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Peer Review Information

Journal: Nature Microbiology
Manuscript Title: **Mucoricin is a Ricin-Like Toxin that is Critical for the Pathogenesis of Mucormycosis**
Corresponding author name(s): Ashraf Ibrahim

Reviewer Comments & Decisions:

Decision Letter, initial version:

Dear Ashraf,

Thank you for your patience while your manuscript "Mucoricin is a Ricin-Like Toxin that is Critical for the Pathogenesis of Mucormycosis" was under peer-review at Nature Microbiology. It has now been seen by 4 referees, whose expertise and comments you will find at the of this email. Although they find your work of some potential interest, and referees 1, 2 and 3 were more positive, they have raised a number of concerns that will need to be addressed before we can consider publication of the work in Nature Microbiology.

In particular, referees 2, 3 and 4 raise several concerns over the lack of sufficient mechanistic insight into the function of mucoricin and ask that you further explore it in more detail. For example, referee 2 suggests that you provide additional evidence to support the claims that mucoricin inactivates ribosomes and inhibits host cell protein synthesis, is physically associated with host ribosomes, and is secreted. Similarly, referee 3 also asks about toxin secretion; and referee 4 asks for additional support on whether depurination activity is observed and needed. Referee 1 and 3 also raise some concerns about the phylogenetic modelling of mucoricin, and referee 3 and 4 point out that additional replicates and statistical analyses are needed to substantiate some of the main claims.

Should additional experimental work allow you to address these criticisms, we would be happy to take a look at a revised version of the manuscript.

We are committed to providing a fair and constructive peer-review process. Please do not hesitate to

contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

We strongly support public availability of data. Please place the data used in your paper into a public data repository, if one exists, or alternatively, present the data as Source Data or Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. For some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories can be found at https://www.nature.com/nature-research/editorial-policies/reporting-standards#availability-of-data.

Please include a data availability statement as a separate section after Methods but before references, under the heading "Data Availability". This section should inform readers about the availability of the data used to support the conclusions of your study. This information includes accession codes to public repositories (data banks for protein, DNA or RNA sequences, microarray, proteomics data etc…), references to source data published alongside the paper, unique identifiers such as URLs to data repository entries, or data set DOIs, and any other statement about data availability. At a minimum, you should include the following statement: "The data that support the findings of this study are available from the corresponding author upon request", mentioning any restrictions on availability. If DOIs are provided, we also strongly encourage including these in the Reference list (authors, title, publisher (repository name), identifier, year). For more guidance on how to write this section please see: http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf

If revising your manuscript:

* Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

* If you have not done so already we suggest that you begin to revise your manuscript so that it conforms to our Article format instructions at http://www.nature.com/nmicrobiol/info/final-submission. Refer also to any guidelines provided in this letter.

* Include a revised version of any required reporting checklist. It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review. A revised checklist is essential for re-review of the paper.

Please use the link below to submit a revised paper:

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Nature Microbiology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. This applies to primary research papers only. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

If you wish to submit a suitably revised manuscript we would hope to receive it within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision, even if a similar study has been accepted for publication at Nature Microbiology or published elsewhere (up to a maximum of 6 months). Should your manuscript be substantially delayed without notifying us in advance and your article is eventually published, the received date would be that of the revised, not the original, version.

In the meantime we hope that you find our referees' comments helpful.

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Reviewer Expertise:

Referee #1: Mucormycosis
Referee #2: Fungal pathogenesis
Referee #3: Fungal pathogenesis
Referee #4: Ricin toxin

Reviewer Comments:

Reviewer #1 (Remarks to the Author):

This is a landmark study and the authors have found a new toxin protein in the Mucorales that has

direct impact on cells. The work is elegant and demonstrates the discovery, validation, cell free expression and testing of the function, RNAi knock down to test for loss of phenotypes.

This is an advance in the field and important to disseminate to the research community.

My only concern is the methodology for molecular phylogenetics and evolution of the protein seems flawed. I re-analyzed these data and did

There is also a homolog of the toxin protein the plant-associated Glomeromycotina fungus Rhizophagus - https://fungidb.org/fungidb/app/record/gene/RO3G_06568#category:evolutionary-biology
It would be useful for the authors to acknowledge that the protein is not Mucorales / Mucormycotina-specific and provide some speculation on the role in other fungi or the implications of it being a gene that has been around for a while in this group of fungi, even if the animal disease properties are more recently emerged.

The other criticism about this section (extended figure 7) are that clustalOmega dendrograms are not good phylogenetic trees for reconstructing history. The neighbor-joining approach does not seem to fare well here for the sequences. I have reanalyzed the data (see attached figure) and find the Pseudomonas exotoxin is much more similar phylogenetically to the Rhizopus toxin protein than in fact Ricin is. So it would be important to clarify this in the text and interpretation. Generally a maximum likelihood analysis is more appropriate for resolving the phylogenetic tree.

Searches of the Rhizopus toxin against the listed dataset of toxin proteins did not find any significant sequence similarity - perhaps the authors focused more on results that came from protein model analyses which could have rescued more alignable regions due to threading through protein structures? Otherwise I think a revision of this figure and restating the interpretations of homology need to be made. I do think the protein modeling is appropriately performed and I think is still providing evidence for a distance connection between these toxin proteins but I think the authors could more clearly state the data.

MInor:
Line 71 Rhizopus delemar is spelled out but I think you can abbreviate to R. delemar now.

Reviewer #2 (Remarks to the Author):

In this paper, Soliman et al. identify and characterise the RO3G_06568/RLT1 gene product in the human fungal pathogen Rhizopus delemar. They demonstrate that RLT1 encodes a 17 kDa protein (which they

name mucoricin) that is hypha-associated and required to damage host cells in vitro and in vivo. The protein appears heat stable since live and heat-killed hyphae are still capable of causing cellular damage. The authors demonstrate that polyclonal antibodies raised to mucoricin are capable of attenuating cellular damage and prolonging the life of infected mice. RNAi knockdown of RLT1 expression induces similar effects. Analogs of R. delemar mucoricin are also identified in additional species of Mucorales fungi. Computational modelling reveals that R. delemar mucoricin shares some structural characteristics of the castor bean toxin ricin. The authors suggest that mucoricin functions similarly to ricin, in that it depurinates ribosomal RNA thereby inhibiting host protein synthesis, which leads to cell death. Epithelial and endothelial cells exposed to mucoricin initiate a pro-inflammatory response resulting in the secretion of IL-8. This is a very nice and important piece of work. However, there are multiple critical issues that need attention and, if taken on board, will considerably strengthen the manuscript.

Major comments and critical issues:

The key message of this manuscript is that mucoricin is a ricin-like toxin, which has ricin-like functions such as the ability to inactivate ribosomes and possessing an LDV motif that mediates vascular leakage. However, apart from showing damage data, none of these functions are actually shown in the manuscript.

Ricin A chain depurinates 28S ribosomal RNA, thereby inhibiting protein synthesis. Mucoricin was combined with “template” RNA from A549 cells and release of adenine was demonstrated. However, demonstrating the depurination of template RNA is not robust enough to support a model of ribosomal inactivation through specific depurination of 28S ribosomal RNA. For instance, if mRNA transcripts were the only RNA species to be depurinated following mucoricin treatment this may also lead to cessation of protein synthesis but the functionality of the host ribosome would remain unaltered (i.e. the ribosomes would still be translationally competent). Therefore, mucoricin-induced depurination of 28S rRNA must be demonstrated. Furthermore, the inhibition of host protein synthesis by mucoricin has not been demonstrated in vitro or in vivo. Experimental techniques such as polysome profiling and/or the incorporation of 35S-labelled methionine into proteins will help address this critical issue. The authors must show, through experimentation, that mucoricin inactivates ribosomes and inhibits host cell protein synthesis.

Should mucoricin depurinate host ribosomal RNA, this would imply a physical association between mucoricin and the host ribosome. The physical association of mucoricin with ribosomes needs to be demonstrated experimentally.

R. delemar is endocytosed into HUVEC cells through the interaction between CotH3 and host GRP78. Presumably, endocytosed fungus is contained within an endocytic vesicle. This being the case, how does mucoricin exert its effect on the host ribosome? Or does the fungus escape and does mucoricin

physically associate with ribosomes (see above).

The authors speculate that mucoricin possesses an LDV lectin binding motif, like ricin, that may mediate vascular leakage. However, in Fig 4a ricin is shown to have a VDV motif, not LDV? Please clarify. Following on, the authors provide no experimental evidence that the LDV-motif is responsible for damage or vascular leakage. The authors need to mutate the LDV motif and demonstrate this. The galactose data is not sufficient. These data, together with the ribosomal data mentioned above, will be sufficient to demonstrate that mucoricin indeed functions similarly to ricin.

The antibody that recognises mucoricin from R. delemar must be characterised thoroughly against whole spore, germling, hyphal and host cell extracts in order to rule out non-specific binding. This required to have confidence in the western, immunofluorescence and IHC data.

Data derived from in vitro experimentation should be a minimum of three biological repeats. It appears that only two biological repeats are presented in some data sets.

Other important comments:

Is mucroricin secreted or cell associated only? The summary model at the end of the manuscript states that both live and dead hyphae “release” mucoricin. However, mucoricin is also described as being hypha-associated elsewhere in the manuscript, which appears to be supported by immunofluorescent analysis of R. delemar hyphae. Ideally, the authors should demonstrate by mass spectrometry whether mucoricin is secreted or not.

Mucoricin-induced host cell damage was time dependent. Almost complete damage occurs after 3 hours of toxin addition, at which point mucoricin was observed around the host cells. However, after 48 hours the toxin was observed inside the cell. Therefore, is the damage caused by mucoricin at 3 hours a surface mediated event that relies upon host cell signalling? (as the toxin is not observed inside the host cell at 3 hours). If endocytosis is blocked, does this prevent damage/ribosome inactivation from occurring?

Unfractionated extracts from R. oryzae, R. delemar, Lichtheimia corymbifera and Cunninghamella bertholletiae all damaged A549 cells, but not all to the same extent. Can the damage caused by these orthologs be titrated by the anti R. delemar mucoricin antibody?

Does the anti R. delemar mucoricin antibody block release of IL-8? i.e. is host recognition of the toxin required in order to induce cytokine release? If not, is IL-8 released as a consequence of apoptosis/necrosis? Following on, detecting IL-8 alone is insufficient to assess the immune response, particularly as the authors model states that mucoricin damages epithelial cells, causing them to secrete

IL-8 “and other proinflammatory cytokines”. Since mucoricin is damaging then the damage associated cytokine IL-1a (or similar) should probably be tested. Likewise, GM-CSF and/or G-CSF should also be investigated as they have similar functions to IL-8 in neutrophil recruitment and/or activation. Many other cytokines/chemokines could also probably be investigated but the above should suffice. Does the antibody block the release of all these cytokines?

Western blot analysis of toxin production in RNAi treated cells indicates that toxin production is vastly reduced but not inhibited. The authors state that surviving mice infected with RNAi-treated fungi had no detectable fungi in their lungs at day 21. How was this determined? No CFU data has been presented in the manuscript. I assume CFU will be present for the empty plasmid but absent for the RNAi at 21 days? Please show data or comment. If the fungus is cleared, presumably the host immune response is not induced by the toxin?

The manuscript often refers to necrosis as being the predominant mechanism of cell death during mucormycosis, but uses the Apoptag kit to stain lung tissue as a means of demonstrating cellular damage. The Apoptag kit detects apoptotic cells in situ by labelling and detecting DNA strand breaks. Apoptosis and necrosis are mechanistically distinct. Necrosis is a caspase-independent process, whereas apoptosis requires caspase activity and may proceed through intrinsic and extrinsic signalling pathways. Can the authors please clarify whether mucoricin induces apoptosis or necrosis.

Can the authors speculate whether the mechanism of toxin-damage is the same between HUVECs and red blood cells? There are significant differences between endothelial cells and mature red blood cells, for instance long-term retention of a nucleus and ribosomes.

Line 282: Lack of RLT1 expression in spores is not shown. In addition, to support this analysis, a western blot in which the whole film is shown (and not a cropped image) would rule out the possibility of any non-specific binding events.

Also, this manuscript used male mice. What about female mice? It is well known that male and female mice can respond differently to pathogens and particularly toxins. Ideally, data with female mice should be included in the manuscript.

Extended Data Fig. 6. Immunohistochemistry of a patient’s lung apparently shows association of the toxin with fungal hyphae that has caused necrosis and massive infiltration of tissues. However, there is fluorescence is many places, with limited localisation to the fungus? Can the authors indicate whether there is any non-specific binding by their anti-mucoricin antibody (please see comment relating to Figure 3A). A control biopsy without mucormycosis is also required. This is also the first immunofluorescence picture. Immunofluorescence with the mouse biopsies may be highly beneficial to demonstrate toxin localisation to the fungus.

Extended Data Fig. 11b. These images are insufficient for the conclusions. Also, most of the cells do not appear to have toxin? The toxin may only enter when the cell is dead. Better confocal imaging is required with z-stacking (side/top views) etc. to demonstrate entry and clarity at different time points.

Minor comments:

The manuscript used different mouse models i.e. DKA and immunosuppressed mice etc. Why are different mouse models required for different parts of the manuscript? Also, wild type mice are not used. Do these fungal species and mucoricin not cause 'infection' in healthy mice? Discussion required. Also, in the relevant Figure legends, please indicate which mouse model was used to help the reader.

Mucoricin is clearly damaging but it is also clear that damage still occurs in the absence of mucoricin. I assume this indicates that other potential toxins may exist in these species? Discussion required.

Is the mucoricin encoded by an entire open reading frame, or is it present as a cryptic sequence within a larger protein such as the case with C. albicans candidalysin/ECE1?

Recombinant mucoricin can be expressed in S. cerevisiae. Mucoricin is believed to exert its effect on the host ribosome. Does the expression of mucoricin have any negative impact on S. cerevisiae? Both mammalian cells and S. cerevisiae are eukaryotic and presumably have a reasonable degree of ribosomal conservation.

When mucoricin was injected intravenously, histopathology indicated the presence of "large" macrophages in the lung. What is meant by "large" in this context? Are these a particular type of macrophage? Are they atypical from what would normally be expected? Are the same "large" macrophages observed when the lung is infected with wild type fungus? Were other organs similarly infiltrated? If not, does this suggest a degree of tropism to the lung?

Orthologs of mucoricin are present in 21 previously sequenced species. Do they all possess the LDV sequence for example? A sequence comparison of orthologs would be a nice supplementary addition to the paper.

Mice were infected intratracheally and then given 30 ug of antibody. Does the antibody (or its vehicle) prevent spore germination/hyphae formation?

The amount of mucoricin used in experiments is described as mass per unit volume (i.e. 20 ug/ml). The molar concentration of mucoricin should be used throughout the manuscript.

Reviewer #3 (Remarks to the Author):

Considering the scarcity of knowledge on the molecular basis of Mucorales pathogenicity, the discovery and characterisation of a functional toxin represents a significant step forward in the field of medical mycology.
Although Mucorales species are quite incalcitrant to genetic manipulation, here the authors have used standard biochemistry to identify a protein present in the toxic fraction of hyphal extract. They go on, using a combination of RNAi and a polyclonal antibody, to show that the aptly named “mucoricin” plays a crucial role in the pathogenicity of Mucorales.
Overall, and up to including Figure 3 and Supplementary Figure 5, this is a really solid manuscript, with each next experiment being logical and comprehensively carried out.
After that I feel there is a slight loss of focus, with occasional elements which could be tidied up or reordered to improve the manuscript. I detail these below.
Despite these criticisms, from the perspective of medical mycology, this is probably the most significant manuscript I’ve read on a Mucorales human pathogen.

