

1 Host-pathogen transcriptomics of macrophages, Mucorales and their endosymbionts: a
2 polymicrobial pas de trois

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4 Authors

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14

15 Abstract

16

17 Mucorales spores, the causative agents of mucormycosis, interact with the innate immune
18 system to cause acute, chronic, or resolving infection. Understanding the factors that
19 influence disease initiation and progression is key to understanding mucormycosis and
20 developing new treatments. Complicating this, mucormycosis can be caused by a number of
21 species that span the Mucorales genus and may be host to bacterial endosymbionts. This
22 study sets out to examine the differences between two species in the Mucorales order by
23 characterising their differential interactions with the innate immune system, and their
24 interactions with environmental bacterial endosymbionts. Through a holistic approach, this
25 study examines the transcriptional responses of *Rhizopus delemar* and *Rhizopus*
26 *microsporus*, two of the most commonly diagnosed species, to innate immune cells. This
27 study also examines the immune cell response and assesses the variation in these
28 responses, given the presence or absence of bacterial endosymbionts within the fungi. We
29 see that the fungal response is driven by interaction with innate immune cells. The effect of
30 the bacterial endosymbiont on the fungus is species specific, with a minimal in the absence
31 of stress, but strongly influencing fungal transcripts during interaction with innate immune
32 cells. In contrast, we observe that the macrophage response varies depending on the
33 infecting fungal species, but also depending on endosymbiont status. The most successful
34 macrophages elicit a pro-inflammatory response, and we see that through germination
35 inhibition macrophage survival is enhanced. This work reveals species-specific host
36 responses to related Mucorales spores and shows that bacterial endosymbionts impact the
37 innate immune cell response.

38

39 Introduction

40

41 Mucormycosis is a devastating, environmentally acquired fungal infection caused by a
42 variety of Mucorales species. Understanding the interaction between Mucorales spores and
43 innate immune cells is key to understanding mucormycosis. Studies frequently focus upon
44 the interaction of a single species of the Mucorales order with innate immune cells (Warris

45 et al. 2005; Chamilos et al. 2008; Schmidt et al. 2013; Kraibooj et al. 2014; Inglesfield et al.
46 2018). However, between Mucorales species, and across infecting isolates, numerous
47 phenotypic and genomic differences can be observed (Hoffmann et al. 2013; Mendoza et al.
48 2014; Schwartze et al. 2014). Whether each species within this family employs the same
49 mechanism to cause infection remains unclear, however the range of virulence profiles
50 displayed across the family indicates there may be diversified mechanisms at work (Petraitis
51 et al. 2013). For example, it has been shown that pathogenicity of these species is linked to
52 the variable copy number of the gene encoding CotH, a family of cell surface proteins
53 important for the spores' interactions with and adherence to endothelial cells. Iron-
54 scavenging and melanisation pathways have also been shown to play a role in pathogenicity
55 (Chibucos et al. 2016, Andrianaki et al. 2018). In addition to fungal genome plasticity and
56 transcriptional responses to host stimuli, fungal-host interactions can be further modulated
57 by bacterial endosymbionts (Itabangi et al., 2019; Partida-Martinez & Hertweck 2005). The
58 majority of Mucorales species have been shown to harbour bacterial endosymbionts whose
59 species can vary between Mucorales isolates (Ibrahim et al. 2008; Kobayashi & Crouch 2009;
60 Mondo et al. 2017; Itabangi et al. 2019). We have recently shown that a bacterial
61 endosymbiont influences the outcome of *Rhizopus microsporus* infections in both zebrafish
62 and murine models through modulation of both fungal and phagocyte phenotypes (Itabangi
63 et al. 2019). However, the impact of endosymbiont on fungal and host transcriptional
64 response remains unclear.

65

66 While disease can be caused by several species in the Mucorales order, *Rhizopus delemar*
67 and *Rhizopus microsporus* are responsible for the majority of infections (Liu et al. 2018). Risk
68 factors for developing the disease include, but are not limited to: innate immune cell
69 deficiencies, uncontrolled diabetes, immune suppression, skin barrier breaches and iron
70 overload (Baldin & Ibrahim 2017). With limited antifungal treatments effective against these
71 invasive infections, there is often a need for surgical debridement or amputation (Spellberg
72 et al. 2016). In disseminated cases, mucormycosis may present with a mortality rate of over
73 90% (Mendoza et al. 2014). Reports of chronic mucormycosis have also emerged, affecting
74 both immunocompromised and immunocompetent patients (Alan et al. 2019). The innate
75 immune response to Mucorales spores is key to infection control: macrophages are required
76 to induce a differential immune response on contact with *Rhizopus* spp. and may inhibit
77 germination or kill hyphal forms upon contact in healthy individuals (Ghuman & Voelz 2017;
78 Andrianaki et al. 2018). Single and multi-species studies have shown that phagosome
79 maturation is arrested by melanin within the cell walls of *Aspergillus* spp. and *Rhizopus* spp.,
80 however iron limitation allows macrophages to more effectively kill *Rhizopus* spp. (Liu et al.
81 2015; Ibrahim et al. 2010; Andrianaki et al. 2018). Several works comparing *Aspergillus* spp.
82 to *Rhizopus* spp. have revealed similar immunostimulatory capacities, but differences in
83 their responses to host stress (Warris et al. 2005; Chamilos et al. 2008; Schmidt et al. 2013;
84 Kraibooj et al. 2014). Exploring and understanding fungal responses to the host is essential
85 to improving our understanding of mucormycosis, yet it remains unclear how Mucorales
86 species respond to, and interact with, the innate immune system, and to what extent this
87 varies by species.

