1 **15-keto-prostaglandin E$_2$ activates host peroxisome proliferator-activated receptor**

2 **gamma (PPAR-γ) to promote *Cryptococcus neoformans* growth during infection.**

3

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22 Running Title: Fungal derived 15-keto-prostaglandin E$_2$ and PPAR-$\gamma$ promote *C. neoformans*
23 infection

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**Abstract**

*Cryptococcus neoformans* is one of the leading causes of invasive fungal infection in humans worldwide. *C. neoformans* uses macrophages as a proliferative niche to increase infective burden and avoid immune surveillance. However, the specific mechanisms by which *C. neoformans* manipulates host immunity to promote its growth during infection remain ill-defined. Here we demonstrate that eicosanoid lipid mediators manipulated and/or produced by *C. neoformans* play a key role in regulating pathogenesis. *C. neoformans* is known to secrete several eicosanoids that are highly similar to those found in vertebrate hosts. Using eicosanoid deficient cryptococcal mutants *Δplb1* and *Δlac1*, we demonstrate that prostaglandin $E_2$ is required by *C. neoformans* for proliferation within macrophages and *in vivo* during infection. Genetic and pharmacological disruption of host $PGE_2$ synthesis is not required for promotion of cryptococcal growth by eicosanoid production. We find that $PGE_2$ must be dehydrogenated into 15-keto-$PGE_2$ to promote fungal growth, a finding that implicated the host nuclear receptor PPAR-$\gamma$. *C. neoformans* infection of macrophages activates host PPAR-$\gamma$ and its inhibition is sufficient to abrogate the effect of 15-keto-$PGE_2$ in promoting fungal growth during infection. Thus, we describe the first mechanism of reliance on pathogen-derived eicosanoids in fungal pathogenesis and the specific role of 15-keto-$PGE_2$ and host PPAR-$\gamma$ in cryptococcosis.

**Author Summary:**

*Cryptococcus neoformans* is an opportunistic fungal pathogen that is responsible for significant numbers of deaths in the immunocompromised population worldwide. Here we address whether eicosanoids produced by *C. neoformans* manipulate host innate immune cells during infection. *Cryptococcus neoformans* produces several eicosanoids that are notable for their similarity to vertebrate eicosanoids, it is therefore possible that fungal-derived eicosanoids may provoke physiological effects in the host. Using a combination of *in vitro* and *in vivo* infection models we identify a specific eicosanoid species - prostaglandin $E_2$ – that is required by *C. neoformans* for

53 growth during infection. We subsequently show that prostaglandin $E_2$ must be converted to 15-

54 keto-prostaglandin $E_2$ within the host before it has these effects. Furthermore, we find that

55 prostaglandin $E_2$/15-keto-prostaglandin $E_2$ mediated virulence is via activation of host PPAR-$\gamma$ –

56 an intracellular eicosanoid receptor known to interact with 15-keto-$PGE_2$.

57 **Introduction**

58 *Cryptococcus neoformans* is an opportunistic pathogen that infects individuals who have severe

59 immunodeficiencies such as late-stage HIV AIDS. *C. neoformans* is estimated to infect 278,000

60 individuals each year resulting in 181,000 deaths (1,2). *C. neoformans* infection begins in the

61 lungs where the fungus is phagocytosed by host macrophages. Macrophages must become

62 activated by further inflammatory signals from the host immune system before they can

63 effectively kill *C. neoformans* (3,4). When this does not occur *C. neoformans* proliferates rapidly

64 intracellularly and may use the macrophage to disseminate to the central nervous system

65 leading to fatal cryptococcal meningitis (5-9).