Comments.
It should be pointed out that the presence of such toxins has been “identified” in the genomes of fungi previously (Revising the taxonomic distribution, origin and evolution of ribosome inactivating protein genes) however I don’t think this detracts from the novel toxic activity reported here.
Actually, I found the Ricin Interpro domain of Rhizopus mucoricin (IPR000772) present in almost 400 sequences using the FungiDB Interpro Domain finder tool indicating the presence of a large number of ricin domain-containing proteins in fungi, well beyond what would be found by BLAST analysis.

The fact that even killed hyphae continue to cause significant cell damage (likely due to persistence of mucoricin activity) is of potentially enormous clinical significance.
i.e. – a patient would continue to suffer tissue necrosis, even after the fungus was fully killed by an antifungal.
It would have been nice to combine the approaches of e.g. Figure 1b with toxin RNAi or antibody to directly demonstrate that the damaging activity of dead hyphae is mucoricin-mediated.

220. “The hyphae-associated toxin has structural features of ricin”
Here the authors claim they “embarked on structural and bioinformatics studies” by directly aligning their mucoricin against known toxin sequences and reporting “Rhizopus delemar toxin, showed the highest homology with ricin produced by the castor bean plant, Ricinus communis”
I don’t think that restricting comparisons of mucoricin to “well-known” toxins is the best way to

interrogate its phylogeny or evolutionary origin. By performing a BLASTp search of RO3G_06568 at NCBI and excluding the fungal kingdom, it's apparent that mucoricin shares high similarity with ricin sequences from CNS and cyanobacteria. In fact, the similarity between mucoricin and these bacterial sequences is higher than to most non-Mucorales fungi.
So, whilst the conclusion "Phylogenetically, the toxin is most similar to ricin…" is true, it would appear to be far more closely related to bacterial ricin than to plant.
Whether this is due to HGT acquisition of a bacterial ricin by fungi (e.g. Metazoan Ribosome Inactivating Protein encoding genes acquired by Horizontal Gene Transfer) or loss of RLT1 by other eukaryotic lineages (e.g. Revising the taxonomic distribution, origin and evolution of ribosome inactivating protein genes) is anyone's guess at this point!
Along with this point, Extended Data Figure 7b is not particularly useful. It would be much more informative to generate a tree based on mucoricin and proteins with actual phylogenetic similarity (i.e. BLASTp reciprocal best hits). Not candidalysin!

If mucoricin is associated with the hyphal cell surface as is shown in the manuscript – is it also being released? Was there any evidence for it being shed from the fungus? This would be important for it to get into host cells.

303-332. "Mucoricin, like ricin, induces inflammation". Overall, this section is a bit weak and feels more like the pilot data for the next study.
I would personally integrate the IL-8 observation into an earlier section rather than have it as a standalone result. e.g. it would fit alongside histology/PMN infiltration.

Extended Data Figure 11 – for me, this would be the nice initial observation in a future study looking at the mechanism of mucoricin entry into host cells. It's a neat observation but doesn't sit right as the final data set in what is otherwise a really solid and logically narrated story.

Minor comments.
"This organism also damages the A549 alveolar epithelial cell line and primary
alveolar epithelial cells, but only after 30 h of incubation (Fig. 1a)."
Is there really damage to A549 at 30 h?

Was the purified and/or recombinant toxin heated and shown to still be active (to fit with heat killed hyphae conclusion)?

124 "act similarly in damaging A549 cells in a rapidly time dependent manner" Remove "rapidly". To many, rapid would mean seconds.

Extended Data Fig. 6 – whilst it's nice to indicate mucoricin is part of the human pathology, I feel this

single observation is a little weak as is.

236 – “We generated a 3-D structural model” – insert “predicted”

295 “Concordant with these results, it has been shown that HUVECs are rapidly damaged by the LDV-motif (Fig. 4a) than the sequences responsible for ribosome inhibition” – rephrase

321 – “microscopy of epithelial cells treated with mucoricin showed instant rounding of cells” – I’m not sure I follow what’s being said here.

607 “Expression and purification of mucoricin” was the construct sequenced?

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Reviewer #4 (Remarks to the Author):

My name is Robert Spooner, University of Warwick. I have been asked to review this manuscript because of my interest in ricin.

An interesting toxin: it has some modelling similarity to lectin binding domains of ricin B chain, and some degree of sequence similarity. Consistent with this anti-ricin antibodies show a degree of cross reaction, suggesting a real structural and sequence similarity. It would be nice to see how these antibodies see this toxin (epitope mapping?).
N-terminal to the putative lectin binding domain are 16 amino acids with some (gapped) similarity to the C-terminal part of ricin A chain. This part of ricin A chain has the 5 amino acid active site motif SEAAR associated with the depurination activity of ricin A chain (and which is identical in Shiga toxins which also depurinate ribosomes). However, the fungal toxin FEEGR, I would suggest, is a very poor copy of this, so it would be an unlikely candidate for any putative deprivation activity. The depurination activity of this would surely be important for this manuscript since the title of the manuscript specifically points to the similarities with ricin. So why is this activity assay relegated to the subordinate Supplementary data section in (Ext Fig 8)? I am not familiar with this assay. I would very much like to see this assay validated using ricin A chain, and also with heat inactivated ricin A chain (no depurination) and heat-inactivated fungal toxin.
If there is a confirmed quantifiable depurination activity, where is the evidence that this is part of the mechanism of cell damage by this toxin? There are no data here that suggest that the toxin is

internalised. And a statement that the spores bind GRP78 makes little sense. GRP78 is BiP, a chaperone that resides in the endoplasmic reticulum. How would a spore see this? Unless of course, this is BiP released from ruptured cells – which is not the same as internalisation. The reference that supports this (reference 37) is Alqahiri, IDweek. IDweek is an annual meeting of IDSA, and is not peer-reviewed: this whole section should be removed.

Statistics.
1. There is some ambiguity in description: for example, in the legend to Fig. 1c, there is a description of n=6/group from two experiments. But if there are two experiments, then n = 2. There is a danger of pseudoreplication here that must be avoided so that P-values are not artificially lowered. And if there are two experiments with 6 observations, is this three observations per experiment? This really is not clear.
Fig 2b legend, Fig 3C, Ext Fig 3b, Ext Fig 9c, 9d and Extended Fig 10 all have this same ambiguous writing.
2. Error bars in Ext Fig 3b, Ext Fig 1 and Fig 2b. What are they? SD? SEM?
3. Why are some experiments shown with individual points, median and interquartile range, whereas others are summary statistics (mean plus or minus something undefined)? Lack of uniformity.
4. Fig 3e and 3f. Mouse survival is typically displayed in a Kaplan-Meier step plot (declining horizontal steps with vertical risers) and the analysis is really a modified chi-squared. However, the authors have just joined the dots.
5. How was Fig 3d normalised? The data presentation suggest that toxin-treated cells can have up to 135% cell damage – this should alert the authors to a normalisation problem.

Other comments.
There is a two hour window between hours 1 and 3 in Fig 2b with no data points, between all and nothing, so the graph is uninformative. Why not just show this as an end-point assay?
Line 161: mice appeared healthy – a somewhat qualitative statement.
Line 241: why is (e.g RIP) needed?
Line 256 – and Fig 4e: Western blot. There is only one polypeptide identified on the Western I'm guessing the anti-ricin antibody was really anti-ricin B chain?
Line 271. Diphtheria toxin and Pseudomonas exotoxin are not RIP: they inactivate elongation factors, not ribosomes
Line 273. Vague writing: what does 'it is cell-associated' mean in this context? Fungal cell? Host cell? Necrotic cell?
HUVEC (line 292-293) and endothelial cell s (Ext Fig 9d). Please use consistent labels/descriptions.
Line 299: a very vague mechanistic statement. Please remove – it adds nothing to the story.
Line 303 to 316. Mucoricin (…) induces inflammation. Since the comparison is with ricin, and therefore presumably important for this story, why are all the inflammatory data confined to Extended Data sections? The following section/ lines raise the mechanistic prospect of a time lag caused by internalisation. However, the authors present no data to suggest internalisation, and in their model

(initiation and pathogenesis model section) do not even mention internalisation.

Display.
Scale bar Fig 2e is likely to be unreadable when data are displayed by the journal – way too small: and the white arrow on the lung tissue is almost invisible.
The scale bar on Fig 3 b will also become invisible when displayed.
RNAi knockdown section: Down-regulation was measured by qRT-PCR (line 142-143) – but not shown.

Author Rebuttal to Initial comments

1. **My only concern is the methodology for molecular phylogenetics, and evolution of the protein seems flawed. I re-analyzed these data and did There is also a homolog of the toxin protein the plant associated Glomeromycotan fungus Rhizophagus. It would be useful for the authors to acknowledge that the protein is not Mucorales/Mucormycotina-specific and provide some speculation on the role in other fungi or the implications of it being a gene that has been around for a while in this group of fungi, even if the animal disease properties are more recently emerged.**

   Answer: We do not claim that mucoricin is unique to Mucorales/Mucormycotina. Our most recent BLAST search of the toxin ORFs retrieved many orthologies, the majority of which belong to the order Mucorales with a high % predicted amino acid identity (ranging from 50-90%) (**see revised Extended Data Table 1**). Many of these Mucorales fungi are known human pathogens (e.g. *Rhizopus, Mucor, Cunninghamella, Lichtheimia,* etc.), animal pathogens (e.g. *Mortierella*) or plant pathogens (e.g. *Choanephora cucurbitarum*). On numerus occasions, these retrieved ORFs are annotated as ricin domain-containing proteins or ricin B-like lectins (including in *Rhizopus*). However, and as stated by the reviewer, less identical ORFs from other fungi such as, *Rhizophagus* (30-40% identity) were retrieved (**Lines 115-117**). *Rhizophagus* lives symbiotically with plants and is recognized as an integral part of the natural ecosystem and was shown to delay plant disease symptoms caused by *Phytophthora infestans*. We also retrieved ORFs belonging to the bacterial genera of *Streptomyces* and *Paenibacillus* (30% identity), both are known inhabitants of soil and present in rhizosphere of various plants. These two bacterial genera are known to be producers of antibiotics and can be used as biological control agents for crops. We now include a paragraph on these possibilities in the Discussion (**Lines 312-318)**.

2. **The other criticism about this section (extended figure 7) are that clustalOmega dendrograms are not good phylogenetic trees for reconstructing history. The neighbor-joining approach does not seem to fare well here for the sequences. I have reanalyzed the data (see attached figure) and find the *Pseudomonas* exotoxin is much more similar phylogenetically to the *Rhizopus* toxin protein than in fact Ricin is. So, it would be important to clarify this in the text and interpretation. Generally a maximum likelihood analysis is more appropriate for resolving the phylogenetic tree.**

   Searches of the Rhizopus toxin against the listed dataset of toxin proteins did not find any significant sequence similarity - perhaps the authors focused more on results that came from protein model analyses which could have rescued more alienable regions due to threading through protein structures? Otherwise I think a revision of this figure and restating the interpretations of homology need to be made. I do think the protein modeling is appropriately performed and I think is still providing evidence for a distance connection between these toxin proteins, but I think the authors could more clearly state the data.

Answer:

i. As suggested by the reviewer, we re-analyzed the phylogenetic tree using the maximum likelihood method and indeed found that *Pseudomonas* exotoxin A to be more related to our toxin than ricin despite the lower sequence similarity compared to ricin (mucoricin and ricin have ~ 26% overall amino acid sequence identity *vs.*16% for mucoricin and *Pseudomonas* exotoxin A).

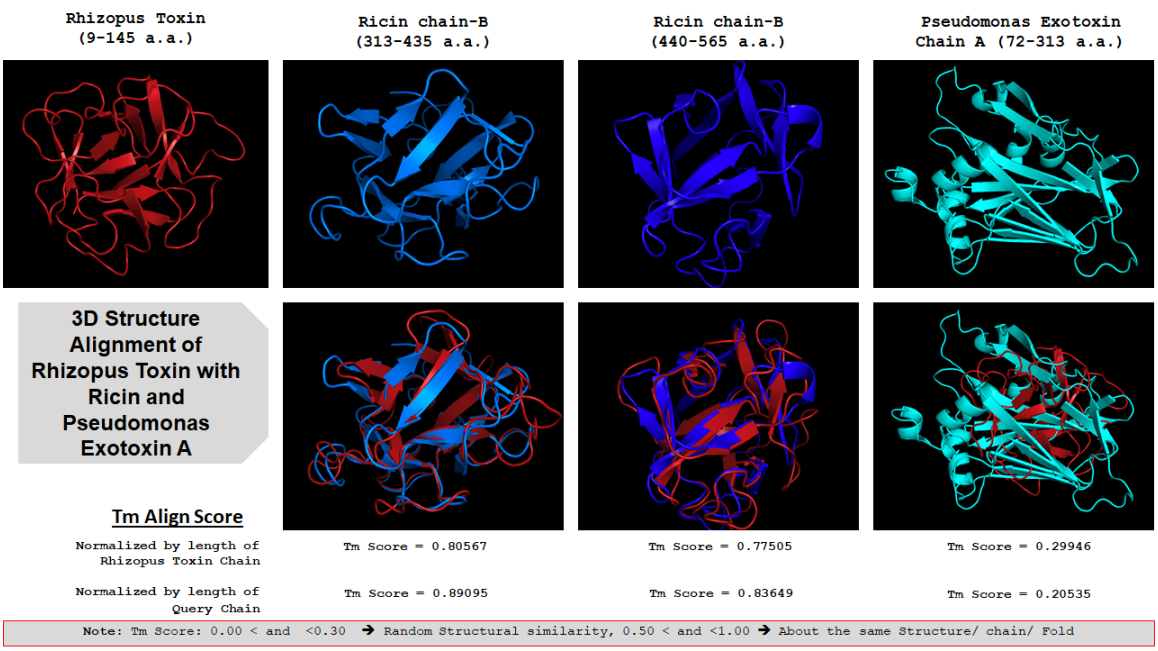
ii. However, after consulting with Dr. Julie Dunning Hotopp (an expert in genomics/sequence analysis/phylogenetics), we concluded that we should remove any phylogeny or discussion of ancestry from the paper. This is because the sequence divergence is too great to align the sequences rigorously.

iii. We have tried downloading sequences using PFAM and also to use BLAST searches but could not produce good alignments that we trust with anything outside the fungal clade. As the editor and reviewer know, if the compared sequences are not aligned well, the phylogenetic inference will be flawed. That said, there are clearly conserved motifs, that we have already identified. However, these motifs alone are not phylogenetically informative.

iv. From the BLAST search the most we can say is that mucoricin has the hallmarks of vertical inheritance in the eukaryotic lineage. The closest sequences are mainly fungal. A few are bacterial (**Extended Data Table 1**).

v. Importantly, the new data using the cell free rabbit reticulocyte assay to measure inhibition of protein synthesis and add further evidence of the similarities between mucoricin and ricin. These points are discussed below in response to specific questions to other Referees.

vi. Finally, the low confidence in the phylogenetic similarity of *Pseudomonas* exotoxin A to mucoricin is consistent with our 3 dimensional modeling showing the weak alignment of this toxin with mucoricin compared with the strong alignment between mucoricin and ricin B-chain (see images blow).



3. **Minor: Line 71 *Rhizopus delemar* is spelled out but I think you can abbreviate to *R. delemar* now.**

Answer: It is now abbreviated (**Lines 75-76**).