88

89 Our work explores the interplay between the innate immune system, *R. delemar*, and *R.*
90 *microsporus* using isolates of both species found to harbour bacterial endosymbionts
91 (Itabangi et al. 2019). We investigate the differences between these two fungal species, how

92 they respond transcriptionally to innate immune cells, and how their respective bacterial
93 endosymbionts affect this interaction. We also investigate the transcriptional response of
94 innate immune cells to these infectious spores and determine how this interaction is
95 influenced by the presence of an endosymbiont. We reveal a large difference between the
96 fungal transcriptional profiles of *R. delemar* and *R. microsporus* during *in vitro* monoculture.
97 There is a small conserved response to exposure to innate immune cells, including key
98 changes in cell wall genes, consistent with germination. Conversely, we see that the host
99 innate immune response differs significantly between fungal species and is also influenced
100 by the presence or lack of an endosymbiont. The innate immune response to *R. delemar* and
101 *R. microsporus* mirrors the relative aggressiveness of infection between these two species.
102 We also observe that through the activation of innate immune cells, or upon inhibition of
103 chitin synthase, we can improve the ability of innate immune cells to control the fungal
104 spores. Our work represents a broad analysis of the transcriptional interplay between innate
105 immune responders and infectious Mucorales spores, revealing species-species differences
106 which question the current model of ‘one species represents all’, when it comes to
107 mucormycosis.

108

109 Results

110

111 Experimental Design

112

113 We set out to investigate paired transcriptional responses of host and fungal cells, whilst
114 also exploring the influence of the endosymbiont on this interaction (Figure 1a). Fungal
115 spores from *Rhizopus delemar* and *Rhizopus microsporus* were either cured via ciprofloxacin
116 treatment to remove the bacterial endosymbiont (cured) or maintained in media permissive
117 to bacterial endosymbiosis (wt). Cured spores were passaged twice in the absence of
118 ciprofloxacin to limit the impact of the drug on transcriptional responses. The cured and wt
119 spores of both *R. delemar* and *R. microsporus* were allowed to swell in sabouraud broth
120 until 95% of the population had reached mid isotropic phase. Due to the differences in
121 germination rates between the species (Figure 1b), this occurred at 2 hours for *R. delemar*
122 and 4 hours for *R. microsporus*. Swollen spores were then used to infect the J774.1 murine
123 macrophage-like cell line. Fungal spores were co-cultured with macrophages for one hour,
124 after which unengulfed spores were removed, and phagocytosed spores were incubated
125 within the macrophages for a further two hours. The cells from the resulting infection were
126 processed to explore their transcriptional response to this infection scenario (Figure 1a).
127 Macrophages that had phagocytosed fungal spores were isolated and sequenced via the
128 10X Genomics Chromium Single Cell Sequencing platform. Macrophages left unexposed to
129 the fungi were used as a negative control. RNA was also isolated from fungal spores (cured
130 and wt) which had been engulfed by macrophages, and this was sequenced with a bulk
131 RNA-Seq approach. Unexposed fungi (cured and wt) were incubated in macrophage media
132 for a matched time and used as a negative control. The data shows the fungal response to
133 phagocytosis by macrophages, as well as the fungal response to the presence of its
134 endosymbiont. The macrophage response to the two species is also revealed, a response
135 which appears to differ when the endosymbiont is present for both fungal species.

136

137 Comparative genomics predicts alternative transcriptional responses

138

139 In order to better understand differential disease progression, we chose to compare *R.*
140 *delemar* and *R. microsporus*, as they cause a large proportion of mucormycosis infections
141 but appear morphologically dissimilar. *R. delemar* germinates more quickly than *R.*
142 *microsporus*, with 50% of spores germinating by 3 hours, and a spore body size which
143 reaches 12.8 μm over the course of germination (Figure 1b, c). *R. microsporus* germinates at
144 a significantly slower rate, taking 6 hours for 50% of spores to germinate, with a final spore
145 body size of 13.9 μm (Figure 1b, c). To better understand their relationship to one another,
146 we compared the gene content to explore the similarity and differences between the two
147 species. Previous work has established that the *R. delemar* genome (45.3 Mb, 17,513 genes)
148 is larger, and contains an increased number of genes, compared to *R. microsporus* genome
149 (26 Mb, 10,959 genes) (Ma et al. 2009; Mondo et al. 2017). Our results show that, compared
150 to *R. microsporus*, the genome of *R. delemar* is enriched for genes with protein domains
151 (PFAM) associated with ion binding, carbohydrate derivative binding, nucleic acid binding,
152 cytoskeletal protein binding, poly(A) binding, NAD⁺ ADP-ribosyltransferase activity, protein
153 kinase C activity, translation initiation factor binding and inorganic phosphate
154 transmembrane transporter activity (Supplemental Figure S1, Figure 1d). *R. microsporus* is
155 enriched for genes with protein domains corresponding to nucleoside phosphate binding,
156 early endosome activity and DNA repair complex activity (Supplemental Figure S1, Figure
157 1d). The clear differences illustrated by genome size and gene content indicate the
158 likelihood of alternative transcriptional responses.

159

160 Alternative transcriptional profiles are presented by *R. delemar* and *R. microsporus* in
161 response to innate immune cells

162

163 First we examined overall trends in fungal responses to phagocytosis, obtained through our
164 bulk RNA-Seq approach. We analysed the signal obtained from *R. delemar* and *R.*
165 *microsporus* samples with principle component analysis (PCA) (Figure 2). We observed large
166 differences between the transcriptomes of both fungal species, when exposed or
167 unexposed to macrophages, while the presence or absence of their respective
168 endosymbionts had a weak but differential effect on PCA. The presence or absence of the
169 endosymbiont appears to have very little bearing on the transcriptional patterns displayed
170 by *R. delemar*, as samples fell into two distinct clusters, most strongly influenced by
171 macrophage status (Figure 2a). *R. microsporus* exhibits a similar trend upon exposure to
172 macrophages, however the presence of the endosymbiont also influenced clustering (Figure
173 2b). We investigate the phenotypic consequences of this interaction in the companion
174 paper by Itabangi et al., showing that the presence of the endosymbiont *Ralstonia pickettii*
175 impacts fungal cell wall organization, resistance to host-relevant stress, spore germination
176 efficiency, and pathogenesis (Itabangi et al., 2019). Therefore, we focus here on the
177 transcriptional analysis of the host-pathogen-endosymbiont interaction across the two
178 species.