66

67 Eicosanoids are an important group of lipid inflammatory mediators produced by innate immune

68 cells such as macrophages. Eicosanoids are a diverse group of potent signalling molecules that

69 have a short range of action and signal through autocrine and paracrine routes. Macrophages

70 produce large amounts of a particular group of eicosanoids called prostaglandins during

71 microbial infection (10,11). Prostaglandins have a number of physiological effects throughout

72 the body, but in the context of immunity they are known to strongly influence the inflammatory

73 state (12). The prostaglandins $PGE_2$ and $PGD_2$ are the best-studied eicosanoid inflammatory

74 mediators. During infection, macrophages produce both $PGE_2$ and $PGD_2$ to which, via autocrine

75 routes, they are highly responsive (12). In vertebrate immunity, the synthesis of eicosanoids

76 such as $PGE_2$ is carefully regulated by feedback loops to ensure that the potent effects of these

77 molecules are properly constrained. Exogenous sources of eicosanoids within the body, such as

4

78    from eicosanoid-producing parasites (13) or tumours that overproduce eicosanoids (14,15), can

79    disrupt host inflammatory signaling as they are not subject to the same regulation.

80

81    It is well known that *C. neoformans* produces its own eicosanoid species. These fungal-derived

82    eicosanoids are indistinguishable from those produced by vertebrates (16-18). Only two

83    *Cryptococcus* enzymes are known to be associated with cryptococcal eicosanoid synthesis -

84    phospholipase B1 and laccase (18,19). Deletion of phospholipase B1 reduces secreted levels of

85    all eicosanoids produced by *C. neoformans* suggesting that it has high level role in eicosanoid

86    synthesis (19), perhaps fulfilling the role of phospholipase $A_2$ in higher organisms. Deletion of

87    laccase results in reduced levels of $PGE_2$ but other eicosanoids are unaffected suggesting that

88    laccase has putative $PGE_2$ synthase activity (18). *C. neoformans* produces eicosanoids during

89    infection, these eicosanoids are indistinguishable from host eicosanoids so it is possible that *C.*

90    *neoformans* is able to manipulate the host inflammatory state during infection by directly

91    manipulating host eicosanoid signaling.

92

93    It has previously been reported that the inhibition of prostaglandin $E_2$ receptors EP2 and EP4

94    during murine pulmonary infection leads to better host survival accompanied by a shift towards

95    Th1/M1 macrophage activation, however it was not determined if $PGE_2$ was derived from the

96    host or the fungus (20). Therefore, a key aspect of *C. neoformans* pathogenesis remains

97    unanswered: do eicosanoids produced by *C. neoformans* manipulate host innate immune cells

98    function during infection?

99

100    We have previously shown that the eicosanoid deficient strain *Δplb1* has reduced proliferation

101    and survival within macrophages (21). We hypothesised that eicosanoids produced by *C.*

102    *neoformans* support intracellular proliferation within macrophages and subsequently promote

103    pathogenesis. To address this hypothesis, we combined *in vitro* macrophage infection assays

104    with our previous published *in vivo* zebrafish model of cryptococcosis (22). We found that $PGE_2$

105    was sufficient to promote growth of *Δplb1* and *Δlac1* independent of host $PGE_2$ production*, in*

106    *vitro* and *in vivo*. We show that the effects of $PGE_2$ in cryptococcal infection are mediated by its

107    dehydrogenated form, 15-keto-$PGE_2$. Finally, we determine that 15-keto-$PGE_2$ promotes *C.*

108    *neoformans* infection via the activation of the host nuclear transcription factor PPAR-$\gamma$,

109    demonstrating that 15-keto-$PGE_2$ and PPAR-$\gamma$ are new factors in cryptococcal infection

110

111    **Results**

112    *Prostaglandin $E_2$ is required for C. neoformans growth in macrophages*

113    We have previously shown that the *C. neoformans* mutant strain *Δplb1* has impaired

114    proliferation and survival within J774 murine macrophages *in vitro* (21). The *Δplb1* strain has a

115    deletion in the *PLB1* gene which codes for the secreted enzyme phospholipase B1 (23). The

116    *Δplb1* strain is known to produce lower levels of fungal eicosanoids indicating that

117    phospholipase B1 is involved in fungal eicosanoid synthesis (19). It has been proposed that the

118    attenuation of this strain within macrophages could be because it cannot produce eicosanoids

119    (19). A previous study has identified $PGE_2$ as an eicosanoid that promotes cryptococcal

120    virulence and manipulates macrophage activation, however this study did not determine if $PGE_2$

121    was produced by the host or *C. neoformans* (20). We hypothesised that $PGE_2$ - or other

122    phospholipase B1 derived eicosanoid species - are produced by *C. neoformans* during infection

123    and promote macrophage infection.