Referee # 2 (Fungal pathogenesis)

Major comments and critical issues:

1. **The key message of this manuscript is that mucoricin is a ricin-like toxin, which has ricin-like functions such as the ability to inactivate ribosomes and possessing an LDV motif that mediates vascular leakage. However, apart from showing damage data, none of these functions are actually shown in the manuscript.**

Answer:

i. In the revised version of the manuscript, we compared the ability of ricin and mucoricin to inactivate protein synthesis using the standard cell-free rabbit reticulocyte assay. In this assay, mucoricin inhibited protein synthesis, albeit at higher concentrations than ricin (IC_{50} of 1.7×10^{-8} M and 2.2×10^{-11} M for mucoricin and ricin, respectively) **(Fig. 6a,b**). The weaker activity of mucoricin is expected since ricin is the second most toxic protein known.

ii. Baluna *et al.* (**reference 25** of revised manuscript) have shown that small peptides containing this LDV sequence but **not** the amino acids in the active site of ricin A chain, caused vascular leak. Therefore, the active site of ricin (or any molecule) is not involved in compromising the integrity of endothelial cell monolayers. We neglected to discuss this in our original paper and have now done so **(Lines 358-363**).

2. Ricin A chain depurinates 28S ribosomal RNA, thereby inhibiting protein synthesis. Mucoricin was combined with "template" RNA from A549 cells and release of adenine was demonstrated. However, demonstrating the depurination of template RNA is not robust enough to support a model of ribosomal inactivation through specific depurination of 28S ribosomal RNA. For instance, if mRNA transcripts were the only RNA species to be depurinated following mucoricin treatment this may also lead to cessation of protein synthesis, but the functionality of the host ribosome would remain unaltered (i.e. the ribosomes would still be translationally competent). Therefore, mucoricin-induced depurination of 28S rRNA must be demonstrated. Furthermore, the inhibition of host protein synthesis by mucoricin has not been demonstrated in vitro or in vivo. Experimental techniques such as polysome profiling and/or the incorporation of 35S-labelled methionine into proteins will help address this critical
issue. The authors must show, through experimentation, that mucoricin inactivates ribosomes and inhibits host cell protein synthesis. Should mucoricin depurinate host ribosomal RNA, this would imply a physical association between mucoricin and the host ribosome. The physical association of mucoricin with ribosomes needs to be demonstrated experimentally.

Answer: We have deleted the depurination data as suggested by Referees 2 and 4 and added the inhibition of cell-free protein synthesis as suggested (**Fig. 6a,b**). In future studies we will investigate the molecular events involved in the ribotoxicity of mucoricin in more detail.

3. ***R. delemar*** **is endocytosed into HUVEC cells through the interaction between CotH3 and host GRP78. Presumably, endocytosed fungus is contained within an endocytic vesicle. This being the case, how does mucoricin exert its effect on the host ribosome? Or does the fungus escape and does mucoricin physically associate with ribosomes (see above).**

Answer: It is quite possible that like ricin, mucoricin in the endocytic vesicle translocates into the cytosol and catalytically inactivates ribosomes. As noted above, the intracellular trafficking pathway of mucoricin remains to be studied in more detail. This will be the subject of future work since it is well beyond the scope of this paper.

4. **The authors speculate that mucoricin possesses an LDV lectin binding motif, like ricin, that may mediate vascular leakage. However, in Fig 4a ricin is shown to have a VDV motif, not LDV? Please clarify**.

Answer: We apologize for the confusion caused by the alignment.

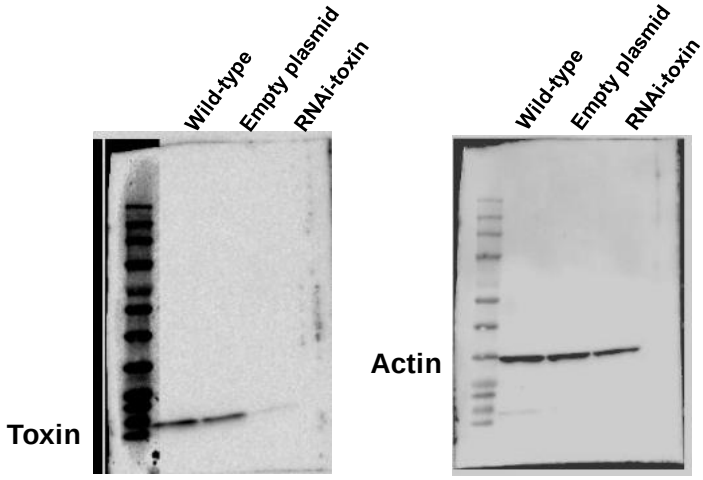
i. Indeed, ricin toxin A chain contains an LDV motif (amino acid 109-111) that is required for vascular leak (**Baluna *et al.* References # 25 and 27** of the revised manuscript**).**
ii. The VDV motif (335-337) is in ricin B chain and happened to align with the LDV motif since the highest sequence resemblance between mucoricin and ricin is within ricin chain B. This issue is clarified in the revised manuscript by inclusion of the full sequence alignment (**Fig. 5a**)

5. **Following on, the authors provide no experimental evidence that the LDV-motif is responsible for damage or vascular leakage. The authors need to mutate the LDV motif and demonstrate this. The galactose data is not sufficient. These data, together with the ribosomal data mentioned above, will be sufficient to demonstrate that mucoricin indeed functions similarly to ricin.**

Answer: We have addressed this above (**Referee 2, point 1ii**).

6. **The antibody that recognizes mucoricin from R. delemar must be characterized thoroughly against whole spore, germling, hyphal and host cell extracts in order to rule out non-specific binding. This required to have confidence in the western, immunofluorescence and IHC data.**

Answer: In the revised manuscript, we show that the anti-mucoricin antibodies do not recognize hyphae or the surrounding tissues of a patient with invasive pulmonary aspergillosis (IPA) (**Extended Data Fig. 10).** The diagnosis with IPA was confirmed by qPCR using the GeneProof Kit for *Aspergillus* and by H&E histology (thin, septate hyphae [**Extended Data Fig 9**]). This confirms the toxin specificity of our antibodies. Additionally, and more importantly, our Western blot data show a single band with the correct size of 17 kDa. Please see the Figure below for a full western blot. To conserve the space of the figure, we truncated this figure to show the mucoricin, and actin bands. Consistent with the qPCR data showing lack of expression of the mucoricin ORF (**Extended Data Figure 4a**), the antibodies do not bind to spores (**Extended Data Figure 4b**).



7. **Data derived from in vitro experimentation should be a minimum of three biological repeats. It appears that only two biological repeats are presented in some data sets.**

Answer: As noted above, all data are now presented as a replicate of three independent experiments.

Other important comments:

8. **Is mucoricin secreted or cell associated only? The summary model at the end of the manuscript states that both live and dead hyphae "release" mucoricin. However, mucoricin is also described as being hypha-associated elsewhere in the manuscript, which appears to be supported by immunofluorescent analysis of R. delemar hyphae. Ideally, the authors should demonstrate by mass spectrometry whether mucoricin is secreted or not.**

Answer: The toxin is mainly cell-associated but can also be detected in cell-free supernatant of growing hyphae (**Fig. 4a**). We do not know if it is actively secreted or shed. The answer to this question will require further experiments *in vitro* and in mice. These results are also supported by the IHC data showing the presence of the toxin on broad aspetate hyphae and in the surrounding tissues (**Extended Data Figure 10**). The revised manuscript includes these important new findings.

9. **Mucoricin-induced host cell damage was time dependent. Almost complete damage occurs after 3 hours of toxin addition, at which point mucoricin was observed around the host cells. However, after 48 hours the toxin was observed inside the cell. Therefore, is the damage caused by mucoricin at 3 hours a surface mediated event that relies upon host cell signaling? (as the toxin is not observed inside the host cell at 3 hours). If endocytosis is blocked, does this prevent damage/ribosome inactivation from occurring?**

Answer: Although the exact mechanisms underlying damage to host cells is yet to be deciphered and is currently the topic of active investigation in our laboratory, given the resemblance to ricin B and the presence of RIP activity, it is possible that mucoricin functions in a manner similar to ricin. It is known that ricin damages tissues by two different mechanisms:

1) The LDV motif in ricin A chain compromises the integrity of endothelial cell monolayers (**Baluna *et al.* Reference 25** of the revised manuscript); and

2) The (ribotoxic) active site sequences in ricin A chain inhibit protein synthesis after series of events involving binding of the holotoxin to cells by the B chain, internalization, separation of the A and B chains, translocation of the A chain into the cytoplasm and catalytic inactivation of ribosomes. The EAARF motif in Ricin A chain is known to be responsible for the RIP activity through depurination of adenosine 4324 in 28S rRNA with the glutamic acid residue (yellow highlight) responsible for this activity **(Hovde *et al.* Reference 38** of the revised manuscript**).** Furthermore, the arginine residue (cyan highlight) is also required for the activity of Shiga toxin (a ricin-like toxin) (**Basu *et al.* Reference 39** of the revised manuscript**).** We have rewritten the discussion to indicate that mucoricin contains both the LDV motif and has a EEAAN that aligns with the ricin EAARF, with the glutamic residue conserved. Mucoricin also contains the EEGRL in which the glutamic acid and arginine residues conserved (**Lines 325-349**).

10. Unfractionated extracts from R. oryzae, R. delemar, Lichtheimia corymbifera and Cunninghamella bertholletiae all damaged A549 cells, but not all to the same extent. Can the damage caused by these orthologs be titrated by the anti R. delemar mucoricin antibody?

Answer: As suggested by the reviewer, we have conducted the suggested studies and we now show that addition of anti-mucoricin antibodies to dead hyphae of different Mucorales blocks damage to A549 cells, albeit to different degrees (50-70% see **Fig. 1e, Lines 123-128**).

11. Does the anti R. delemar mucoricin antibody block release of IL-8? i.e. is host recognition of the toxin required in order to induce cytokine release? If not, is IL-8 released as a consequence of apoptosis/necrosis? Following on, detecting IL-8 alone is insufficient to assess the immune response, particularly as the authors model states that mucoricin damages epithelial cells, causing them to secrete IL-8 "and other proinflammatory cytokines". Since mucoricin is damaging then the damage associated cytokine IL-1a (or similar) should probably be tested. Likewise, GM-CSF and/or G-CSF should also be investigated as they have similar functions to IL-8 in neutrophil recruitment and/or activation. Many other cytokines/chemokines could also probably be investigated but the above should suffice. Does the antibody block the release of all these cytokines?

Answer: Following the comment by Referee 3, and further thoughts on this issue, we have opted to take this entire section out since the current paper should be focused on the contribution of the toxin to pathogenesis and analogies to the structure and function of ricin. The effect of the toxin on the host immune response will be addressed in a separate and more detailed investigation.

12. **Western blot analysis of toxin production in RNAi treated cells indicates that toxin production is vastly reduced but not inhibited. The authors state that surviving mice infected with RNAi-treated fungi had no detectable fungi in their lungs at day 21. How was this determined? No CFU data has been presented in the manuscript. I assume CFU will be present for the empty plasmid but absent for the RNAi at 21 days? Please show data or comment. If the fungus is cleared, presumably the host immune response is not induced by the toxin?**

Answer: The conclusion that the lungs of surviving mice had no residual infection was gleaned by harvesting the lungs from surviving mice on Day 21 (when the survival experiment is terminated) cutting the lungs into pieces and plating them on PDA plates **(Gebremariam *et al.* Reference 12** of the revised manuscript). Using this technique, no growth was detected. This is now explained in the results (**Lines 184-185).** The absence of growth is for all strains used (empty plasmid 1 mouse and the RNAi strain 12 mice) since the evaluation was conducted at Day 21 post infection when mice appeared healthy and unlikely to die from infection.

13. **The manuscript often refers to necrosis as being the predominant mechanism of cell death during mucormycosis but uses the Apoptag kit to stain lung tissue as a means of demonstrating cellular damage. The Apoptag kit detects apoptotic cells in situ by labelling and detecting DNA strand breaks. Apoptosis and necrosis are mechanistically distinct. Necrosis is a caspase-independent process, whereas apoptosis requires caspase activity and may proceed through intrinsic and extrinsic signaling pathways. Can the authors please clarify whether mucoricin induces apoptosis or necrosis.**

Answer: We agree with the Referee. It is known that ricin and ricin-like toxins induce host cell death by both necrosis and apoptosis. Consistent with these published data, we used a kit that differentiate between the two mechanisms of cell death and compared both ricin and mucoricin in their ability to induce death of A549 cells. Both toxins induced comparable cell death by necrosis and apoptosis. These data are now presented in **Fig. 6c and discussed (Lines 298-304)**.

14. **Can the authors speculate whether the mechanism of toxin-damage is the same between HUVECs and red blood cells? There are significant differences between endothelial cells and mature red blood cells, for instance long-term retention of a nucleus and ribosomes.**

Answer: This point was indeed confusing. Such studies are beyond the scope of the current manuscript and will be the focus of future studies.

15. ***Line 282:*** **Lack of RLT1 expression in spores is not shown. In addition, to support this analysis, a western blot in which the whole film is shown (and not a cropped image) would rule out the possibility of any non-specific binding events.**

Answer: The requested data are now presented in **Extended Data Fig. 4b**. Please see above for the whole Western blot. To preserve space, a cropped western blot from the image above is provided in **Fig. 3a.**

16. **Also, this manuscript used male mice. What about female mice? It is well known that male and female mice can respond differently to pathogens and particularly toxins. Ideally, data with female mice should be included in the manuscript.**

Answer: Dr. Ibrahim is part of a consortium contracted by the NIH to provide small animal (mice) service testing of small molecules, vaccines and immunotherapies for mucormycosis. Included in these studies was the effect of mouse gender on experimental outcome. Although these data have not been published, we found no evidence of any differences in male versus female mice response to infection or treatment. A statement in the Method section has been added to indicate this **(Lines 693-695**).

17. ***Extended Data Fig. 6.*** **Immunohistochemistry of a patient's lung apparently shows association of the toxin with fungal hyphae that has caused necrosis and massive infiltration of tissues. However, there is fluorescence is many places, with limited localization to the fungus? Can the authors indicate whether there is any non-specific binding by their anti-mucoricin antibody (please see comment relating to Figure 3A). A control biopsy without mucormycosis is also required. This is also the first immunofluorescence picture. Immunofluorescence with the mouse biopsies may be highly beneficial to demonstrate toxin localization to the fungus.**

Answer: We now provide data that mucoricin is not only cell-associated but also shed/secreted. The fluorescence that is not associated with the hyphae is due to the antibody reacting to the shed/secreted toxin. In the new image (**Extended Data Fig. 10**) we included an IHC of biopsy of a patient infected with invasive pulmonary aspergillosis (identified by a histopathologist and confirmed by qPCR). We do not see any residual fluorescence in lungs from the patient infected with aspergillosis, thereby indicating that the antibody used is specific. This addresses the reviewer's concern regarding the antibody specificity and is a better control than a biopsy from uninfected patient.

18. ***Extended Data Fig. 11b*****. These images are insufficient for the conclusions. Also, most of the cells do not appear to have toxin. The toxin may only enter when the cell is dead. Better confocal imaging is required with z-stacking (side/top views) etc. to demonstrate entry and clarity at different time points.**

Answer: Following the recommendation of Referee 3 (point 6 below), this Extended data has been deleted and will be the topic of future investigation focusing on the mechanism of host cell entry and damage.

Minor comments:

19. **The manuscript used different mouse models i.e. DKA and immunosuppressed mice etc. Why are different mouse models required for different parts of the manuscript? Also, wild type mice are not used. Do these fungal species and mucoricin not cause 'infection' in healthy mice? Discussion required. Also, in the relevant Figure legends, please indicate which mouse model was used to help the reader.**

Answer: This is a good point. Mucormycosis is primarily a disease of **immunocompromised** hosts. The DKA and immunosuppressed mice are two clinically relevant models representing the two most vulnerable mucormycosis patients. Both models were developed in the Ibrahim's laboratory. They are well-

characterized and considered to be the standard models for testing pathogenesis, evaluating antifungal therapies and immunotherapies. Wild-type mice have normal (robust) immune systems and are resistant to pulmonary infection. These patient categories were described in the introduction of the original version (**opening paragraph**).