179

180 There are 2,493 genes that are significantly differentially expressed (Log fold change > 2;
181 false discovery rate < 0.05) in *R. delemar* across all conditions (Figure 3a), while *R.*
182 *microsporus* only exhibits 40 genes significantly differentially expressed across all conditions
183 (Log fold change > 2; false discovery rate < 0.05) (Figure 3b). The theme of a muted

184 transcriptional response from *R. microsporius* is also seen within pairwise comparisons of
185 conditions. Pairwise comparisons of differential expression across each experimental
186 condition show similar trends in responses between *R. delemar* and *R. microsporius*,
187 however *R. microsporius* responds with a reduced gene set (Figure 4). Pairwise comparisons
188 showed the biggest shift in transcriptional response when comparing phagocytosed fungal
189 spores to those unexposed to macrophages, regardless of endosymbiont status. When
190 phagocytosed (Supplemental Figure S2), *R. microsporius* upregulates genes enriched in GO
191 categories corresponding to thiamine metabolism, sulfur metabolism, glycerol metabolism,
192 alcohol dehydrogenase activity and transmembrane transporter activity (hypergeometric
193 test, corrected P value < 0.05). This is consistent with the fungal response seen to
194 macrophage stress (Parente-Rocha et al. 2015), and the micronutrient scavenging response
195 to nutritional immunity (Ballou and Wilson 2016; Shen et al., 2018; Andrianaki et al., 2018).
196 Phagocytosed *R. microsporius* downregulated genes enriched in GO categories
197 corresponding to rRNA processing, ribosome biogenesis and ribosome localization
198 (hypergeometric test, corrected P value < 0.05) (Supplemental Figure S2), consistent with
199 growth arrest within the phagolysosome (Inglesfield et al. 2018; Andrianaki et al. 2018).

200
201 Comparisons of *R. delemar* conditions reveal that, upon phagocytosis, spores upregulate
202 genes enriched in KEGG classifications corresponding to MAPK signalling, phenylalanine
203 metabolism, tyrosine metabolism, glutathione metabolism and fatty acid synthesis
204 (hypergeometric test, corrected P value < 0.05). Upregulation of these processes is
205 consistent with melanin biosynthesis (Eisenman et al. 2011; Andrianaki et al. 2018) and
206 intra-phagosomal survival (Yadav et al., 2011; Lorenz and Fink, 2005). Unexposed *R. delemar*
207 spores upregulate genes enriched in KEGG classifications corresponding to ketone body
208 synthesis, protein processing via the endoplasmic reticulum, amino sugar and nucleotide
209 sugar metabolism (hypergeometric test, corrected P value < 0.05). This is consistent with
210 metabolic activation and cell wall biogenesis (Figure 5).

211
212 *R. delemar* and *R. microsporius* harbour distinct bacterial endosymbionts (Itabangi et al.,
213 2019). When comparing transcriptional profiles of wt and cured spores incubated in serum-
214 free DMEM (sfDMEM) for 3 hours (time matched to phagocytosis assay), we observed very
215 few transcriptional changes in either species in response to curing (Supplemental Table 1).
216 In *R. delemar*, a single gene, predicted to be a putative protein phosphatase, was repressed.
217 In *R. microsporius*, three genes were induced: an autophagy-related protein, a C2H2 zinc
218 finger transcription factor, and ribosomal protein L2.

219
220 Despite these small changes, loss of the endosymbiont significantly impacted the
221 transcriptional responses of both fungal species to macrophages (Figure 4). Specifically, we
222 observed an overall increase in the number of fungal genes differentially regulated upon
223 phagocytosis for both species. Exposure of wt *R. microsporius* to macrophages induced the
224 expression of one gene with no known function and repressed 6 genes. The repressed genes
225 include an autophagy-related protein and a zinc finger transcription factor, as well as 4 un-
226 annotated genes (Figure 4, Supplemental Table S1). In contrast, when cured *R. microsporius*
227 spores were exposed to macrophages, 277 genes were significantly induced and 82
228 repressed, compared to unexposed cured spores (Figure 4). Induced genes were enriched
229 (hypergeometric test, corrected P value < 0.05) for the following GO categories: organelle
230 organisation, pre-ribosome and ribosome activity, ATPase activity, hydrolase activity,

231 pyrophosphatase activity, helicase activity, nucleic acid binding, RNA metabolism, nitrogen
232 metabolism, chromatin silencing. Repressed genes were enriched (hypergeometric test,
233 corrected P value < 0.05) for the following GO categories: oxidoreductase activity, hydrogen
234 sulphide metabolism, glycolysis, sulphur metabolism, hexose catabolism, siderophore activity,
235 iron assimilation, nitrogen metabolism, carboxylic acid metabolism. This suggests an overall
236 failure to properly respond to host stresses such as iron starvation in the absence of the
237 bacterial endosymbiont.