124    To test if $PGE_2$ promotes the intracellular growth of *C. neoformans* we treated *Δplb1* infected

125    J774 macrophages with exogenous $PGE_2$ and measured intracellular proliferation over 18

126    hours. The addition of exogenous $PGE_2$ to J774 macrophages infected with *Δplb1* was sufficient

127    to recover the intracellular proliferation of *Δplb1* compared to the H99 (parental wild type strain)

128    and *Δplb1:PLB1* (reconstituted strain) strains (Fig 1 p= 0.038). These findings support our initial

129    hypothesis and identify PGE$_2$ as a mediator of cryptococcal virulence during macrophage

130    infection.

131    We have previously shown that *Δplb1* has reduced intracellular proliferation due to a

132    combination of reduced cell division and reduced viability within the phagosome (21). In our

133    intracellular proliferation assay the number of intracellular *Cryptococcus* cells is quantified by

134    lysing infected macrophage and counting the number of *Cryptococcus* cells with a

135    hemocytometer. Due to their structurally stable fungal cell wall dead or dying *Cryptococcus* cells

136    do not look noticeably different on a hemocytometer from viable cells. To quantifiy the viability of

137    *Cryptococcus* cells retrieved from the phagosome we diluted the lysate to give an expected

138    number of CFUs (in this case 200 CFU), spread the diluted lysate on YPD agar and count the

139    actual number of CFUs produced – a difference between the expected CFU count (200 CFU)

140    and the actual CFU count indicates a loss of *Cryptococcus* cell viability. In this case viability

141    assays showed that exogenous PGE$_2$ produced no significant increase in the viability of *Δplb1*

142    cells within the phagosome (Supplementary Fig 1A).

143    *Exogenous prostaglandin E$_2$ rescues in vivo growth of Δplb1-GFP*

144

145    Our *in vitro* data showed that PGE$_2$ promotes the intracellular proliferation of *C. neoformans*

146    within macrophages. First, we injected 2-day post fertilisation (dpf) zebrafish larvae with *Δplb1*-

147    GFP (a constitutively expressed GFP tagged version of the *Δplb1* generated for this study). One

148    of the advantages of this model is that the fungal burden can be non-invasively imaged within

149    infected larvae using fluorescently tagged *C. neoformans* strains and we able measure the

150    growth of the fungus at 1-, 2- and 3-days post-infection (dpi). We found that *Δplb1*-GFP infected

151    larvae had significantly lower fungal burdens at 1, 2- and 3-days post infection (Fig 2D and

152    Supplementary Fig 1B) compared to the parental strain H99-GFP (Fig 2A and Supplementary

7

153     Fig 1B). These data demonstrated that the $\Delta plb1$-GFP mutant had a similar growth deficiency to

154     our *in vitro* phenotype and with previous studies (21,23,24). To confirm that $PGE_2$ promotes

155     cryptococcal infection *in vivo* we infected zebrafish larvae with $\Delta plb1$-GFP or H99-GFP and

156     treated the larvae with exogenous $PGE_2$. In agreement with our *in vitro* findings (Fig 1),

157     exogenous $PGE_2$ increased the growth of both the parental H99 strain (Fig 2B, p = 0.0137,

158     1.35-fold increase vs. DMSO) and the $\Delta plb1$-GFP mutant (Fig 2E, p= 0.0001, 2.15-fold increase

159     vs. DMSO) while $PGD_2$ did not (Fig 2C and 2F). Taken together these data show that $PGE_2$ is

160     sufficient to enhance the virulence of *C. neoformans in vivo*, furthermore our *in vitro* data

161     suggest that this is a result of uncontrolled intracellular proliferation within macrophages (Fig 1).