20. Mucoricin is clearly damaging but it is also clear that damage still occurs in the absence of mucoricin. I assume this indicates that other potential toxins may exist in these species? Discussion required. Is the mucoricin encoded by an entire open reading frame, or is it present as a cryptic sequence within a larger protein such as the case with C. albicans candidalysin/ECE1?

Answer: The reviewer is correct. There is another different and mainly secreted toxin that we are currently working on. The contribution of this toxin to pathogenesis is still under investigation and is the topic of another study. A statement to this effect is now added to the Discussion **(Lines 408-410**). Mucoricin is encoded by the entire ORF since the purified protein size matches the predicted size from the ORF.

21. Recombinant mucoricin can be expressed in S. cerevisiae. Mucoricin is believed to exert its effect on the host ribosome. Does the expression of mucoricin have any negative impact on S. cerevisiae? Both mammalian cells and S. cerevisiae are eukaryotic and presumably have a reasonable degree of ribosomal conservation.

Answer: Most likely (and just like ricin) it does have some toxicity to the encoding host since the level of production is low (0.2-0.5 mg/Liter).

22. When mucoricin was injected intravenously, histopathology indicated the presence of "large" macrophages in the lung. What is meant by "large" in this context? Are these a particular type of macrophage? Are they atypical from what would normally be expected? Are the same "large" macrophages observed when the lung is infected with wild type fungus? Were other organs similarly infiltrated? If not, does this suggest a degree of tropism to the lung?

Answer: We should not have used word 'large' for macrophages. Our intention was to point to the infiltration of megakaryocytes into the lungs and liver, which is abnormal. This is now corrected (**Line 155).**

23. Orthologs of mucoricin are present in 21 previously sequenced species. Do they all possess the LDV sequence for example? A sequence comparison of orthologs would

Answer: All Mucorales sequenced that are known to cause mucormycosis contain the [x]D[y] motif (**See Baluna *et al.* Reference 25**) in the mucoricin orthologs (x=L,I, G, or V, and y=V, L, or S) . The majority contain LDV motif and some have IDV, IDS, GDL, or VDV. This information is now presented in **Extended Data Table 3.**

24. Mice were infected intratracheally and then given 30 ug of antibody. Does the antibody (or its vehicle) prevent spore germination/hyphae formation?

Answer: The antibodies have no effect on spore germination or the growth of the fungus. This is now presented in **Extended Data Figure 3**.

25. The amount of mucoricin used in experiments is described as mass per unit volume (i.e. 20 ug/ml). The molar concentration of mucoricin should be used throughout the manuscript.

Answer: The concentration of the toxin is now presented in molar concentration as suggested by the reviewer.

Referee #3 (Fungal pathogenesis)

Remarks to the Author:

1. Overall, and up to including Figure 3 and Supplementary Figure 5, this is a really solid manuscript, with each next experiment being logical and comprehensively carried out. After that I feel there is a slight loss of focus, with occasional elements which could be tidied up or reordered to improve the manuscript.

Answer: We have added new experimental data and bioinformatics to support the functionality of the toxin and its relation as a ricin-like toxin. We have retained **Fig. 4** since we think it strengthens the study (now **Fig. 5**). Some of the figures beyond **Extended Data Fig. 5**, are also important. However, we do agree with the Referee that some of the Extended Data beyond Fig. 5 reduce the focus of the paper and we opted to take them out (*e.g.* effect of the toxin on IL-8 production, phylogenetic tree, **Extended Data Fig. 7 and 11**).

Comments:

2. It should be pointed out that the presence of such toxins has been "identified" in the genomes of fungi previously (Revising the taxonomic distribution, origin and evolution of ribosome inactivating protein genes) however I don't think this detracts from the novel toxic activity reported here. Actually, I found the Ricin Interpro domain of Rhizopus mucoricin (IPR000772) present in almost 400 sequences using the FungiDB Interpro Domain finder tool indicating the presence of a large number of ricin domain-containing proteins in fungi, well beyond what would be found by BLAST analysis.

Answer: Please see our response to Referee 1 and revised **Extended Data Table 1**.

3. The fact that even killed hyphae continue to cause significant cell damage (likely due to persistence of mucoricin activity) is of potentially enormous clinical significance. i.e. – a patient would continue to suffer tissue necrosis, even after the fungus was fully killed by an antifungal. It would have been nice to combine the approaches of e.g. Figure 1b with toxin RNAi or antibody to directly demonstrate that the damaging activity of dead hyphae is mucoricin-mediated.

Answer: Data with the antibody blocking damage caused by killed hyphae have been added **(Fig 1e and discussed on Lines 123-128).**

4. *Line 220.* "The hyphae-associated toxin has structural features of ricin" Here the authors claim they "embarked on structural and bioinformatics studies" by directly

aligning their mucoricin against known toxin sequences and reporting "Rhizopus delemar toxin, showed the highest homology with ricin produced by the castor bean plant, Ricinus communis" I don't think that restricting comparisons of mucoricin to "well-known" toxins is the best way to interrogate its phylogeny or evolutionary origin. By performing a BLASTp search of RO3G_06568 at NCBI and excluding the fungal kingdom, it's apparent that mucoricin shares high similarity with ricin sequences from CNS and cyanobacteria. In fact, the similarity between mucoricin and these bacterial sequences is higher than to most non-Mucorales fungi. So, whilst the conclusion "Phylogenetically, the toxin is most similar to ricin…" is true, it would appear to be far more closely related to bacterial ricin than to plant. Whether this is due to HGT acquisition of a bacterial ricin by fungi (e.g. Metazoan Ribosome Inactivating Protein encoding genes acquired by Horizontal Gene Transfer) or loss of RLT1 by other eukaryotic lineages (e.g. Revising the taxonomic distribution, origin and evolution of ribosome inactivating protein genes) is anyone's guess at this point! Along with this point, *Extended Data Figure 7b* is not particularly useful. It would be much more informative to generate a tree based on mucoricin and proteins with actual phylogenetic similarity (i.e. BLASTp reciprocal best hits). Not candidalysin!

Answer. After rethinking this, we agree. Please see our response to the phylogeny issue above (Referee 1 point 3). Following the Referee's recommendation, **Extended Data Fig. 7** has been omitted.

5. **If mucoricin is associated with the hyphal cell surface as is shown in the manuscript – is it also being released? Was there any evidence for it being shed from the fungus? This would be important for it to get into host cells.**

Answer: See our response to Referee 2, point 7. Indeed, the toxin is also released (*i.e*. shed or secreted) and these data are now included in **Fig. 4**.

6. ***Line 303-332*. "Mucoricin, like ricin, induces inflammation". Overall, this section is a bit weak and feels more like the pilot data for the next study. I would personally integrate the IL-8 observation into an earlier section rather than have it as a standalone result. e.g. it would fit alongside histology/PMN infiltration.**

Answer: We agree with the Referee. These data have been removed and will be the subject of a future study on the immune response. We now feel that this paper should focus on the structure and function of the toxin.

7. ***Extended Data Figure 11* – for me, this would be the nice initial observation in a future study looking at the mechanism of mucoricin entry into host cells. It's a neat observation but doesn't sit right as the final data set in what is otherwise a really solid and logically narrated story.**

Answer: Following the Referee's recommendation, **Extended Data Figure 11** is omitted from this study and will be the topic of future studies focusing on the mechanism of the toxin entry and trafficking into target cells. It should be noted that in the ricin field, this whole issue took many years to work out.

Minor comments.

8. **"This organism also damages the A549 alveolar epithelial cell line and primary alveolar epithelial cells, but only after 30 h of incubation (Fig. 1a)." Is there really damage to A549 at 30 h?**

Answer: A minimal damage of ~ 5% of the host cells was consistently observed at 30 has now indicated in the text (**Line 85).**

9. **Was the purified and/or recombinant toxin heated and shown to still be active (to fit with heat killed hyphae conclusion)?**

Answer: The purification of the toxin using host cell damage assay as a readout and the inclusion of anti-mucoricin antibody blocking data of heat-killed hyphae as shown in **Fig. 1e** should suffice for the role of the toxin in heat-killed hyphae.

10. ***Line 124* "act similarly in damaging A549 cells in a rapidly time dependent manner" Remove "rapidly". To many, rapid would mean seconds.**

Answer: The word 'rapidly' has been removed.

11. ***Extended Data Fig. 6* – whilst it's nice to indicate mucoricin is part of the human pathology, I feel this single observation is a little weak as is.**

Answer: We feel that these data gives clinical relevance to the identified toxin. These data is now stronger with the inclusion of an IHC from a patient with invasive pulmonary aspergillosis (**Extended Data Fig. 10**) showing lack of detection of mucoricin.

12. ***Line 236* – "We generated a 3-D structural model" – insert "predicted"**

Answer: Done

13. ***Line 295* "Concordant with these results, it has been shown that HUVECs are rapidly damaged by the LDV-motif (Fig. 4a) than the sequences responsible for ribosome inhibition" – rephrase**

Answer: This has been addressed above and changed in the text **(Lines 358-363)**

14. ***Line 321* – "microscopy of epithelial cells treated with mucoricin showed instant rounding of cells" – I'm not sure I follow what's being said here.**

Answer: As in the case of ricin, rounding of cells in these assays are the first step in cell damage. Cells then detach from the matrix and die. This is well accepted in the field of endothelial cell biology. In the case of ricin, this is described by **Baluna *et al.* Reference 25** as noted above. In anyway, this data has been omitted as suggested by the Referee (**Extended Data Figure 11** of the original version).

15. ***Line 607* "Expression and purification of mucoricin" was the construct sequenced?**

Answer: Yes; all cloned genes were sequenced prior to production. This is now mentioned in the revised version (**Lines 540-541**).

Referee #4 (ricin)

Remarks to the Author:

1. **It would be nice to see how these antibodies see this toxin (epitope mapping?).**

Answer: We agree and fully intend to do this. However, this paper is focused on showing the role of the toxin in the pathogenesis of mucormycosis and its resemblance to ricin. Epitope mapping with a panel of MAbs is extremely important but clearly beyond the scope of the current study.

2. **N-terminal to the putative lectin binding domain are 16 amino acids with some (gapped) similarity to the C-terminal part of ricin A chain. This part of ricin A chain has the 5 amino acid active site motif SEAAR associated with the depurination activity of ricin A chain (and which is identical in Shiga toxins which also depurinate ribosomes). However, the fungal toxin FEEGR, I would suggest, is a very poor copy of this, so it would be an unlikely candidate for any putative deprivation activity. The depurination activity of this would surely be important for this manuscript since the title of the manuscript specifically points to the similarities with ricin. So why is this activity assay relegated to the subordinate Supplementary data section in (Ext Fig 8)? I am not familiar with this assay. I would very much like to see this assay validated using ricin A chain, and also with heat inactivated ricin A chain (no depurination) and heat-inactivated fungal toxin.**

Answer: As detailed in our response to Referee 2, we now introduce evidence of ribosome inhibition using the standard cell-free rabbit reticulocyte protein inhibition assay. The concentration of mucoricin required to cause 50% inhibition in protein synthesis (IC_{50}) is about 800 times higher than the concentration of ricin needed to cause similar protein inhibition (2.2×10^{-11} M for ricin vs. 1.7×10^{-8} M for mucoricin). These data are now shown in **Fig. 6a**. This "weaker" activity of Mucoricin as compared to ricin toxin might indeed be explained by the EEGRL version of the active ricin motif of EAARF as stated by the Referee. It is prudent to keep in mind that this fungal toxin does not need to be as potent as ricin or Shiga toxin to be required for pathogenesis. As detailed in our response to Referee 2 (point 9), mucoricin has a EAANQ that aligns with the ricin EAARF, in which the glutamic residue required for the ricin and shiga toxin activity conserved. Finally, WGRLS sequence required for RIP activity in Shiga toxin aligns with the *R. delemar* sequence of EEGRL All these possibilities are now discussed in the revised manuscript (**Lines 325-349**). Since we now show that mucoricin inhibits protein synthesis, we have omitted the depurination data from the revised manuscript.

3. **If there is a confirmed quantifiable depurination activity, where is the evidence that this is part of the mechanism of cell damage by this toxin? There are no data here that suggest that the toxin is internalized. And a statement that the spores bind GRP78 makes little sense. GRP78 is BiP, a chaperone that resides in the endoplasmic reticulum. How would a spore see this? Unless of course, this is BiP released from ruptured cells – which is not the same as internalization. The**

reference that supports this (reference 37) is Alqahiri, IDweek. IDweek is an annual meeting of IDSA and is not peer-reviewed: this whole section should be removed.

Answer:

i. It is well-documented that although the majority of GRP78 are cytoplasmic, to carry out its function as a chaperone, it is also expressed on the cell surface, especially under stressful conditions. It is also well-documented by us and others that GRP78 acts as a receptor to a variety of pathogens during host cell invasion by Mucorales **(Liu *et al.* JCI, 2010:120:1914-1924; Gebremariam *et al.* JCI, 2016;126:2280-2894**) Dengue virus **(Jindadamrongwech *et al.* Arch Virol, 2004;149, 915-927),** MERS and coronavirus HKU9 **(Chu *et al.*, JBC 2018,293;11709-11726)**, Coxsackieviruse A9 (**Triantafiluo *et al.*, J Virol., 2002;76:633-643)**, and zika virus **(Ojha *et al.*, Front. Immunol. 2018;9:2340**). Consequently, the fungus invades the host cell *via* host cell receptors including GRP78 on endothleial cells and nasal epithelial cells and integrins and EGFR on alveolar epithelial cells, Since the toxin is cell-associated, it can exert its effect on the host cell once it is internalized.

ii. With the new data showing that the toxin is also secreted, it can be internalized (by a mechanism yet to be determined) and exert its inhibotry effect on protein synthesis.

iii. The above mentioned refernce is now published in mBio (**Reference 43**). A revised model is introduced in the revised manuscript (**Lines 387-405**).

Statistics.

4. There is some ambiguity in description: for example, in the legend to *Fig. 1c*, there is a description of n=6/group from two experiments. But if there are two experiments, then n = 2. There is a danger of pseudoreplication here that must be avoided so that P-values are not artificially lowered. And if there are two experiments with 6 observations, is these three observations per experiment? This really is not clear. *Fig 2b* legend, *Fig 3C*, *Ext Fig 3b*, *Ext Fig 9c*, *9d* and *Extended Fig 10* all have this same ambiguous writing.

Answer: We apologize for the ambiguity in the Figure legends. All data now represent the combination of data points collected from three independent experiments. This is now explained in all the figure legends.

5. Error bars in *Ext Fig 3b*, *Ext Fig 1* and *Fig 2b*. What are they? SD? SEM?

Answer: All data are presented as median ± interquartile range.

6. Why are some experiments shown with individual points, median and interquartile range, whereas others are summary statistics (mean plus or minus something undefined)? Lack of uniformity.

Answer: See our response above

7. ***Fig 3e*** **and** ***3f*****. Mouse survival is typically displayed in a Kaplan-Meier step plot (declining horizontal steps with vertical risers) and the analysis is really a modified chi-squared. However, the authors have just joined the dots.**

Answer: Both formats are correct, and we routinely use the one depicted. The two figures in question, are correctly presented as Kaplan survival curves.