238

239 A similar impact of the endosymbiont was observed for *R. delemar*. While the overall fungal
240 response to phagocytosis is characterized by a robust transcriptional response, induced
241 genes in wt samples (1137 genes, Figure 4) were enriched for KEGG classifications
242 corresponding to: alanine metabolism, PPAR signalling, aromatic compound biosynthesis
243 and degradation, lysine metabolism, lipid metabolism, MAPK signalling, sugar metabolism,
244 tyrosine metabolism, secondary metabolite biosynthesis (Figure 5). Repressed genes in wt
245 samples (472 genes, Figure 4) were enriched for KEGG classifications corresponding to:
246 carbohydrate metabolism, secondary metabolite biosynthesis, ketone body processing,
247 protein processes, MAPK signalling (Figure 5). In contrast, induced genes in cured samples
248 (1285 genes, Figure 4) were enriched for KEGG classifications corresponding to: Fatty acid
249 metabolism, DNA replication, amino acid metabolism, glycan metabolism, pyruvate
250 metabolism, secondary metabolite processing (Figure 5). Repressed genes in cured samples
251 (878 genes, Figure 4) were enriched for KEGG classifications corresponding to: sugar
252 metabolism, amino acid metabolism, lipid metabolism, MAPK signalling, NOD-like receptor
253 signalling. Again, this suggests that the endosymbiont has an overall suppressive impact on
254 fungal transcription in response to macrophage challenge.

255

256 Next, we directly compared the transcription patterns of genes shared across the two fungal
257 species. When comparing the transcriptional responses of orthologous genes shared by *R.*
258 *delemar* and *R. microsporus*, we saw only a small proportion behave similarly (213 genes).
259 When phagocytosed, wt spores from both species upregulate orthologues genes involved in
260 fatty acid catabolism, transcription, regulation via polymerase II, and organelle organization.
261 Phagocytosed cured spores from both species upregulate orthologues genes involved in
262 RNA processing, chromosome organization and condensed chromosome pathways. When
263 unexposed, we see wt spores upregulate orthologous genes involved in translocation,
264 protein binding, siderophore activity, cobalamin processing, and post-translational protein
265 targeting. Cured unexposed spores upregulate orthologous genes with roles in siderophore
266 activity and transferase activity. Overall, *R. delemar* and *R. microsporus* both respond
267 transcriptionally to the presence of macrophages, however the size and composition of this
268 response differs between species.

269

270 Finally, we examined the regulation of genes predicted to be involved in ferrous iron
271 transport, as previous work has linked iron scavenging to survival within the phagolysosome
272 (Andrianaki et al., 2018). There are 12 genes in the *R. delemar* genome with predicted
273 ferrous iron roles. While 8 showed no significant change over the tested conditions, 3
274 (ROG3_006623, ROG3_007727, ROG3_011864) appeared highly expressed in wt and cured
275 phagocytosed spores, compared to unexposed spores. The last gene, ROG3_009943, is
276 highly expressed in wt spores unexposed to macrophages. Together, this suggests there

277 may be condition dependent specialization in the expression of ferrous iron transport in *R.*
278 *delemar*.
279

280 Innate immune cell transcription varies with fungal species and endosymbiont
281 presence

282
283 To investigate the innate immune response when challenged with *R. delemar* and *R.*
284 *microsporus*, we carried out single cell RNA-Seq of J774.A1 murine macrophages, unexposed
285 and exposed for 3 hours to the four types of pre-swollen spores (Figure 1a). Transcription of
286 both challenged and unchallenged macrophages displayed underlying population
287 heterogeneity (Supplemental Figure S3). To identify the transcriptional patterns of genes
288 responding to the spores, we focused on the expression of a subset of genes previously
289 identified as immune response genes (Muñoz et al. 2018). Principle component analysis of
290 the aggregated transcriptional data shows there is a clear difference in transcription
291 between macrophages that have and have not been exposed to the fungi (Figure 6). Across
292 all exposed conditions, relative to unexposed macrophages, there was a profile consistent
293 with cytokine activation, response to stimulus, and activation of the NF-Kb pathway. This
294 was accompanied by repression of CCL5, which is involved in T-cell recruitment (Figure 7).
295 However, different macrophage profiles can be seen in response to the two fungal species,
296 and these are further influenced by the presence of the endosymbiont. While the response
297 to wt *R. delemar* shows the most deviation from the macrophage-only control, exposure to
298 cured *R. delemar* also elicited a strong and distinct macrophage response (Figures 6,7).
299 Exposure to wt *R. delemar* elicits increased expression of general markers of activation,
300 including GTPase activity and MHC class II protein binding (LAG3 repressor of T-cell
301 activation, H2-M2, IFN-gamma induced IIGP1, MX1, KCTD14, PNP2), growth factor binding,
302 IL1 receptor agonist activity and endocytosis (SERPINE1, ENG, FGFBP3, GM8898, GCNT2,
303 IL1F6). Specifically, we observe modest increases in the expression of IFN- γ responsive
304 CXCL10 (3.1 fold) and IRG1/IRG11 (5.9 fold), pro-inflammatory SAA3 (3.5 fold), and ENPP4
305 (2.8 fold), but also induction of the M2 polarizing PSTPIP2/21 (6.7 fold), the IL-4 responsive
306 signaling modulator CISH (5.4 fold), and the vascular damage responsive F3/F31 (5.9 fold)
307 (Martinez et al. 2013). These latter genes are not as strongly induced during exposure to *R.*
308 *microsporus*, which may reflect the aggressive nature of infection by *R. delemar* relative to
309 *R. microsporus*. In contrast, infection with cured *R. delemar* showed a decrease in the
310 induction of these M2-polarisation markers (PSTPIP2, 3.5 fold relative to uninduced). The
311 transcriptional profile is instead shifted to include increased transcription of genes involved
312 in G protein signaling and phosphoinositide binding (PDE7B, CCL1, SCARF1, RGS16,
313 PLEKHA4) (Figure 7).