162

163     *Prostaglandin $E_2$ must be dehydrogenated into 15-keto-$PGE_2$ to promote C. neoformans growth.*

164

165     $PGE_2$ can be enzymatically and non-enzymatically modified in cells to form a number of distinct

166     metabolites. To distinguish the biological activity of $PGE_2$ rather than its metabolites we used an

167     analogue of $PGE_2$ called 16,16-dimethyl $PGE_2$ that cannot be dehydrogenated but otherwise has

168     comparable activity to $PGE_2$ (25)). We found that unlike $PGE_2$, 16,16-dimethyl $PGE_2$ treatment

169     did not increase the fungal burden of $\Delta plb1$-GFP (Fig 3C p= 0.9782) *or* H99-GFP infected

170     larvae (Fig 3A p= 0.9954). Therefore, the biological activity of $PGE_2$ alone did not appear to

171     promote cryptococcal pathogenesis and that dehydrogenation of $PGE_2$ was required. $PGE_2$ and

172     16,16-dimethyl $PGE_2$ both signal through $PGE_2$ receptors (EP1, EP2, EP3 and EP4) but 15-keto-

173     $PGE_2$ does not. In a murine model of pulmonary cryptococcosis the $PGE_2$ receptors EP2 and

174     EP4 have been identified as promoters of fungal virulence (20). To confirm that $PGE_2$ itself does

175     not promote virulence in our model we treated zebrafish with antagonists against the EP2 and

176     EP4 receptors. In support of our previous experiment we found that EP2 / EP4 inhibition had no

177     effect on fungal burden in zebrafish (Fig 3E). An abundant dehydrogenated form of $PGE_2$ is 15-

178     keto-$PGE_2$ (that has also been isolated from *C. neoformans* (18)) and we tested if 15-keto-$PGE_2$

179    was sufficient to rescue the growth defect of the *Δplb1* mutant during infection. Therefore, we

180    treated infected zebrafish larvae with exogenous 15-keto-PGE$_2$ and found that this was

181    sufficient to significantly increase the fungal burden of zebrafish larvae infected with both *Δplb1*-

182    GFP (Fig 3D, p = 0.0119, 1.56-fold increase vs. DMSO) and H99-GFP (Fig 3B, p = 0.0048,

183    1.36-fold increase vs. DMSO). To explore the *Cryptococcus* eicosanoid synthesis pathway

184    further we used a second eicosanoid deficient *C. neoformans* mutant *Δlac1*. Whereas *Δplb1* is

185    unable to produce any eicosanoid species *Δlac1* is deficient only in PGE$_2$ and 15-keto-PGE$_2$

186    (18). Using a *Δlac1*-GFP strain generated for this study we found that *Δlac1*-GFP also produced

187    low fungal burden during *in vivo* zebrafish larvae infection (Supplementary Fig 2B). As with

188    *Δplb1*-GFP, this defect could be rescued with the addition of exogenous PGE$_2$ (Supplementary

189    Fig 2C), but not with 16,16-dm-PGE$_2$ (Supplementary Fig 2D) or with 15-keto-PGE$_2$

190    (Supplementary Fig 2E). Prostaglandin E$_2$ is known to affect haematopoietic stem cell

191    homeostasis in zebrafish (26). This could affect macrophage number and subsequently fungal

192    burden. We have previously observed that a large depletion of macrophages can lead to

193    increased fungal burden in zebrafish larvae (22). We performed whole body macrophage counts

194    on 2 dpf uninfected larvae treated with PGE$_2$ or 15-keto-PGE$_2$ 2 days post treatment (the same

195    time points used in our infection assay). Following PGE$_2$ and 15-keto PGE$_2$ treatment

196    macrophages were still observable throughout the larvae. For PGE$_2$ treatment we saw on

197    average a 15% reduction in macrophage number while 15-keto PGE$_2$ did not cause any

198    decrease (Supplementary Fig 1C). Due to the fact that fungal burden increased during both

199    PGE$_2$ and 15-keto PGE$_2$ treatments it is highly unlikely that this reduction could account for the

200    increases in burden seen.