8. **How was** ***Fig 3d*** **normalized? The data presentation suggest that toxin-treated cells can have up to 135% cell damage – this should alert the authors to a normalization problem.**

Answer: Each data point is normalized to the median of damage without IgG. This is mentioned on the *y-axis*.

Other comments.

9. **There is a two-hour window between hours 1 and 3 in Fig 2b with no data points, between all and nothing, so the graph is uninformative. Why not just show this as an end-point assay?**

Answer: The point in showing the figure in this manner is to demonstrate that HUVECs are more rapidly damaged than alveolar epithelial cells. This is stated in the Discussion (**Lines 354-358**).

10. ***Line 161*****: mice appeared healthy – a somewhat qualitative statement.**

Answer: The words "appeared healthy" have been deleted.

11. **Line 241: why is (e.g. RIP) needed?**

Answer: RIP has been deleted

12. **Line 256 – and Fig 4e: Western blot. There is only one polypeptide identified on the Western I'm guessing the anti-ricin antibody was really anti-ricin B chain?**

Answer: The figure legend states that the Western blot is carried out using the polyclonal anti-mucoricin polyclonal antibody. As stated, the 8A1 MAb reacts with ricin B chain (**Lines 287, 472, 618, and 620, and Reference 30).**

13. **Line 271. Diphtheria toxin and Pseudomonas exotoxin are not RIP: they inactivate elongation factors, not ribosomes**

Answer: corrected.

14. **Line 273. Vague writing: what does 'it is cell-associated' mean in this context? Fungal cell? Host cell? Necrotic cell?**

Answer: Cell-associated means **not** secreted but rather associated with the fungal cell. We have rephrased this. New data showing that the toxin is also shed/secreted is now included.

15. **HUVEC (*line 292-293*) and endothelial cells (*Ext Fig 9d*). Please use consistent labels/descriptions.**

Answer: HUVECs is now used instead of endothelial cells in the Figure (**Extended Data Figure 4d of the revised version**).

16. **Line 299: a very vague mechanistic statement. Please remove – it adds nothing to the story.**

Answer: The last sentence has been removed.

17. **Line 303 to 316. Mucoricin (…) induces inflammation. Since the comparison is with ricin, and therefore presumably important for this story, why are all the inflammatory data confined to Extended Data sections? The following section/ lines raise the mechanistic prospect of a time lag caused by internalization. However, the authors present no data to suggest internalization, and in their model (initiation and pathogenesis model section) do not even mention internalization.**

Answer: As suggested by Referee 3, the section on inflammation has been removed and will be the topic of future investigations. The paper now focuses on the structural and functional comparisons between ricin and mucoricin. These data are presented as actual figures and not supplementary data.

18. **Display. Scale bar Fig 2e is likely to be unreadable when data are displayed by the journal – way too small: and the white arrow on the lung tissue is almost invisible. The scale bar on Fig 3 b will also become invisible when displayed. RNAi knockdown section: Down-regulation was measured by qRT-PCR (*line 142-143*) – but not shown.**

Answer: All these issues have been fixed and the RNAi data which resulted in >90% inhibition of the gene expression are now presented in **Extended Data Figure 5**

Decision Letter, first revision:

Dear Ashraf,

Thank you for your patience while your manuscript "Mucoricin is a Ricin-Like Toxin that is Critical for the Pathogenesis of Mucormycosis" was under peer review at Nature Microbiology. It has now been seen by our referees, whose expertise and comments you will find at the end of this email. In the light of their advice, we have decided that we unfortunately cannot offer to publish your manuscript in Nature Microbiology.

From the reports, you will see that while they find your work of some potential interest, and referees #1 and #3 are satisfied with the revised manuscript, referees # 2 and #4 still raise important concerns about the insufficient experimental demonstration that mucoricin functions similarly to ricin. In particular, referee #2 says "the authors must show whether (i) mucoricin inactivates ribosomes or elongation factors to inhibit protein synthesis, and (ii) the proposed motif (EEGRL) is required for RIP activity. It would also be beneficial but not essential to show that the LDV/VDV motif is required for damage activity." Specifically, referee #2 says "A key experiment that needs to be performed is to

express and purify a mutant mucoricin that does not contain the proposed motif required for RIP activity (EEGRL) and compare it with wild type toxin in the cell-free system." Furthermore, referee #2 says "the authors must show the mechanism of mucoricin function i.e. does it inactive ribosomes or elongation factors to inhibit protein synthesis and is the proposed motif (EEGRL) required for RIP activity. Without these data, the final conclusions as to whether mucoricin does in fact function similarly to ricin are too speculative." Referee #4 is concerned over the insufficient experimental support for ricin A chain like activity, stating that "inhibition of rabbit reticulocyte lysate is not a specific assay for ricin activity". Given the length of time that it would likely take to address these concerns thoroughly, these criticisms are sufficiently important as to preclude further consideration of your work in Nature Microbiology.

However, editorially we remain very interested in this study, and if you do feel that you would be able to include additional work to address these points, we would be willing to consider an appeal, although please note that we would reassess novelty with respect to existing literature at the time of appeal and would be unlikely to engage the referees again unless we felt that their concerns had been satisfied in full. In the case of a successful appeal and eventual publication, the received date would be that of the revised paper.

I am sorry that we cannot be more positive on this occasion, but hope that you find the referees' comments helpful.

Author Rebuttal, first revision:

Our answers to the specific Referees' critiques are listed below:

Referee # 1 (Mucormycosis)

4. **My only concern is the methodology for molecular phylogenetics, and evolution of the protein seems flawed. I re-analyzed these data and did There is also a homolog of the toxin protein the plant associated Glomeromycotan fungus Rhizophagus. It would be useful for the authors to acknowledge that the protein is not Mucorales/Mucormycotina-specific and provide some speculation on the role in other fungi or the implications of it being a gene that has been around for a while in this group of fungi, even if the animal disease properties are more recently emerged.**

Answer: We do not claim that mucoricin is unique to Mucorales/Mucormycotina. Our most recent BLAST search of the toxin ORFs retrieved many orthologies, the majority of which belong to the order Mucorales with a high % predicted amino acid identity (ranging from 50-90%) (**see revised Extended Data Table 1**). Many of these Mucorales fungi are known human pathogens (e.g. *Rhizopus, Mucor, Cunninghamella, Lichtheimia,* etc.), animal pathogens (e.g. *Mortierella*) or

plant pathogens (e.g. *Choanephora cucurbitarum*). On numerus occasions, these retrieved ORFs are annotated as ricin domain-containing proteins or ricin B-like lectins (including in *Rhizopus*). However, and as stated by the reviewer, less identical ORFs from other fungi such as, *Rhizophagus* (30-40% identity) were retrieved (**Lines 115-117**). *Rhizophagus* lives symbiotically with plants and is recognized as an integral part of the natural ecosystem and was shown to delay plant disease symptoms caused by *Phytophthora infestans*. We also retrieved ORFs belonging to the bacterial genera of *Streptomyces* and *Paenibacillus* (30% identity), both are known inhabitants of soil and present in rhizosphere of various plants. These two bacterial genera are known to be producers of antibiotics and can be used as biological control agents for crops. We now include a paragraph on these possibilities in the Discussion (**Lines 312-318)**.

5. **The other criticism about this section (extended figure 7) are that clustalOmega dendrograms are not good phylogenetic trees for reconstructing history. The neighbor-joining approach does not seem to fare well here for the sequences. I have reanalyzed the data (see attached figure) and find the *Pseudomonas* exotoxin is much more similar phylogenetically to the *Rhizopus* toxin protein than in fact Ricin is. So, it would be important to clarify this in the text and interpretation. Generally a maximum likelihood analysis is more appropriate for resolving the phylogenetic tree.**

Searches of the Rhizopus toxin against the listed dataset of toxin proteins did not find any significant sequence similarity - perhaps the authors focused more on results that came from protein model analyses which could have rescued more alienable regions due to threading through protein structures? Otherwise I think a revision of this figure and restating the interpretations of homology need to be made. I do think the protein modeling is appropriately performed and I think is still providing evidence for a distance connection between these toxin proteins, but I think the authors could more clearly state the data.

Answer:

vii. As suggested by the reviewer, we re-analyzed the phylogenetic tree using the maximum likelihood method and indeed found that *Pseudomonas* exotoxin A to be more related to our toxin than ricin despite the lower sequence similarity compared to ricin (mucoricin and ricin have ~ 26% overall amino acid sequence identity *vs.*16% for mucoricin and *Pseudomonas* exotoxin A).

viii. However, after consulting with Dr. Julie Dunning Hotopp (an expert in genomics/sequence analysis/phylogenetics), we concluded that we should remove any phylogeny or discussion of ancestry from the paper. This is because the sequence divergence is too great to align the sequences rigorously.

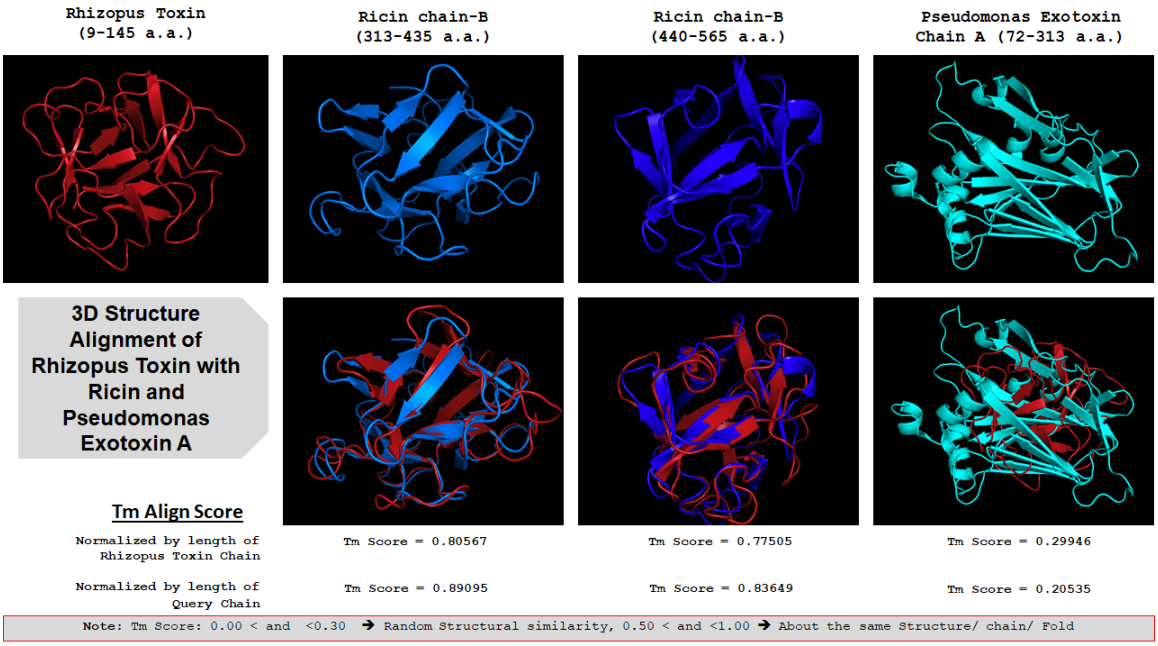
ix. We have tried downloading sequences using PFAM and also to use BLAST searches but could not produce good alignments that we trust with anything outside the fungal clade. As the

editor and reviewer know, if the compared sequences are not aligned well, the phylogenetic inference will be flawed. That said, there are clearly conserved motifs, that we have already identified. However, these motifs alone are not phylogenetically informative.

x. From the BLAST search the most we can say is that mucoricin has the hallmarks of vertical inheritance in the eukaryotic lineage. The closest sequences are mainly fungal. A few are bacterial (**Extended Data Table 1**).

xi. Importantly, the new data using the cell free rabbit reticulocyte assay to measure inhibition of protein synthesis and add further evidence of the similarities between mucoricin and ricin. These points are discussed below in response to specific questions to other Referees.

xii. Finally, the low confidence in the phylogenetic similarity of *Pseudomonas* exotoxin A to mucoricin is consistent with our 3 dimensional modeling showing the weak alignment of this toxin with mucoricin compared with the strong alignment between mucoricin and ricin B-chain (see images blow).



6. **Minor: Line 71 *Rhizopus delemar* is spelled out but I think you can abbreviate to *R. delemar* now.**

Answer: It is now abbreviated (**Lines 75-76**).

Referee # 2 (Fungal pathogenesis)

Major comments and critical issues:

1. **The key message of this manuscript is that mucoricin is a ricin-like toxin, which has ricin-like functions such as the ability to inactivate ribosomes and possessing an LDV motif that mediates vascular leakage. However, apart from showing damage data, none of these functions are actually shown in the manuscript.**

Answer:

iii. In the revised version of the manuscript, we compared the ability of ricin and mucoricin to inactivate protein synthesis using the standard cell-free rabbit reticulocyte assay. In this assay, mucoricin inhibited protein synthesis, albeit at higher concentrations than ricin (IC_{50} of 1.7×10^{-8} M and 2.2×10^{-11} M for mucoricin and ricin, respectively) **(Fig. 6a,b**). The weaker activity of mucoricin is expected since ricin is the second most toxic protein known.

iv. Baluna *et al.* (**reference 25** of revised manuscript) have shown that small peptides containing this LDV sequence but **not** the amino acids in the active site of ricin A chain, caused vascular leak. Therefore, the active site of ricin (or any molecule) is not involved in compromising the integrity of endothelial cell monolayers. We neglected to discuss this in our original paper and have now done so **(Lines 358-363**).

2. **Ricin A chain depurinates 28S ribosomal RNA, thereby inhibiting protein synthesis. Mucoricin was combined with "template" RNA from A549 cells and release of adenine was demonstrated. However, demonstrating the depurination of template RNA is not robust enough to support a model of ribosomal inactivation through specific depurination of 28S ribosomal RNA. For instance, if mRNA transcripts were the only RNA species to be depurinated following mucoricin treatment this may also lead to cessation of protein synthesis, but the functionality of the host ribosome would remain unaltered (i.e. the ribosomes would still be translationally competent). Therefore, mucoricin-induced depurination of 28S rRNA must be demonstrated. Furthermore, the inhibition of host protein synthesis by mucoricin has not been demonstrated in vitro or in vivo. Experimental techniques such as polysome profiling and/or the incorporation of 35S-labelled methionine into proteins will help address this critical**
issue. The authors must show, through experimentation, that mucoricin inactivates ribosomes and inhibits host cell protein synthesis. Should mucoricin depurinate host ribosomal RNA, this would imply a physical association between mucoricin and the host ribosome. The physical association of mucoricin with ribosomes needs to be demonstrated experimentally.

Answer: We have deleted the depurination data as suggested by Referees 2 and 4 and added the inhibition of cell-free protein synthesis as suggested (**Fig. 6a,b**). In future studies we will investigate the molecular events involved in the ribotoxicity of mucoricin in more detail.

10. ***R. delemar* is endocytosed into HUVEC cells through the interaction between CotH3 and host GRP78. Presumably, endocytosed fungus is contained within an endocytic vesicle. This being the case, how does mucoricin exert its effect on the host ribosome? Or does the fungus escape and does mucoricin physically associate with ribosomes (see above).**

Answer: It is quite possible that like ricin, mucoricin in the endocytic vesicle translocates into the cytosol and catalytically inactivates ribosomes. As noted above, the intracellular trafficking pathway of mucoricin remains to be studied in more detail. This will be the subject of future work since it is well beyond the scope of this paper.

11. **The authors speculate that mucoricin possesses an LDV lectin binding motif, like ricin, that may mediate vascular leakage. However, in Fig 4a ricin is shown to have a VDV motif, not LDV? Please clarify**.