314
315 A similar change in macrophage polarization was observed during exposure to wt and cured
316 *R. microsporus*. The phenotypic analysis of this is discussed more fully in Itabangi et al. 2019.
317 For both wt and cured *R. microsporus*, expression of IFN- γ responsive CXCL10, SAA3, and
318 ENPP4 was comparable to unexposed macrophages. Compared to *R. delemar*, exposure to
319 wt *R. microsporus* induced a more limited expression of genes with roles in cytokine
320 activation, ERK1 and ERK2 regulation, and regulation of NF-kB cascade (Figure 7). There was
321 also a weaker induction of the vascular damage responsive F3/F31 genes, and a relatively
322 stronger upregulation of the PLA2G16 phospholipase, TRIM30D, SLC1A2 and the M2
323 polarizing IL-6. However, other key polarizing genes, particularly PSTPIP2/21, SAA3, and

324 ENPP4 were only weakly induced (Supplemental Table S2). Overall, this profile suggests a
325 weak M1-like activation consistent with poor phagocytosis and reduced overall antifungal
326 activity that we observe in macrophages interacting with endosymbiont-harboring spores
327 (Itabangi et al., 2019).

328
329 Finally, cured *R. microsporus* induced a strong pro-inflammatory response, which included
330 upregulation of CXCL3, the neutrophil chemoattractant, consistent with our observations of
331 differences in phagocyte recruitment in zebrafish upon infection with wt vs. cured spores
332 (Itabangi et al., 2019). Markers of NF- κ B activation were also strongly induced in this
333 population. Cured *R. microsporus* also strongly induced the expression of TNFRSF8 (CD30), a
334 marker of lymphocyte activation occasionally associated with subcutaneous fungal
335 infections. Overall, this is suggestive of a shift to a more pro-inflammatory profile. In our
336 companion paper, we observed that cured *R. microsporus* is more sensitive to phagocyte-
337 mediated killing and phagocyte recruitment compared to wt (Itabangi et al., 2019). We
338 therefore went on to test whether a more successful response to the spores could be
339 mounted via the induction of a pro-inflammatory response.

340

341 Chitin synthase inhibition and pro-inflammatory priming regulate infection outcome

342

343 *R. delemar* exhibits rapid germination followed by hyphal extension (Sephton-Clark et al.,
344 2018). The transcriptional profile we observe here in macrophages exposed to *R. delemar* is
345 consistent with a strong damage response, likely prompted by the germination of *R.*
346 *delemar* spores. We therefore hypothesized that macrophages may be better able to
347 control the infection if the fungi were slowed in their developmental progress. The necessity
348 of genes involved in chitin synthesis and regulation appeared important for both *R. delemar*
349 and *R. microsporus* in response to phagocytosis (Supplemental Figure S4). In previous work
350 we also highlighted the importance of chitin synthase for germination (Sephton-Clark et al.
351 2018). When fungal spores were pre-treated with the chitin synthesis inhibitor Nikkomycin Z
352 (24 μ g/ml), spores failed to germinate, displayed less chitin/chitosan in their outer cell wall
353 (Supplemental Figure S5) and macrophage survival was increased at 7.5 hours post infection
354 (Figure 8). At Nikkomycin Z concentrations lower than those used to pre-treat spores for
355 phagocytosis, we see the spores are able to swell, however development appears halted
356 after swelling (Supplemental Figure S5). As the macrophages are better able to control
357 these spores, this suggests that spores undergoing the initial stages of germination may
358 offer less of a challenge for the macrophages.

359

360 Our transcriptional data show a strong M2 alternative activation signal during *R. delemar*-
361 macrophage interaction, but a weaker M2 polarisation during *R. microsporus*-macrophage
362 interaction that was further shifted towards NF- κ B-mediated M1 upon endosymbiont cure.
363 We therefore hypothesized that shifting the macrophage polarization towards M1 classical
364 activation might have a protective effect upon Mucorales infection. Consistent with this, the
365 pre-treatment of macrophages with NF- κ B activating lipopolysaccharide (LPS) significantly
366 improved the ability of macrophages to control *R. microsporus*. At 7.5 hours post infection,
367 59.7% of macrophages survived when pre-treated with LPS, compared to 24.6% without
368 (Figure 8).

369

370 Discussion

371

372 In this work we show the fungal response to innate immune cells differs by species in the
373 Rhizopodaceae family. Although *R. delemar* and *R. microsporus* share a small conserved
374 response to exposure to macrophages, the majority of their response differs. We show that
375 the ability to germinate prior to phagocyte control appears to be key to virulence, as
376 blocking spore germination with the chitin synthase inhibitor Nikkomycin Z improves
377 macrophage survival. A range of germination and virulence phenotypes can be seen
378 throughout the Mucorales, and this highlights a need for further investigation into these
379 differences, to better understand the infections they cause.

380

381 We have also shown that the fungal transcriptional response appears largely unperturbed
382 by the presence, or lack, of an endosymbiont, in the absence of stress. However, the
383 presence of an endosymbiont greatly effects the response of and to the host. It has been
384 shown that endosymbionts influence asexual development and sporulation through the
385 regulation of Ras2 (Mondo et al. 2017). While we observed limited changes in fungal
386 transcription when comparing wt and cured spores, we did observe changes in the
387 expression of fungal genes upon exposure to macrophages. In our companion paper,
388 Itabangi et al show that the presence of an endosymbiont is important for virulence of *R.*
389 *microsporus*. The endosymbiont enhances virulence through the secretion of an anti-
390 phagocytic factor. The endosymbiont also impacts organisation of the fungal plasma
391 membrane, as well as environmental stress resistance and resistance to macrophage-
392 mediated killing (Itabangi et al., 2019). We show here that this is mirrored by the
393 transcriptional response of the WT and cured spores of both *Rhizopus* species to
394 macrophages, and in the macrophage response to infection.

395

396 As anticipated, activation of pro-inflammatory pathways increased macrophage survival in
397 response to the spores. This consolidates several studies which demonstrate improved
398 innate immune cell response to fungal pathogens when primed (Rogers et al. 2013; Municio
399 et al. 2013; Blasi et al. 1995). It has been shown that the early immune response to a *Mucor*
400 *circinelloides* infection is dependent on the formation of a pro-inflammatory TNF- α -
401 expressing granuloma-like structure that controls but does not kill spores in the zebrafish
402 model (Inglesfield et al. 2018). Ungerminated *Rhizopus* spores are highly resistant to
403 ROS/RNS which may mediate survival within granulomata. Consistent with this, the
404 induction of a strong proinflammatory response by cured *R. microsporus*, hypersensitive to
405 ROS/RNS stress, allows macrophages to better control the spores.