201

202    *Host derived prostaglandins are not required for growth of C. neoformans*

203

204    After determining that $PGE_2$ promotes the growth of *C. neoformans in vitro* and *in vivo* via its

205    metabolite 15-keto-$PGE_2$, we wanted to determine whether the source of these prostaglandins

206    was the host or the fungus. Our *in vitro* data show that the *C. neoformans* strain *Δplb1* has a

207    growth deficiency *in vitro* and *in vivo* that can be rescued with the addition of $PGE_2$, because

208    this phenotype is mediated by cryptococcal phospholipase B1 it indicates that pathogen-derived

209    rather than host-derived prostaglandins are required. A previous study reports that *C.*

210    *neoformans* infection can induce higher $PGE_2$ levels in the lung during *in vivo* pulmonary

211    infection of mice (20), although it was not determined if the $PGE_2$ was host or pathogen derived.

212    Therefore, we tested the hypothesis that host prostaglandin synthesis was not required for

213    cryptococcal virulence.

214

215    To block host prostaglandin synthesis *in* vitro we inhibited host cyclooxygenase activity because

216    it is essential for prostaglandin synthesis in vertebrates (27). We treated H99 and *Δplb1* infected

217    J774 macrophages with aspirin at a concentration we determined was sufficient to block host

218    $PGE_2$ synthesis (Supplementary Fig 3A). Aspirin is a non-reversible inhibitor of cyclooxygenase

219    1 (COX-1) and cyclooxygenase 2 (COX-2) enzymes, we therefore included a condition where

220    J774 cells were pretreated with aspirin only prior to infection and then at a condition where

221    aspirin was present throughout infection. We found that aspirin treatment did not affect the

222    intracellular proliferation of H99 or *Δplb1* (Fig 4A), suggesting that host cyclooxygenase activity

223    is not required for the phospholipase B1 dependent virulence of *C. neoformans* during

224    macrophage infection. To confirm these *in vivo,* we pharmacologically blocked zebrafish

225    cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). We used separate cyclooxygenase

226    inhibitors with zebrafish larvae instead of aspirin because we found that aspirin treatment led to

227    lethal developmental defects in zebrafish larvae (unpublished observation). We infected 2 dpf

228    zebrafish larvae with H99-GFP and *Δplb1*-GFP and treated with inhibitors for COX-1 (NS-398,

229    15 μM) and COX-2 (SC-560, 15 μM). We found that both inhibitors decreased the fungal burden

230  of H99-GFP, but not *Δplb1*-GFP infected zebrafish larvae (Fig 4B i and 4B ii, H99-GFP - NS-

231  398, p= 0.0002, 1.85-fold decrease vs. DMSO. SC-560 p= <0.0001, 3.14-fold decrease vs

232  DMSO). These findings were different to what we had observed *in vitro* but because this

233  phenotype was phospholipase B1 dependent we reasoned that these inhibitors could be having

234  off target effects on *C. neoformans* - although *C. neoformans* does not have a homolog to

235  cyclooxygenase other studies have tried to inhibit eicosanoid production in *Cryptococcus* using

236  cyclooxygenase inhibitors but their efficacy and target remain uncertain (17,28). To support our

237  pharmacological evidence, we used a CRISPR/Cas9-mediated knockdown of the prostaglandin

238  E$_2$ synthase gene (*ptges)* (29). We used a knockdown of tyrosinase (*tyr*) – a gene involved in

239  the conversion of tyrosine into melanin as a control because *tyr*^{-/-} crispants are easy to identify

240  because they do not produce any pigment. We infected 2 dpf *ptges*^{-/-} and *tyr*^{-/-} zebrafish larvae

241  with H99-GFP or *Δplb1*-GFP and measured the fungal burden at 3 dpi. We found that *ptges*^{-/-}

242  zebrafish infected with H99-GFP had a higher fungal burden at 3 dpi compared to *tyr*^{-/-} zebrafish

243  infected with H99-GFP whereas there was no difference between *ptges*^{-/-} and *tyr*^{-/-} zebrafish

244  larvae infected with *Δplb1*-GFP (Fig 4C).  Thus, both pharmacological and genetic inhibitions of

245  host prostaglandin synthesis were not determinants of C. neoformans growth.