Answer: We apologize for the confusion caused by the alignment.

iii. Indeed, ricin toxin A chain contains an LDV motif (amino acid 109-111) that is required for vascular leak (**Baluna *et al.* References # 25 and 27** of the revised manuscript**).**

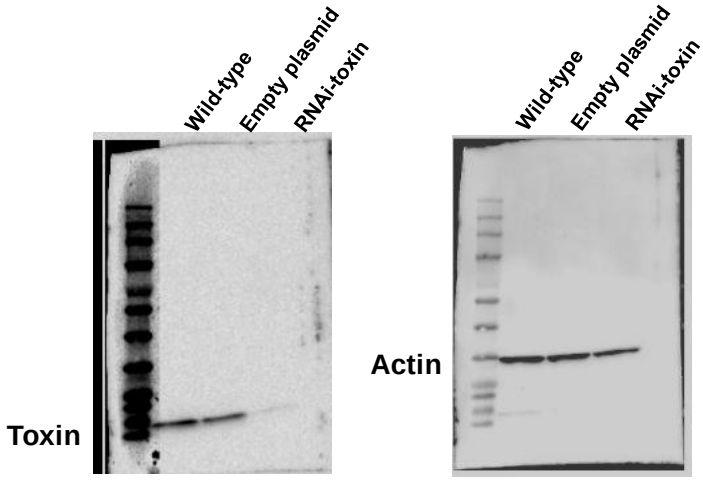
iv. The VDV motif (335-337) is in ricin B chain and happened to align with the LDV motif since the highest sequence resemblance between mucoricin and ricin is within ricin chain B. This issue is clarified in the revised manuscript by inclusion of the full sequence alignment (**Fig. 5a**)

12. **Following on, the authors provide no experimental evidence that the LDV-motif is responsible for damage or vascular leakage. The authors need to mutate the LDV motif and demonstrate this. The galactose data is not sufficient. These data, together with the ribosomal data mentioned above, will be sufficient to demonstrate that mucoricin indeed functions similarly to ricin.**

Answer: We have addressed this above (**Referee 2, point 1ii**).

13. **The antibody that recognizes mucoricin from R. delemar must be characterized thoroughly against whole spore, germling, hyphal and host cell extracts in order to rule out non-specific binding. This required to have confidence in the western, immunofluorescence and IHC data.**

Answer: In the revised manuscript, we show that the anti-mucoricin antibodies do not recognize hyphae or the surrounding tissues of a patient with invasive pulmonary aspergillosis (IPA) (**Extended Data Fig. 10).** The diagnosis with IPA was confirmed by qPCR using the GeneProof Kit for *Aspergillus* and by H&E histology (thin, septate hyphae [**Extended Data Fig 9**]). This confirms the toxin specificity of our antibodies. Additionally, and more importantly, our Western blot data show a single band with the correct size of 17 kDa. Please see the Figure below for a full western blot. To conserve the space of the figure, we truncated this figure to show the mucoricin, and actin bands. Consistent with the qPCR data showing lack of expression of the mucoricin ORF (**Extended Data Figure 4a**), the antibodies do not bind to spores (**Extended Data Figure 4b**).



14. **Data derived from in vitro experimentation should be a minimum of three biological repeats. It appears that only two biological repeats are presented in some data sets.**

Answer: As noted above, all data are now presented as a replicate of three independent experiments.

Other important comments:

15. **Is mucoricin secreted or cell associated only? The summary model at the end of the manuscript states that both live and dead hyphae "release" mucoricin. However, mucoricin is also described as being hypha-associated elsewhere in the manuscript, which appears to be**

supported by immunofluorescent analysis of R. delemar hyphae. Ideally, the authors should demonstrate by mass spectrometry whether mucoricin is secreted or not.

Answer: The toxin is mainly cell-associated but can also be detected in cell-free supernatant of growing hyphae (**Fig. 4a**). We do not know if it is actively secreted or shed. The answer to this question will require further experiments *in vitro* and in mice. These results are also supported by the IHC data showing the presence of the toxin on broad aspetate hyphae and in the surrounding tissues (**Extended Data Figure 10**). The revised manuscript includes these important new findings.

16. **Mucoricin-induced host cell damage was time dependent. Almost complete damage occurs after 3 hours of toxin addition, at which point mucoricin was observed around the host cells. However, after 48 hours the toxin was observed inside the cell. Therefore, is the damage caused by mucoricin at 3 hours a surface mediated event that relies upon host cell signaling? (as the toxin is not observed inside the host cell at 3 hours). If endocytosis is blocked, does this prevent damage/ribosome inactivation from occurring?**

Answer: Although the exact mechanisms underlying damage to host cells is yet to be deciphered and is currently the topic of active investigation in our laboratory, given the resemblance to ricin B and the presence of RIP activity, it is possible that mucoricin functions in a manner similar to ricin. It is known that ricin damages tissues by two different mechanisms:

1) The LDV motif in ricin A chain compromises the integrity of endothelial cell monolayers (**Baluna *et al.* Reference 25** of the revised manuscript); and

2) The (ribotoxic) active site sequences in ricin A chain inhibit protein synthesis after series of events involving binding of the holotoxin to cells by the B chain, internalization, separation of the A and B chains, translocation of the A chain into the cytoplasm and catalytic inactivation of ribosomes. The EAARF motif in Ricin A chain is known to be responsible for the RIP activity through depurination of adenosine 4324 in 28S rRNA with the glutamic acid residue (yellow highlight) responsible for this activity **(Hovde *et al.* Reference 38** of the revised manuscript**).** Furthermore, the arginine residue (cyan highlight) is also required for the activity of Shiga toxin (a ricin-like toxin) (**Basu *et al.* Reference 39** of the revised manuscript**).** We have rewritten the discussion to indicate that mucoricin contains both the LDV motif and has a EEAAN that aligns with the ricin EAARF, with the glutamic residue conserved. Mucoricin also contains the EEGRL in which the glutamic acid and arginine residues conserved (**Lines 325-349**).

10. Unfractionated extracts from R. oryzae, R. delemar, Lichtheimia corymbifera and Cunninghamella bertholletiae all damaged A549 cells, but not all to the same extent. Can the damage caused by these orthologs be titrated by the anti R. delemar mucoricin antibody?

Answer: As suggested by the reviewer, we have conducted the suggested studies and we now show that addition of anti-mucoricin antibodies to dead hyphae of different Mucorales blocks damage to A549 cells, albeit to different degrees (50-70% see **Fig. 1e, Lines 123-128**).

26. Does the anti R. delemar mucoricin antibody block release of IL-8? i.e. is host recognition of the toxin required in order to induce cytokine release? If not, is IL-8 released as a consequence of apoptosis/necrosis? Following on, detecting IL-8 alone is insufficient to assess the immune response, particularly as the authors model states that mucoricin damages epithelial cells, causing them to secrete IL-8 "and other proinflammatory cytokines". Since mucoricin is damaging then the damage associated cytokine IL-1a (or similar) should probably be tested. Likewise, GM-CSF and/or G-CSF should also be investigated as they have similar functions to IL-8 in neutrophil recruitment and/or activation. Many other cytokines/chemokines could also probably be investigated but the above should suffice. Does the antibody block the release of all these cytokines?

Answer: Following the comment by Referee 3, and further thoughts on this issue, we have opted to take this entire section out since the current paper should be focused on the contribution of the toxin to pathogenesis and analogies to the structure and function of ricin. The effect of the toxin on the host immune response will be addressed in a separate and more detailed investigation.

27. Western blot analysis of toxin production in RNAi treated cells indicates that toxin production is vastly reduced but not inhibited. The authors state that surviving mice infected with RNAi-treated fungi had no detectable fungi in their lungs at day 21. How was this determined? No CFU data has been presented in the manuscript. I assume CFU will be present for the empty plasmid but absent for the RNAi at 21 days? Please show data or comment. If the fungus is cleared, presumably the host immune response is not induced by the toxin?

Answer: The conclusion that the lungs of surviving mice had no residual infection was gleaned by harvesting the lungs from surviving mice on Day 21 (when the survival experiment is terminated) cutting the lungs into pieces and plating them on PDA plates **(Gebremariam *et al.* Reference 12** of the revised manuscript). Using this technique, no growth was detected. This is now explained in the results (**Lines 184-185).** The absence of growth is for all strains used (empty plasmid 1 mouse and the RNAi strain 12 mice) since the evaluation was conducted at Day 21 post infection when mice appeared healthy and unlikely to die from infection.

28. The manuscript often refers to necrosis as being the predominant mechanism of cell death during mucormycosis but uses the Apoptag kit to stain lung tissue as a means of demonstrating cellular damage. The Apoptag kit detects apoptotic cells in situ by labelling and detecting DNA strand breaks. Apoptosis and necrosis are mechanistically distinct. Necrosis is a caspase-independent process, whereas apoptosis requires caspase activity and may proceed through intrinsic and extrinsic signaling pathways. Can the authors please clarify whether mucoricin induces apoptosis or necrosis.

Answer: We agree with the Referee. It is known that ricin and ricin-like toxins induce host cell death by both necrosis and apoptosis. Consistent with these published data, we used a kit that differentiate between the two mechanisms of cell death and compared both ricin and mucoricin in their ability to

induce death of A549 cells. Both toxins induced comparable cell death by necrosis and apoptosis. These data are now presented in **Fig. 6c and discussed (Lines 298-304)**.

29. **Can the authors speculate whether the mechanism of toxin-damage is the same between HUVECs and red blood cells? There are significant differences between endothelial cells and mature red blood cells, for instance long-term retention of a nucleus and ribosomes.**

Answer: This point was indeed confusing. Such studies are beyond the scope of the current manuscript and will be the focus of future studies.

30. ***Line 282:*** **Lack of RLT1 expression in spores is not shown. In addition, to support this analysis, a western blot in which the whole film is shown (and not a cropped image) would rule out the possibility of any non-specific binding events.**

Answer: The requested data are now presented in **Extended Data Fig. 4b**. Please see above for the whole Western blot. To preserve space, a cropped western blot from the image above is provided in **Fig. 3a.**

31. **Also, this manuscript used male mice. What about female mice? It is well known that male and female mice can respond differently to pathogens and particularly toxins. Ideally, data with female mice should be included in the manuscript.**

Answer: Dr. Ibrahim is part of a consortium contracted by the NIH to provide small animal (mice) service testing of small molecules, vaccines and immunotherapies for mucormycosis. Included in these studies was the effect of mouse gender on experimental outcome. Although these data have not been published, we found no evidence of any differences in male versus female mice response to infection or treatment. A statement in the Method section has been added to indicate this **(Lines 693-695**).

32. ***Extended Data Fig. 6.*** **Immunohistochemistry of a patient's lung apparently shows association of the toxin with fungal hyphae that has caused necrosis and massive infiltration of tissues. However, there is fluorescence is many places, with limited localization to the fungus? Can the authors indicate whether there is any non-specific binding by their anti-mucoricin antibody (please see comment relating to Figure 3A). A control biopsy without mucormycosis is also required. This is also the first immunofluorescence picture. Immunofluorescence with the mouse biopsies may be highly beneficial to demonstrate toxin localization to the fungus.**

Answer: We now provide data that mucoricin is not only cell-associated but also shed/secreted. The fluorescence that is not associated with the hyphae is due to the antibody reacting to the shed/secreted toxin. In the new image (**Extended Data Fig. 10**) we included an IHC of biopsy of a patient infected with invasive pulmonary aspergillosis (identified by a histopathologist and confirmed by qPCR). We do not see any residual fluorescence in lungs from the patient infected with aspergillosis, thereby indicating that the antibody used is specific. This addresses the reviewer's concern regarding the antibody specificity and is a better control than a biopsy from uninfected patient.

33. ***Extended Data Fig. 11b*****. These images are insufficient for the conclusions. Also, most of the cells do not appear to have toxin. The toxin may only enter when the cell is dead. Better confocal**

imaging is required with z-stacking (side/top views) etc. to demonstrate entry and clarity at different time points.

Answer: Following the recommendation of Referee 3 (point 6 below), this Extended data has been deleted and will be the topic of future investigation focusing on the mechanism of host cell entry and damage.

Minor comments:

34. The manuscript used different mouse models i.e. DKA and immunosuppressed mice etc. Why are different mouse models required for different parts of the manuscript? Also, wild type mice are not used. Do these fungal species and mucoricin not cause 'infection' in healthy mice? Discussion required. Also, in the relevant Figure legends, please indicate which mouse model was used to help the reader.

Answer: This is a good point. Mucormycosis is primarily a disease of **immunocompromised** hosts. The DKA and immunosuppressed mice are two clinically relevant models representing the two most vulnerable mucormycosis patients. Both models were developed in the Ibrahim's laboratory. They are well-characterized and considered to be the standard models for testing pathogenesis, evaluating antifungal therapies and immunotherapies. Wild-type mice have normal (robust) immune systems and are resistant to pulmonary infection. These patient categories were described in the introduction of the original version (**opening paragraph**).

35. Mucoricin is clearly damaging but it is also clear that damage still occurs in the absence of mucoricin. I assume this indicates that other potential toxins may exist in these species? Discussion required. Is the mucoricin encoded by an entire open reading frame, or is it present as a cryptic sequence within a larger protein such as the case with C. albicans candidalysin/ECE1?

Answer: The reviewer is correct. There is another different and mainly secreted toxin that we are currently working on. The contribution of this toxin to pathogenesis is still under investigation and is the topic of another study. A statement to this effect is now added to the Discussion **(Lines 408-410**). Mucoricin is encoded by the entire ORF since the purified protein size matches the predicted size from the ORF.

36. Recombinant mucoricin can be expressed in S. cerevisiae. Mucoricin is believed to exert its effect on the host ribosome. Does the expression of mucoricin have any negative impact on S. cerevisiae? Both mammalian cells and S. cerevisiae are eukaryotic and presumably have a reasonable degree of ribosomal conservation.

Answer: Most likely (and just like ricin) it does have some toxicity to the encoding host since the level of production is low (0.2-0.5 mg/Liter).

37. When mucoricin was injected intravenously, histopathology indicated the presence of "large" macrophages in the lung. What is meant by "large" in this context? Are these a particular type of macrophage? Are they atypical from what would normally be expected? Are the same "large" macrophages observed when the lung is infected with wild type fungus? Were other organs similarly infiltrated? If not, does this suggest a degree of tropism to the lung?

Answer: We should not have used word 'large' for macrophages. Our intention was to point to the infiltration of megakaryocytes into the lungs and liver, which is abnormal. This is now corrected (**Line 155).**

38. Orthologs of mucoricin are present in 21 previously sequenced species. Do they all possess the LDV sequence for example? A sequence comparison of orthologs would

Answer: All Mucorales sequenced that are known to cause mucormycosis contain the [x]D[y] motif (**See Baluna *et al.* Reference 25**) in the mucoricin orthologs (x=L,I, G, or V, and y=V, L, or S) . The majority contain LDV motif and some have IDV, IDS, GDL, or VDV. This information is now presented in **Extended Data Table 3.**

39. Mice were infected intratracheally and then given 30 ug of antibody. Does the antibody (or its vehicle) prevent spore germination/hyphae formation?

Answer: The antibodies have no effect on spore germination or the growth of the fungus. This is now presented in **Extended Data Figure 3**.

40. The amount of mucoricin used in experiments is described as mass per unit volume (i.e. 20 ug/ml). The molar concentration of mucoricin should be used throughout the manuscript.

Answer: The concentration of the toxin is now presented in molar concentration as suggested by the reviewer.

Referee #3 (Fungal pathogenesis)

Remarks to the Author:

6. Overall, and up to including Figure 3 and Supplementary Figure 5, this is a really solid manuscript, with each next experiment being logical and comprehensively carried out. After that I feel there is a slight loss of focus, with occasional elements which could be tidied up or reordered to improve the manuscript.

