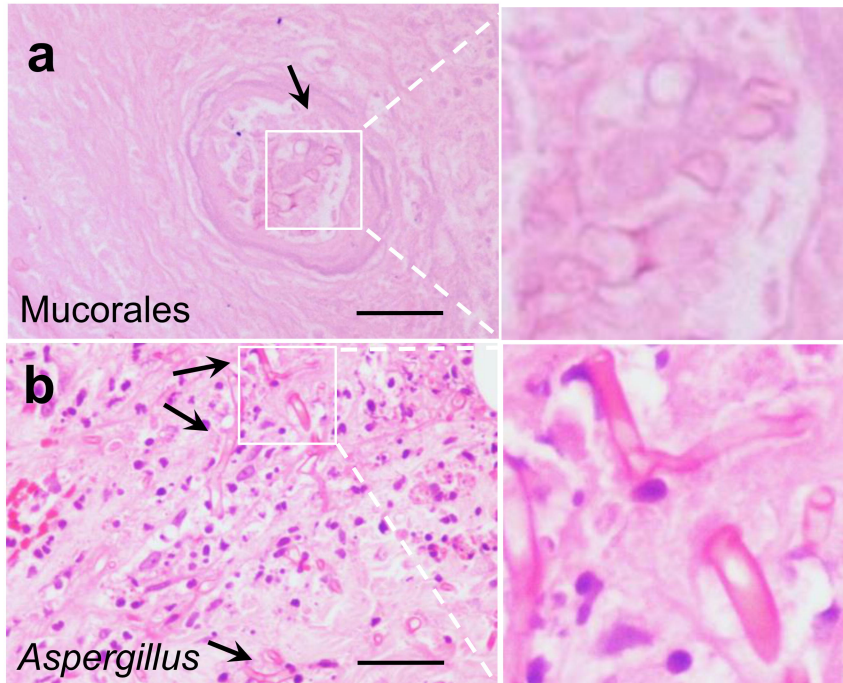
b

Normal rabbit IgG

IgG anti-*R. delemar* toxin

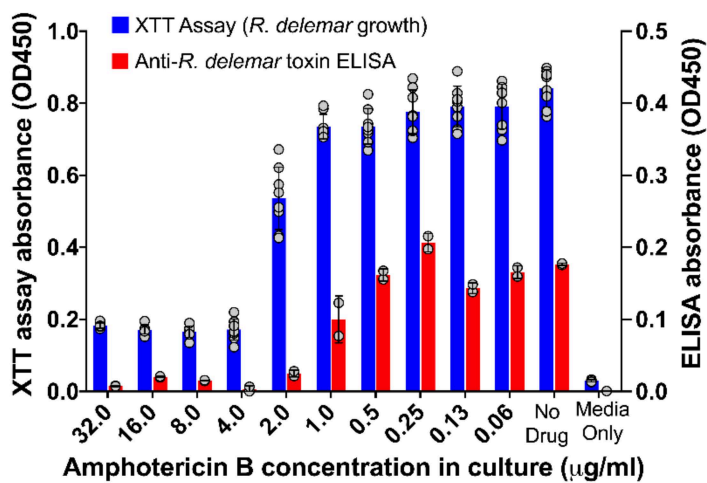


Extended Data Figure 9



Extended Data Figure 10

a



b