246

247  *Phospholipase B1 dependent factors are sufficient to support Δplb1 growth in macrophages*

248  To further evidence that *C. neoformans* was the source of PGE$_2$ during macrophage infection

249  we used a co-infection assay which has previously been used to investigate the interaction of

250  different *C. gattii* strains within the same macrophage (30). We hypothesised that if *C.*

251  *neoformans* derived prostaglandins promoted fungal growth, co-infection between H99 and

252  *Δplb1* would support mutant growth within macrophages because the parental strain H99 would

253  produce growth promoting prostaglandins that are lacking in *Δplb1*. To produce co-infection,

254  J774 murine macrophages were infected with a 50:50 mixture of *Δplb1* and H99-GFP (31) (Fig

11

255    4E i; as described previously for *C. gattii* (30)). This approach allowed us to differentiate

256    between *Δplb1* (GFP negative) and H99 (GFP positive) *Cryptococcus* strains within the same

257    macrophage and to score their proliferation separately. The intracellular proliferation of *Δplb1*

258    was calculated by counting the change in number of GFP negative *Δplb1* cells over an 18hr

259    period from time-lapse movies of infected cells. We found that co-infected macrophages did not

260    always contain an equal ratio of each strain at the start of the 18hr period so we scored the

261    proliferation of *Δplb1* for a range of initial burdens (1:2, 1:3 and 1:4). We found that *Δplb1*

262    proliferated better when accompanied by two H99-GFP yeast cells in the same macrophage

263    (Fig 4E ii, 1:2 p = 0.014) as opposed to when two *Δplb1* yeast cells were accompanied by one

264    H99-GFP yeast cell (Fig 4E ii, 2:1). We observed a similar effect for ratios of 1:3 and 1:4, but

265    these starting burden ratios are particularly rare, under powering our analysis (Supplementary

266    Fig 3). This effect was also recapitulated for J774 macrophages co-infected with *Δlac1* and H99-

267    GFP (Supplementary Fig 2A). These data indicate that a phospholipase B1 dependent factor

268    found in H99 infected macrophages, but absent in *Δplb1* and *Δlac1* infected macrophages, is

269    required for intracellular proliferation within macrophages.

270

271    *Production of PGE$_2$ by macrophages is not altered by C. neoformans infection.*

272

273    Our data indicate that host cells are not the source of virulence promoting prostaglandins, and

274    that secreted factors produced by wild type - but not *Δplb1* or *Δlac1 Cryptococcus* strains –

275    promote fungal growth within macrophages. We wanted to test if there were detectable

276    difference in PGE$_2$ levels between infected and uninfected macrophages caused by

277    cryptococcal prostaglandin synthesis. To do this we performed ELISA analysis to detect PGE$_2$

278    concentrations in supernatants from *C. neoformans* infected J774 macrophages (Fig 4D i). We

279    found that J774 macrophages produced detectable levels of PGE$_2$ (mean concentration 4.10

280    ng/$1x10^6$ cells), however we did not see any significant difference between infected or

281  uninfected macrophages (infected with H99, *Δplb1* or *Δplb1:PLB1* strains). To confirm our

282  ELISA results, we performed LC MS/MS analysis of lysed J774 macrophages using a $PGE_2$

283  standard for accurate quantification (Fig 4D ii). The concentrations detected were similar to

284  those measured by our ELISA (mean concentration 6.35 ng/1X10$^6$ cells) and also did not show

285  any significant differences between conditions. Taken together these data suggest that any

286  *Cryptococcus*-derived prostaglandins present during infection were likely to be contained within

287  the macrophage in low, localized concentrations and that the host receptor targeted by these

288  eicosanoids is therefore likely to be intracellular.