Answer: We have added new experimental data and bioinformatics to support the functionality of the toxin and its relation as a ricin-like toxin. We have retained **Fig. 4** since we think it strengthens the study (now **Fig. 5**). Some of the figures beyond **Extended Data Fig. 5**, are also important. However, we do agree with the Referee that some of the Extended Data beyond Fig. 5 reduce the focus of the paper and we opted to take them out (*e.g.* effect of the toxin on IL-8 production, phylogenetic tree, **Extended Data Fig. 7 and 11**).

Comments:

7. It should be pointed out that the presence of such toxins has been "identified" in the genomes of fungi previously (Revising the taxonomic distribution, origin and evolution of ribosome inactivating protein genes) however I don't think this detracts from the novel toxic activity reported here. Actually, I found the Ricin Interpro domain of Rhizopus mucoricin (IPR000772)

present in almost 400 sequences using the FungiDB Interpro Domain finder tool indicating the presence of a large number of ricin domain-containing proteins in fungi, well beyond what would be found by BLAST analysis.

Answer: Please see our response to Referee 1 and revised **Extended Data Table 1**.

8. **The fact that even killed hyphae continue to cause significant cell damage (likely due to persistence of mucoricin activity) is of potentially enormous clinical significance. i.e. – a patient would continue to suffer tissue necrosis, even after the fungus was fully killed by an antifungal. It would have been nice to combine the approaches of e.g. Figure 1b with toxin RNAi or antibody to directly demonstrate that the damaging activity of dead hyphae is mucoricin-mediated.**

Answer: Data with the antibody blocking damage caused by killed hyphae have been added **(Fig 1e and discussed on Lines 123-128).**

9. ***Line 220.*** **"The hyphae-associated toxin has structural features of ricin" Here the authors claim they "embarked on structural and bioinformatics studies" by directly aligning their mucoricin against known toxin sequences and reporting "Rhizopus delemar toxin, showed the highest homology with ricin produced by the castor bean plant, Ricinus communis" I don't think that restricting comparisons of mucoricin to "well-known" toxins is the best way to interrogate its phylogeny or evolutionary origin. By performing a BLASTp search of RO3G_06568 at NCBI and excluding the fungal kingdom, it's apparent that mucoricin shares high similarity with ricin sequences from CNS and cyanobacteria. In fact, the similarity between mucoricin and these bacterial sequences is higher than to most non-Mucorales fungi. So, whilst the conclusion "Phylogenetically, the toxin is most similar to ricin…" is true, it would appear to be far more closely related to bacterial ricin than to plant. Whether this is due to HGT acquisition of a bacterial ricin by fungi (e.g. Metazoan Ribosome Inactivating Protein encoding genes acquired by Horizontal Gene Transfer) or loss of RLT1 by other eukaryotic lineages (e.g. Revising the taxonomic distribution, origin and evolution of ribosome inactivating protein genes) is anyone's guess at this point! Along with this point, *Extended Data Figure 7b* is not particularly useful. It would be much more informative to generate a tree based on mucoricin and proteins with actual phylogenetic similarity (i.e. BLASTp reciprocal best hits). Not candidalysin!**

Answer. After rethinking this, we agree. Please see our response to the phylogeny issue above (Referee 1 point 3). Following the Referee's recommendation, **Extended Data Fig. 7** has been omitted.

10. **If mucoricin is associated with the hyphal cell surface as is shown in the manuscript – is it also being released? Was there any evidence for it being shed from the fungus? This would be important for it to get into host cells.**

Answer: See our response to Referee 2, point 7. Indeed, the toxin is also released (*i.e*. shed or secreted) and these data are now included in **Fig. 4**.

6. ***Line 303-332*****. "Mucoricin, like ricin, induces inflammation". Overall, this section is a bit weak**

and feels more like the pilot data for the next study. I would personally integrate the IL-8 observation into an earlier section rather than have it as a standalone result. e.g. it would fit alongside histology/PMN infiltration.

Answer: We agree with the Referee. These data have been removed and will be the subject of a future study on the immune response. We now feel that this paper should focus on the structure and function of the toxin.

7. ***Extended Data Figure 11*** **– for me, this would be the nice initial observation in a future study looking at the mechanism of mucoricin entry into host cells. It's a neat observation but doesn't sit right as the final data set in what is otherwise a really solid and logically narrated story.**

Answer: Following the Referee's recommendation, **Extended Data Figure 11** is omitted from this study and will be the topic of future studies focusing on the mechanism of the toxin entry and trafficking into target cells. It should be noted that in the ricin field, this whole issue took many years to work out.

Minor comments.

8. **"This organism also damages the A549 alveolar epithelial cell line and primary alveolar epithelial cells, but only after 30 h of incubation (Fig. 1a)." Is there really damage to A549 at 30 h?**

Answer: A minimal damage of ~ 5% of the host cells was consistently observed at 30 has now indicated in the text (**Line 85).**

16. **Was the purified and/or recombinant toxin heated and shown to still be active (to fit with heat killed hyphae conclusion)?**

Answer: The purification of the toxin using host cell damage assay as a readout and the inclusion of anti-mucoricin antibody blocking data of heat-killed hyphae as shown in **Fig. 1e** should suffice for the role of the toxin in heat-killed hyphae.

17. ***Line 124*** **"act similarly in damaging A549 cells in a rapidly time dependent manner" Remove "rapidly". To many, rapid would mean seconds.**

Answer: The word 'rapidly' has been removed.

18. ***Extended Data Fig. 6*** **– whilst it's nice to indicate mucoricin is part of the human pathology, I feel this single observation is a little weak as is.**

Answer: We feel that these data gives clinical relevance to the identified toxin. These data is now stronger with the inclusion of an IHC from a patient with invasive pulmonary aspergillosis (**Extended Data Fig. 10**) showing lack of detection of mucoricin.

19. ***Line 236*** **– "We generated a 3-D structural model" – insert "predicted"**

Answer: Done

20. ***Line 295*** **"Concordant with these results, it has been shown that HUVECs are rapidly damaged by the LDV-motif (Fig. 4a) than the sequences responsible for ribosome inhibition" – rephrase**

Answer: This has been addressed above and changed in the text **(Lines 358-363)**

21. ***Line 321*** **– "microscopy of epithelial cells treated with mucoricin showed instant rounding of cells" – I'm not sure I follow what's being said here.**

Answer: As in the case of ricin, rounding of cells in these assays are the first step in cell damage. Cells then detach from the matrix and die. This is well accepted in the field of endothelial cell biology. In the case of ricin, this is described by **Baluna *et al.* Reference 25** as noted above. In anyway, this data has been omitted as suggested by the Referee (**Extended Data Figure 11** of the original version).

22. ***Line 607*** **"Expression and purification of mucoricin" was the construct sequenced?**

Answer: Yes; all cloned genes were sequenced prior to production. This is now mentioned in the revised version (**Lines 540-541**).

Referee #4 (ricin)

Remarks to the Author:

1. **It would be nice to see how these antibodies see this toxin (epitope mapping?).**

Answer: We agree and fully intend to do this. However, this paper is focused on showing the role of the toxin in the pathogenesis of mucormycosis and its resemblance to ricin. Epitope mapping with a panel of MAbs is extremely important but clearly beyond the scope of the current study.

2. **N-terminal to the putative lectin binding domain are 16 amino acids with some (gapped) similarity to the C-terminal part of ricin A chain. This part of ricin A chain has the 5 amino acid active site motif SEAAR associated with the depurination activity of ricin A chain (and which is identical in Shiga toxins which also depurinate ribosomes). However, the fungal toxin FEEGR, I would suggest, is a very poor copy of this, so it would be an unlikely candidate for any putative deprivation activity. The depurination activity of this would surely be important for this manuscript since the title of the manuscript specifically points to the similarities with ricin. So why is this activity assay relegated to the subordinate Supplementary data section in (Ext Fig 8)? I am not familiar with this assay. I would very much like to see this assay validated using ricin A chain, and also with heat inactivated ricin A chain (no depurination) and heat-inactivated fungal toxin.**

Answer: As detailed in our response to Referee 2, we now introduce evidence of ribosome inhibition using the standard cell-free rabbit reticulocyte protein inhibition assay. The concentration of mucoricin required to cause 50% inhibition in protein synthesis (IC_{50}) is about 800 times higher than the concentration of ricin needed to cause similar protein inhibition (2.2×10^{-11} M for ricin vs. 1.7×10^{-8} M for mucoricin). These data

are now shown in **Fig. 6a**. This "weaker" activity of Mucoricin as compared to ricin toxin might indeed be explained by the EEGRL version of the active ricin motif of EAARF as stated by the Referee. It is prudent to keep in mind that this fungal toxin does not need to be as potent as ricin or Shiga toxin to be required for pathogenesis. As detailed in our response to Referee 2 (point 9), mucoricin has a EAANQ that aligns with the ricin EAARF, in which the glutamic residue required for the ricin and shiga toxin activity conserved. Finally, WGRLS sequence required for RIP activity in Shiga toxin aligns with the *R. delemar* sequence of EEGRL All these possibilities are now discussed in the revised manuscript (**Lines 325-349**). Since we now show that mucoricin inhibits protein synthesis, we have omitted the depurination data from the revised manuscript.

11. If there is a confirmed quantifiable depurination activity, where is the evidence that this is part of the mechanism of cell damage by this toxin? There are no data here that suggest that the toxin is internalized. And a statement that the spores bind GRP78 makes little sense. GRP78 is BiP, a chaperone that resides in the endoplasmic reticulum. How would a spore see this? Unless of course, this is BiP released from ruptured cells – which is not the same as internalization. The reference that supports this (reference 37) is Alqahiri, IDweek. IDweek is an annual meeting of IDSA and is not peer-reviewed: this whole section should be removed.

Answer:

iv. It is well-documented that although the majority of GRP78 are cytoplasmic, to carry out its function as a chaperone, it is also expressed on the cell surface, especially under stressful conditions. It is also well-documented by us and others that GRP78 acts as a receptor to a variety of pathogens during host cell invasion by Mucorales **(Liu *et al.* JCI, 2010:120:1914-1924; Gebremariam *et al.* JCI, 2016;126:2280-2894**) Dengue virus **(Jindadamrongwech *et al.* Arch Virol, 2004;149, 915-927),** MERS and coronavirus HKU9 **(Chu *et al.*, JBC 2018,293;11709-11726)**, Coxsackieviruse A9 (**Triantafiluo *et al.*, J Virol., 2002;76:633-643)**, and zika virus **(Ojha *et al.*, Front. Immunol. 2018;9:2340**). Consequently, the fungus invades the host cell *via* host cell receptors including GRP78 on endothleial cells and nasal epithelial cells and integrins and EGFR on alveolar epithelial cells, Since the toxin is cell-associated, it can exert its effect on the host cell once it is internalized.

v. With the new data showing that the toxin is also secreted, it can be internalized (by a mechanism yet to be determined) and exert its inhibotry effect on protein synthesis.

vi. The above mentioned refernce is now published in mBio (**Reference 43**). A revised model is introduced in the revised manuscript (**Lines 387-405**).

Statistics.

12. There is some ambiguity in description: for example, in the legend to *Fig. 1c*, there is a description of n=6/group from two experiments. But if there are two experiments, then n = 2. There is a danger of pseudoreplication here that must be avoided so that P-values are not artificially lowered. And if there are two experiments with 6 observations, is these three

observations per experiment? This really is not clear. *Fig 2b* legend, *Fig 3C*, *Ext Fig 3b*, *Ext Fig 9c*, *9d* and *Extended Fig 10* all have this same ambiguous writing.

Answer: We apologize for the ambiguity in the Figure legends. All data now represent the combination of data points collected from three independent experiments. This is now explained in all the figure legends.

13. Error bars in *Ext Fig 3b*, *Ext Fig 1* and *Fig 2b*. What are they? SD? SEM?

Answer: All data are presented as median ± interquartile range.

14. Why are some experiments shown with individual points, median and interquartile range, whereas others are summary statistics (mean plus or minus something undefined)? Lack of uniformity.

Answer: See our response above

15. *Fig 3e* and *3f*. Mouse survival is typically displayed in a Kaplan-Meier step plot (declining horizontal steps with vertical risers) and the analysis is really a modified chi-squared. However, the authors have just joined the dots.

Answer: Both formats are correct, and we routinely use the one depicted. The two figures in question, are correctly presented as Kaplan survival curves.

16. How was *Fig 3d* normalized? The data presentation suggest that toxin-treated cells can have up to 135% cell damage – this should alert the authors to a normalization problem.

Answer: Each data point is normalized to the median of damage without IgG. This is mentioned on the *y-axis*.

Other comments.

17. There is a two-hour window between hours 1 and 3 in Fig 2b with no data points, between all and nothing, so the graph is uninformative. Why not just show this as an end-point assay?

Answer: The point in showing the figure in this manner is to demonstrate that HUVECs are more rapidly damaged than alveolar epithelial cells. This is stated in the Discussion (**Lines 354-358**).

18. *Line 161*: mice appeared healthy – a somewhat qualitative statement.

Answer: The words “appeared healthy” have been deleted.

11. Line 241: why is (e.g. RIP) needed?

Answer: RIP has been deleted

12. **Line 256 – and Fig 4e: Western blot. There is only one polypeptide identified on the Western I'm guessing the anti-ricin antibody was really anti-ricin B chain?**

Answer: The figure legend states that the Western blot is carried out using the polyclonal anti-mucoricin polyclonal antibody. As stated, the 8A1 MAb reacts with ricin B chain (**Lines 287, 472, 618, and 620, and Reference 30).**

13. **Line 271. Diphtheria toxin and Pseudomonas exotoxin are not RIP: they inactivate elongation factors, not ribosomes**

Answer: corrected.

14. **Line 273. Vague writing: what does 'it is cell-associated' mean in this context? Fungal cell? Host cell? Necrotic cell?**

Answer: Cell-associated means **not** secreted but rather associated with the fungal cell. We have rephrased this. New data showing that the toxin is also shed/secreted is now included.

16. **HUVEC (*line 292-293*) and endothelial cells (*Ext Fig 9d*). Please use consistent labels/descriptions.**

Answer: HUVECs is now used instead of endothelial cells in the Figure (**Extended Data Figure 4d of the revised version**).

16. **Line 299: a very vague mechanistic statement. Please remove – it adds nothing to the story.**

Answer: The last sentence has been removed.

17. **Line 303 to 316. Mucoricin (…) induces inflammation. Since the comparison is with ricin, and therefore presumably important for this story, why are all the inflammatory data confined to Extended Data sections? The following section/ lines raise the mechanistic prospect of a time lag caused by internalization. However, the authors present no data to suggest internalization, and in their model (initiation and pathogenesis model section) do not even mention internalization.**

Answer: As suggested by Referee 3, the section on inflammation has been removed and will be the topic of future investigations. The paper now focuses on the structural and functional comparisons between ricin and mucoricin. These data are presented as actual figures and not supplementary data.

18. **Display. Scale bar Fig 2e is likely to be unreadable when data are displayed by the journal – way too small: and the white arrow on the lung tissue is almost invisible. The scale bar on Fig 3 b will also become invisible when displayed. RNAi knockdown section: Down-regulation was measured by qRT-PCR (*line 142-143*) – but not shown.**

Answer: All these issues have been fixed and the RNAi data which resulted in >90% inhibition of the gene expression are now presented in **Extended Data Figure 5**

We thank the reviewers and the editor for their time and effort in reviewing this manuscript and for the high quality of the reviews! We think that the revised paper is both stronger and more focused.