406

407 Interestingly, we see the chitin synthase inhibitor, Nikkomycin Z, is able to inhibit
408 germination in both *R. delemar* and *R. microsporus*. This demonstrates the requirement of
409 chitin synthase for spore development in these species. Treated spores swell, but do not
410 polarise. The ability to germinate also has consequences for virulence. The ability of
411 macrophages to control swollen spores, but not subsequently polarised ones, highlights that
412 the developmental stage of the spore is key to innate immune success and control. Whilst
413 many fungal pathogens are virulent in an ungerminated form, germination appears a key
414 virulence factor for these filamentous fungi. This is confirmed by work which shows that the
415 developmental stage of both *R. microsporus* and *Rhizopus oryzae* spores impacts
416 pathogenesis in zebrafish and murine models (Itabangi et al., 2019; Andrianaki et al., 2018).

417 Specifically, infection with pre-swollen spores leads to evasion of macrophage-mediated
418 immunity and increased pathogenesis (Itabangi et al., 2019; Andrianaki et al., 2018). In both
419 models, spore clearance is dependent on phagocyte recruitment, and we show that
420 phagocyte recruitment (in Itabangi et al.) and activation (Figure 7) is influenced by
421 endosymbiont status (Itabangi et al., 2019; Andrianaki et al., 2018). In addition, we
422 extended this analysis by revealing profound differences in the host response to two closely
423 related *Rhizopus* species. In particular, we observe a M2/damage-associated response
424 during infection with wt *R. delemar* spores that is shifted towards an M1 protective
425 response upon infection with cured *R. microsporus* spores. We reinforce this finding through
426 experimental modulation of macrophage polarization, showing that exposure to strongly
427 M1-polarizing LPS is sufficient to reduce macrophage killing by wt *R. delemar* spores.
428 Therefore, our data provide a framework for beginning to understand differences in the
429 relative virulence of pathogenic Mucorales species and underpin our finding of cross-
430 kingdom fungal-bacterial symbiosis influencing mammalian disease.

431

432 Methods

433

434 Fungal Culture

435 *R. delemar* and *R. microsporus* were cultured with Sabouraud dextrose agar (SDA) or broth
436 (10 g/liter mycological peptone, 20 g/liter dextrose), sourced from Sigma-Aldrich, at room
437 temperature. Spores were harvested, 10 days after plating, with phosphate-buffered saline
438 (PBS), centrifuged for 3 min at 3,000 rpm, and washed. Appropriate concentrations of
439 spores were used for further experiments as indicated. To cure spores of their respective
440 bacterial endosymbionts, spores were cultured with ciprofloxacin, as described in Itabangi
441 et al. 2019. Once cured, spores were subcultured at least twice in ciprofloxacin-free media
442 before use.

443 Macrophage Culture

444 Macrophages from the J774.A1 cell line were cultured in Dulbecco's Modified Eagle
445 Medium, (complemented with 10% foetal bovine serum, 1% penicillin, 1% streptomycin and
446 1% L-glutamine). Macrophages were grown at 37°C, in 5% CO₂.

447

448 Phagocytosis Assay

449 Macrophages were incubated for one hour in serum-free DMEM prior to infection. Spores
450 were pre-swollen in SAB (2 hr for *R. delemar*, 4 hr for *R. microsporus*). Washed spores were
451 incubated at 5:1 MOI with 1×10^5 macrophages as described in Itabangi et al, to ensure that
452 >95% of macrophages contained one spore or more. After a 1 hour of incubation, excess
453 spores were washed off the surface and the macrophages were incubated for a further 2
454 hours, before processing for RNA-Seq experiments. For live cell imaging experiments,
455 images were taken starting immediately after the excess spores were removed.

456

457 Live Cell Imaging

458 Time course images were taken to determine how LPS pre-treatment (100ng/ml) (Myers et
459 al. 2010) of macrophages, and Nikkomycin Z pre-treatment (120ug/ml over the course of
460 swelling in SAB) of spores effected phagocytic outcome. Images were taken at 20x on a Zeiss
461 Axio Observer, with images taken every 5 minutes. Bright-field and fluorescent images were

462 then analysed using ImageJ V1.

463 Comparative Genomics and Enrichment Analysis

464 Fishers exact test was used to detect enrichment of Pfam terms between *R. delemar* and *R.*
465 *microsporus*, terms with a corrected P value of < 0.01 were considered significant.
466 Orthologue genes of *R. delemar* and *R. microsporus* were identified using blast+. R (version
467 3.3.3) was used to carry out hypergeometric testing of KEGG and GO terms to determine
468 enrichment.

469

470 Bulk RNA-Seq

471 RNA was extracted from spores which had either been incubated with the macrophages, or
472 incubated in DMEM for the equivalent time period. To remove the macrophages, triton at
473 1% was used to lyse the macrophages, the resulting solution was then centrifuged for 3 min
474 at 3,000 rpm, and washed, leaving only spores. The DMEM control also received the same
475 treatment. To extract total RNA, the washed samples were immediately immersed in TRIzol
476 and lysed via bead beating at 6,500rpm for 60s. Samples were then either immediately
477 frozen at -20°C and stored for RNA extraction or placed on ice for RNA extraction. After lysis,
478 0.2 ml of chloroform was added for every 1 ml of TRIzol used in the sample preparation.
479 Samples were incubated for 3 min and then spun at 12,000 g at 4°C for 15 min. To the
480 aqueous phase, an equal volume of 100% ethanol (EtOH) was added, before the samples
481 were loaded onto RNeasy RNA extraction columns (Qiagen). The manufacturer's
482 instructions were followed from this point onwards. RNA quality was checked by Agilent,
483 with all RNA integrity number (RIN) scores above 7 (Schroeder et al. 2006). One microgram
484 of total RNA was used for cDNA library preparation. Library preparation was done in
485 accordance with the NEBNext pipeline, with library quality checked by Agilent. Samples
486 were sequenced using the Illumina HiSeq platform; 100-bp paired-end sequencing was
487 employed (2 x 100 bp) (>10 million reads per sample).