Decision Letter, second revision:

Dear Ashraf,

Thank you for your letter asking us to reconsider our decision on your Article entitled "Mucoricin is a Ricin-Like Toxin that is Critical for the Pathogenesis of Mucormycosis". After careful consideration we have decided that we would be willing to consider a revised version of your manuscript.

Specifically, we feel that it will be important to add the new data on vascular leak and to carry out the glycosidase experiments, as you suggested.

Along with your revised manuscript, you should also submit a separate point-by-point response to all of the concerns raised by the referees, in each case describing what changes have been made to the manuscript or, alternatively, if no action has been taken, providing a compelling argument for why that is the case. If we feel that a substantial attempt has been made to address the referees' comments, this response will be sent back to the referees - along with the revised manuscript - so that they can judge whether their concerns have been addressed satisfactorily or otherwise.

I should stress, however, that we would be reluctant to engage our referees again unless we thought that their comments had been addressed in full.

When revising your paper:

- ensure it complies with our format requirements for Letters as set out in our guide to authors at www.nature.com/nmicrobiol/authors/index.html

- state in a cover note the length of the text, methods and legends; the number of references and the number of display items.

Please ensure that all correspondence is marked with your Nature Microbiology reference number in the subject line.

Please use the following link to submit your revised manuscript:

{REDACTED]

I would appreciate it if you could tell me if you think you will be able to submit a revised manuscript, and also the likely timescale.

I look forward to hearing from you soon.

Author Rebuttal, Second Revision

Our answers to the remaining critiques from the referees follow:

Referee #2

Comment: Currently, the precise mechanism of action of mucoricin remains unclear. There are many ribosome-independent ways in which protein synthesis can be inhibited; for instance, by stimulating the phosphorylation of eukaryotic initiation factor one-alpha (eIF1α) on serine 51, and by interfering with the function of elongation factors.

Answer: As described above, these data have been added to **Figure 4, panels c,d**. Both ricin and mucoricin act as N-glycosidases and are therefore RIPs.

Comment: A key experiment that needs to be performed is to express and purify a mutant mucoricin that does not contain the proposed motif required for RIP activity (EEGRL) and compare it with wild type toxin in the cell-free system. Furthermore, other reviewers pointed out that mucoricin may have more 'similarity' to Pseudomonas exotoxin than ricin. Pseudomonas exotoxin is not a RIP but functions to inactivate elongation factors, not ribosomes. This is precisely the point this and other reviewers are trying to make to the authors. Given this, the authors must show the mechanism of mucoricin function i.e. does it inactive ribosomes or elongation factors to inhibit protein synthesis and is the proposed motif (EEGRL) required for RIP activity.

Without these data, the final conclusions as to whether mucoricin does in fact function similarly to ricin are too speculative. Lastly, despite the apparent importance of the LDV/VDV motif for ricin damage activity, this sequence still has not been mutated either. This is also highly recommended. The inclusion of these additional data will significantly strengthen the manuscript and make it exceptional.

Answer:

1. As noted above we have now shown that mucoricin is indeed a RIP.
2. As we argued previously, the referee's recommendation of linking function to certain motifs (*i.e.* mutating the EEGRL and LDV/VDV motifs), as well as epitope mapping are important next steps and these experiments are currently in progress. However, it will take several years to complete this work; it is well beyond the scope of this report. In fact, in the case of ricin, the structure-

function studies required over fifteen years of studies by multiple laboratories and there are still gaps!

3. In summary, at this point in time, we feel that the paper presents a complete story regarding the role of mucoricin in pathogenesis and its similarity to ricin with regard to:

    i. cross-reactivity
    ii. homology
    iii. ability to inhibit protein synthesis in a cell free assay
    iv. activity as an N-glycosidase resulting in depurination
    v. ability to induce vascular leak in a published *in vitro* model.

A critical and important point is the effect of anti-toxin antibodies in reversing vascular leak and cellular toxicity in vitro and its important effect on pathogenesis in mice. This points to the possible future use of anti-toxin antibodies or vaccines as potential therapeutics.

Referee #4

Comment: Furthermore, 3D modelling underscores the similarity with ricin B chain domains. However, ricin B chain folds/domains are not particularly unusual.

Answer: We do not claim that the folding as a ricin B chain is unusual. In fact, we state that many of the orthologues are predicted to be ricin B chain (Lines 343-352). Mucoricin is structurally related to ricin B chain (as demonstrated by sequence identity, homology modeling and antibody cross-reactivity). Finally, it is also functionally related to ricin since it is both a RIP and an inducer of vascular permeability **(new data Figure 4c-f**).

Comment: There is a statement on page 13 that rRNA glycosylase activity is a feature of ricin, and to test this, the toxin was added to rabbit reticulocyte lysate. Addition of the Rhizopus toxin inhibits translation in a rabbit reticulocyte but it does so ~800 fold less efficiently than ricin A chain. However, this is no more than a first step in defining the enzyme activity of this toxin, because inhibition of rabbit reticulocyte lysate is not a specific assay for ricin activity. It can also be inhibited by diphtheria toxin A chain (although much less efficiently than by ricin A chain) and diphtheria toxin does not depurination ribosomes – it modifies eEF-2 by ADP-ribosylation. The inhibition could be non-specific (adding 800 times more protein than ricin A chain may add sufficient amounts of a co-purified

molecule that is inhibitory). The prescriptive, definitive assay for ricin A chain-like activity can be performed on rabbit reticulocyte lysates after treatment with toxin: if the inhibition is caused by ricin-like activity, the depurinated 28S rRNA should be cleaved with acetic aniline at the a basic site to generate a diagnostic RNA fragment (Osborn and Hartley 1990, Eur J Biochem, 193, 401-407).

The enzyme activity of ricin A chain depends on the stacking of the relevant adenine (A4324) between ricin A chain residues Tyr80 and Tyr123, followed by protonation of the leaving group by Arg180, breaking the N-glcosidic bond, and the deprotonation of the hydrolytic water by Glu177. There is also a role for Trp211. There are no equivalent amino acids in the Rhizopus toxin to Tyr80, Tyr2123 and Trp211, so the bioinformatics seems to rely upon a GluXXArg motif. I imagine these could be quite common. If there really is defined ricin-like depurination, I would not expect efficiency of action since there is nothing obvious to hold the adenine in the correct place. But realistically, is an activity of 0.125% of ricin A chain really ricin-like?

Answer:

1. The cell-free inhibition of protein synthesis (**Figure 4a,b**), the depurination (**Figure 4c**) and the activity of mucoricin as an N-glycosidase (**Figure 4d**) data are now shown as noted above.
2. With regard to the structure-function studies with panels of mutants, as noted above, these studies will take several years to complete and are well beyond the scope of this report.
3. We have now demonstrated that mucoricin is ricin-like with regard to its structural and functional similarities but not necessarily its potency, even though it is quite potent (evident by the severely diminished virulence in mice infected with the knockdown mutant or in those infected with *R. delemar* wild-type and treated with anti-mucoricin Abs)! There are other RIPs that are also much less potent than ricin (*e.g.* the ribosome-inactivating protein TRIP from tobacco leaves with IC_{50} of 100 ng/ml Reference 40 in the revised manuscript). In fact, we would not expect mucoricin to be as potent as ricin (the second most potent toxin known) since the pathogen has several other features that are involved in infection as detailed in the introduction (e.g. CotH invasins [Gebremariam et al. JCI 2014, 2016]). It is also important that mucormycosis is a disease of the immunosuppressed host (the majority of mucormycosis patients) or the immunocompetent host, the latter mainly through direct inoculation into the bloodstream in severe trauma (e.g. blast injuries, auto-accidents and burn patients). *Most importantly (and as noted above), anti-mucoricin antibodies are effective in preventing disease and this is clinically important and highly significant.*

Comment: One of my earlier comments was about the error bars in some of the extended Figures: and the answer was that in all cases what was shown were medians and interquartile ranges. However, there is a lack of uniformity in display, so a reader can easily be misled. If you show individual points

in the main body of the work, why not show them in the extended Figures (which in some cases, really do look like mean +/- some error)?

Answer: The data have always been presented as median ± interquartile range. All the data (Figures or Extended Data Figures) are now shown as individual points. The only exception is the growth curve in Extended Figure 6b because individual points with three organisms make the curve unclear.

Again, we thank the editors and reviewers for their time and for their many insightful comments! We hope that our manuscript is now acceptable for publication.

Decision Letter, Third revision:

Dear Ashraf,

Thank you for your patience while your manuscript "Mucoricin is a Ricin-Like Toxin that is Critical for the Pathogenesis of Mucormycosis" was under peer review at Nature Microbiology. It has now been seen by our referees, and in the light of their advice I am delighted to say that we can in principle offer to publish it.

Editorially, we will need you to make some changes so that the paper complies with our Guide to Authors at http://www.nature.com/nmicrobiol/info/gta.

Nature Microbiology offers a transparent peer review option for new original research manuscripts submitted from 1st December 2019. We encourage increased transparency in peer review by publishing the reviewer comments, author rebuttal letters and editorial decision letters if the authors agree. Such peer review material is made available as a supplementary peer review file. Please state in the cover letter 'I wish to participate in transparent peer review' if you want to opt in, or 'I do not wish to participate in transparent peer review' if you don't. Failure to state your preference will result in delays in accepting your manuscript for publication.

Please note: we allow redactions to authors' rebuttal and reviewer comments in the interest of confidentiality. If you are concerned about the release of confidential data, please let us know specifically what information you would like to have removed. Please note that we cannot incorporate redactions for any other reasons. Reviewer names will be published in the peer review files if the reviewer signed the comments to authors, or if reviewers explicitly agree to release their name. For more information, please refer to our FAQ page.

In recognition of the time and expertise our reviewers provide to Nature Microbiology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "Mucoricin is a Ricin-Like Toxin that is Critical for the Pathogenesis of Mucormycosis". For those reviewers who give their assent, we will be publishing their names alongside

the published article.

I appreciate this email is long and recommend that you print it and use it as a checklist, reading it carefully to the end, in order to avoid delays to publication down the line.

Please note that we will be considering your paper for publication as an ARTICLE in our pages.

Specific points:
In particular, while checking through the manuscript and associated files, we noticed the following specific points which we will need you to address:

1. Main text display items and supplementary information. Please note that we have recently started publishing additional figures as "Extended Data". These figures appear online in the html version of the manuscript in the place they are referred to and greatly increase discoverability of the data that is presented in them.
All Supplementary Information must be submitted in accordance with the instructions in the attached Inventory of Supporting Information, and should fit into one of three categories: EXTENDED DATA (ED); SUPPLEMENTARY INFORMATION (SI); and SOURCE DATA. Below are detailed instructions on how to format each category. For your paper, we suggest that you do the following:

a. Main figures: please maintain the current 4 main figures that illustrate the main findings of the paper.

b. Extended data (ED): There is a limit of 10 Extended Data (ED) figures. Please convert 2 ED figures into supplementary figures. ED figures are an integral part of the paper (presented online in the online version) and are meant to be multipanel A4 size figures. More information on file formats and how the legends should be supplied can be found below and in the attached Inventory of Supporting Information.

c. Supplementary information (SI): your study will have the 'Supplementary online data' as SI. Please submit all SI as a separate pdf file. All supplementary materials need to be assembled into a single file, including all tables (excluding those that are excessively large). In the Supplementary Information file, figure legends should be immediately below each figure and the pages should be numbered.

d. Source data: this format should be used to display source data linked to the main figures and ED figures.
We strongly encourage you include as much additional raw data underlying the graphs in the main and ED figures as possible. These data should be supplied as Excel tables, one file per main or ED figure, and should be clearly labeled and presented in a way that individual experiments are identifiable (for example, across a time course if applicable).

2. Data Availability statement. The data availability statement should clearly refer to all of the source data provided in the manuscript (more instructions on how to write this section can be found in the general formatting guidelines below).

3. Reporting checklist. Please revise this document according to the instructions found in the annotated PDF attached to this message and send in a final version with your article. The final reporting checklist will be published with your manuscript.

4. Competing interest statement. The competing interest statement needs to be included in the manuscript text (before or after the Acknowledgements).

5. New/Novel. There is an instance of the use of the word novel in the text (line 461), and of the use of the word new (line 40). Please remove them, except if it is strictly necessary. It is journal policy to limit unnecessarily hyperbolic use of terms related to novelty.

6. Scale bars/size markers. Please indicate scale bars in the figures, and define their length in the figure legend (not in the figure itself). For Extended Data Figure 6a and Extended Data Figure 9a,b, please add a scale bar and define the length in the legend. For Extended Data Figure 2, please include labelling of the marker size.

7. Source data. Full-length, unprocessed gels and blots must be provided as source data for any relevant figures, and should be provided as individual PDF files for each figure containing all supporting blots and/or gels with the linked figure noted directly in the file.

8. ORCID. We now require corresponding authors to provide an ORCID identifier, and would ask that you please provide one with your final submission (please also see below). There is a step during the upload of the information to our online system in which the number can be introduced.

9. Replicates and statistics. While carefully checking the figures, we noted a few things that need to be revised so that they comply with our style guidelines and accurately report on the number of replicates, statistical testing, etc. As general rules, please note that:

General comments:

Wherever statistics have been derived (e.g., error bars, box plots, statistical significance), the legend needs to provide and define the n number (i.e., the sample size used to derive statistics) as a precise value (not a range), using the wording "n=X biologically independent samples/animals/independent experiments," etc. as applicable.

All error bars need to be defined in the legends (e.g., SD, SEM) together with a measure of centre (e.g. mean, median), and should be accompanied by their precise n number defined as noted above.

All violin plots need to be defined in the legends in terms of minima, maxima, centre, and percentiles, and should be accompanied by their precise n number defined as noted above.

The figure legends must indicate the statistical test used and if applicable, whether the test was one- or two-sided. A description of any assumptions or corrections such as tests of normality and adjustment for multiple comparisons must also be included.

For null hypothesis testing, please indicate the test statistic (e.g., F, t, r) with confidence intervals, effect sizes, degrees of freedom and P values noted.

Test results (e.g., p-values, q-values) should be given as exact values whenever possible and appropriate, and confidence intervals noted.

Please indicate how estimates of effect sizes were calculated (e.g., Cohen's d, Pearson's r).

Please state in the legends how many times each experiment was repeated independently with similar results. This is needed for all experiments but is particularly important wherever representative experiments are shown. If space in the legends is limiting, this information can be included in a section titled "Statistics and Reproducibility".

For all bar graphs, the corresponding dot plot must be overlaid.

Specific comments to address:

Please see the attached "Extended_Comments" file.

General points:

Please read carefully through all of the following general formatting points when preparing the final version of your manuscript, as submitting the manuscript files in the required format will greatly speed the process to final acceptance of you work.

Titles should give an idea of the main finding of the paper and ideally not exceed 90 characters (including spaces). We discourage the use of active verbs and do not allow punctuation.

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We hope to hear from you within two weeks; please let us know if the revision process is likely to take longer.

****
Reviewer Expertise:
Referee #4: ricin toxin

Reviewer Comments:

Reviewer #4 (Remarks to the Author):

Figure 4 is convincing. The data in Fig 4c are now shown in context (not hidden in supplementary data) and provide a lead into Fig 4d, which shows release of a fragment of RNA following aniline treatment of ricin A/mucoricin treated rabbit reticulocyte lysate.
Figures 4e and 4f also form a convincing pair: anti ricin antibodies block R delamar toxicity and anti-mucoricin antibodies block ricin toxicity.
This is a very nice set of experiments.

Final Decision Letter:

Dear Ashraf,

I am pleased to accept your Article "Mucoricin is a Ricin-Like Toxin that is Critical for the Pathogenesis of Mucormycosis" for publication in Nature Microbiology. Thank you for having chosen to submit your work to us and many congratulations.

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