488 Single Cell RNA-Seq

489 For single cell sequencing experiments, macrophages were infected with fungal spores, as
490 outlined above. Uninfected macrophages, used as a negative control, were treated in the
491 same manner and underwent mock washes and media changes at identical time points to
492 infected macrophages. Macrophages were isolated and released from the bottom of their
493 wells with accutase, as per manufacturer's instructions (Technologies n.d.). Once in solution,
494 the macrophages were loaded onto the 10X genomics single cell RNA sequencing pipeline
495 for single cell isolation and library preparation. In total, 1082 single cells were sequenced.
496 The libraries were sequenced on the Illumina Sequencing Platform.

497

498 Data Analysis

499 For the bulk RNA-Seq data, FastQC (version 0.11.5) was employed to ensure the quality of
500 all samples. Hisat2 (version 2.0.5) was used to align reads to the indexed genome of
501 *Rhizopus delemar* RA 88-880 (PRJNA13066, Ma et al. 2009) and the indexed genome of
502 *Rhizopus microsporus* (Mondo et al. 2017). HTSeq (version 0.8.0) was used to quantify the
503 output (Anders et al. 2015). Trinity and edgeR (Robinson et al. 2009)(version 3.16.5) were
504 then used to analyse differential expression (Grabherr et al. 2013). For the single cell RNA-
505 Seq data, the 10X genomics analysis pipeline (Loupe Cell Browser V 2.0.0, Cell Ranger
506 Version V 2.0.0) was used to align reads to the *mus musculus* genome (version MM10), and

507 quantify the output. For single cell analysis, the samples were then aggregated using this
508 pipeline, to allow comparisons between samples.

509 Data Availability

510 Data will be available upon request.

511

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515

516 Author contributions

517 PSC conceived and designed the experiments, collected the data, performed the analysis
518 and interpretation, and wrote the manuscript. JFM, KV and CAC contributed to
519 interpretation of the data, and contributed to the manuscript. ERB conceived and designed
520 the experiments, contributed to interpretation of the data, and wrote the manuscript.

521

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531

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649

650 Figure legends

651

652 **Figure 1 Phenotypic and genomic comparisons.** A) Experimental setup: Pre-swollen (Sab)
653 WT or cured fungal spores from *Rhizopus delemar* (2hr) and *Rhizopus microsporus* (4hr) in
654 mid-isotropic phase were co-cultured with J774.1 murine macrophages (sfDMEM) at 5:1
655 MOI for 1hr, washed to remove unengulfed spores, then co-incubated for a further 2 hr and
656 RNA for fungi and host cells harvested. Pre-swollen spores (Sab) incubated in sfDMEM for
657 3hr served as the negative control. B) Germination percentage over time for *R. delemar* and
658 *R. microsporus* grown in Sab. C) Spore sizes for *R. delemar* and *R. microsporus* grown in SAB.
659 D) Pfam terms enriched (corrected P < 0.01) in *R. delemar* vs *R. microsporus* genomes, with

660 colour indicating the extent of enrichment (Log₁₀ Count).

661 **Figure 2 Principal component analysis of fungal genes differentially expressed across all**
662 **samples.** A) *R. delemar* wt and cured, macrophage engulfed or sfDMEM control B) *R.*
663 *microsporus* wt and cured, macrophage engulfed or sfDMEM control. Replicates (n=3) are
664 shown for each sample.

665 **Figure 3 Clustering of fungal transcriptional changes.** A) Heatmap displaying differentially
666 expressed genes in *R. delemar*. Expression levels are plotted in Log₂, space and mean-
667 centered (FDR < 0.001) B) Heatmap displaying differentially expressed genes in *R.*
668 *microsporus*. Expression levels are plotted in Log₂, space and mean-centered (FDR < 0.001)

669 **Figure 4 Differential expression of fungal genes in compared conditions.** A) The number of
670 genes significantly differentially expressed (multiple corrected P value < 0.05) between
671 samples. Blue bars indicate genes with an increase in expression (LogFC > 2), whilst orange
672 bars indicate genes with a decrease in expression (LogFC < -2).

673 **Figure 5 Gene functions of genes differentially expressed in *R. delemar*.** Enriched KEGG
674 categories for the up/down regulated genes over sample comparisons. The enrichment of
675 the category is indicated by the colour bar. White corresponds to no enrichment, and yellow
676 to red corresponds to the given P value of the enrichment.

677 **Figure 6 Principal component analysis of macrophage genes differentially expressed**
678 **across all samples.** Single cell sequencing was performed on uninfected and infected
679 macrophages. Transcriptional data from the experiment was analysed with the 10X
680 genomics analysis pipeline, and aggregated prior to principle component analysis.

681 **Figure 7 Clustering of macrophage transcription.** Heatmap displaying immune response
682 genes significantly differentially expressed between macrophage populations. Expression
683 levels are plotted in Log₂ (FDR < 0.001)

684 **Figure 8 Macrophage survival following exposure to *R. delemar* and *R. microsporus* spores.**
685 Macrophages +/- LPS pre-treatment were infected (MOI 5:1) with fungal spores, pre-swollen
686 in SAB consistent with single cell experiments. Macrophages were infected with fungal
687 spores that were pre-treated with +/- Nikkomycin Z (24 µg/ml; n=3 for each sample).
688 Macrophage survival was determined 7 hr post infection. Significant differences between
689 samples is indicated by (*= p < 0.01).

690 **Supplementary Figure 1** Table of Pfam terms found to be enriched (corrected P < 5 x 10⁻⁸) in
691 *R. delemar* and *R. microsporus* upon genome comparison.

692 **Supplementary Figure 2 Clustering of fungal transcription with GO annotation.** Heatmap
693 displaying all differentially expressed genes, across all conditions, in *R. microsporus*.
694 Expression levels are plotted in Log₂, space and mean-centered (FDR < 0.001).

695 **Supplementary Figure 3 Clustering and heterogeneity in single cell analysis of J774.1**
696 **macrophages.** Single cell plot generated by loupe cell browser. Colors indicate experimental
697 conditions as indicated. Spacial distribution indicates relative similarity across all detected
698 transcripts. 1082 single cells represented.

699 **Supplementary Figure 4 Expression of chitin synthase genes.** A) Heatmap showing the
700 expression of chitin synthase genes in *R. delemar* B) Heatmap showing the expression of
701 chitin synthase genes in *R. microsporus*

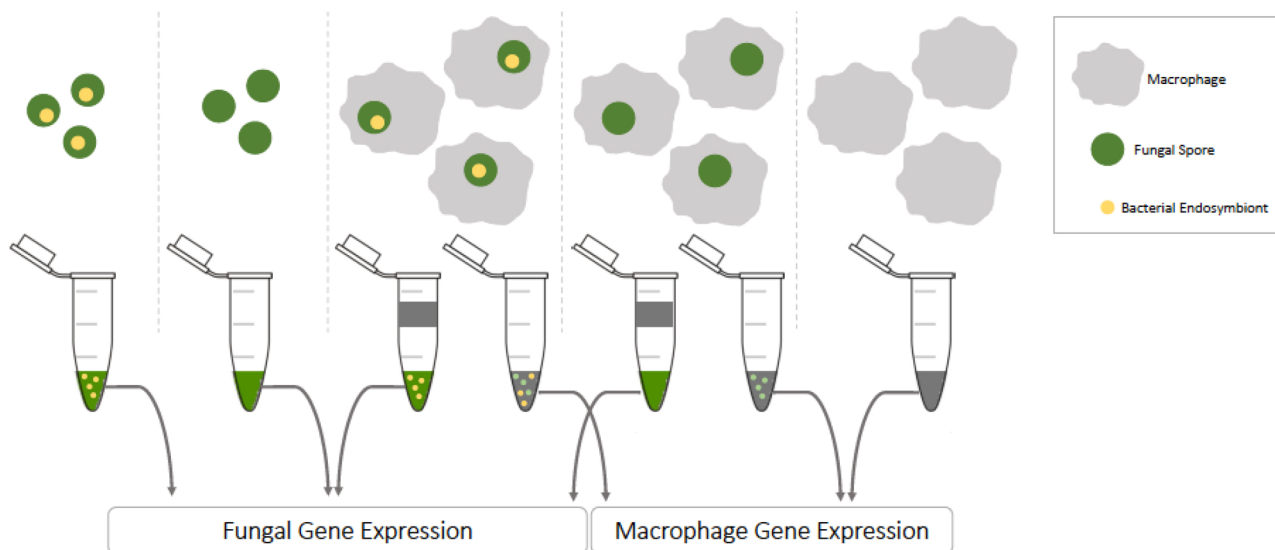
702 **Supplementary Figure 5 Chitin synthase inhibition of *R. delemar* and *R. microsporus***
703 **germination.** *R. delemar* and *R. microsporus* treated with Nikkomycin Z in SAB at indicated
704 concentrations for the indicated times. Fluorescence indicates calcofluor white staining, and
705 thus the availability of chitin/chitosan in the cell wall. Labels indicate time and
706 concentration of inhibitor.

707 **Supplemental Table 1** Selected genes differentially regulated in pairwise comparisons, with
708 Pfam annotations provided where available.

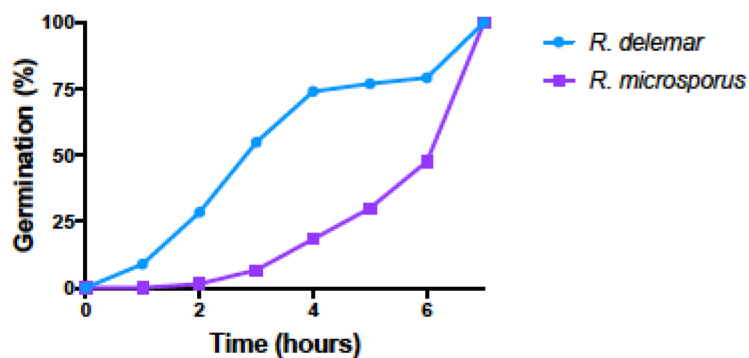
709 **Supplementary Table 2** Table displaying the LogFC values for a subset of genes displayed in
710 Figure 7.

711

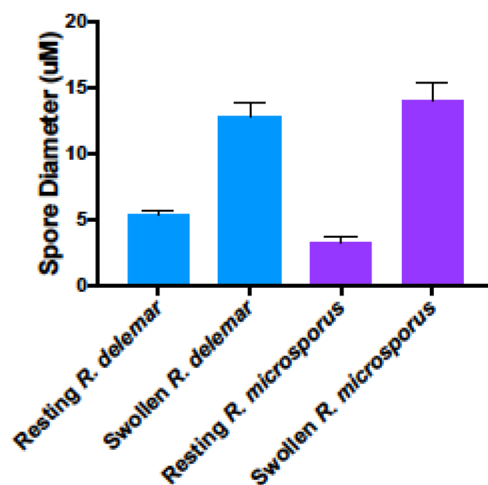
A



B



C



D