289

290  *15-keto-PGE$_2$ promotes C. neoformans growth by activating host PPAR-$\gamma$*

291

292  We next wanted to determine how $PGE_2$ / 15-keto-$PGE_2$ promotes *C. neoformans* infection. We

293  hypothesized that these prostaglandins were interfering with inhibition of fungal growth via a

294  host receptor. Our experiments inhibiting EP2 / EP4 suggest that 15-keto-$PGE_2$ must signal

295  through a different receptor to $PGE_2$ (Fig 3E). 15-keto-$PGE_2$ is a known agonist of the

296  peroxisome proliferation associated receptor gamma (PPAR-$\gamma$) (32); a transcription factor that

297  controls expression of many inflammation related genes (33-35). We first tested if PPAR-$\gamma$

298  activation occurs within macrophages during *C. neoformans* infection by performing

299  immunofluorescent staining for PPAR-$\gamma$ in macrophages infected with H99 and *Δplb1*. To

300  quantify PPAR-$\gamma$ activation we measured its nuclear translocation by comparing nuclear and

301  cytoplasmic fluorescence intensity in infected cells. PPAR-$\gamma$ is a cytosolic receptor that

302  translocates to the nucleus upon activation, therefore cells where PPAR-$\gamma$ is activated should

303  have increased nuclear staining for PPAR-$\gamma$. We found that J774 macrophages infected with

304  H99 had significantly higher levels of nuclear staining for PPAR-$\gamma$ compared to *Δplb1* infected

13

305     and uninfected cells  (Fig 5A i and Supplementary Fig 3C). This confirmed that *C. neoformans*

306     activates PPAR-$\gamma$ and that this phenotype is phospholipase B1 dependent.

307

308     To test the activation of PPAR-$\gamma$ during infection we first wanted to confirm that exogenous 15-

309     keto-PGE$_2$ activates zebrafish PPAR-$\gamma$ *in vivo* by using transgenic PPAR-$\gamma$ reporter zebrafish

310     larvae (36,37). We treated these larvae at 2 dpf with 15-keto-PGE$_2$ and Troglitazone (TLT) which

311     is a specific agonist of PPAR-$\gamma$. TLT treatment was performed with a concentration (0.55 µM)

312     previously shown to strongly activate PPAR-$\gamma$ in these zebrafish larvae (36). We found that TLT

313     treatment at 2 dpf strongly activated GFP reporter expression in the larvae. We employed a

314     receptor competition assay as a sensitive measurement of binding by simultaneously treating

315     zebrafish with with 15-keto-PGE$_2$ and TLT. We observed a reduction in GFP expression

316     compared to TLT treatment alone (Fig 5 Aii), demonstrating competition for the same receptor.

317     This could mean that 15-keto-PGE$_2$ was a partial agonist (38,39) or an antagonist to PPAR-$\gamma$ in

318     this experiment. Existing studies suggest 15-keto-PGE$_2$  is an agonist to PPAR-$\gamma$ (32) but to

319     confirm this in our model we treated *Δplb1*-GFP infected zebrafish larvae with exogenous 15-

320     keto-PGE$_2$ as before but at the same time treated fish with the PPAR-$\gamma$ antagonist GW9662. We

321     found that 15-keto-PGE$_2$ treatment significantly improved the growth of *Δplb1*-GFP during

322     infection but that inhibition of PPAR-$\gamma$ was sufficient to reverse this effect (Fig 5C). Therefore,

323     we could demonstrate that that 15-keto-PGE$_2$ was an agonist to PPAR-$\gamma$, and that PPAR-$\gamma$

324     activation was sufficient to promote a permissive environment for *C. neoformans* growth during

325     infection.

326

327     To determine if PPAR-$\gamma$ activation by *C. neoformans* facilitates growth within macrophages we

328     treated H99 and *Δplb1* infected J774 murine macrophages with the PPAR-$\gamma$ antagonist

329     GW9662. GW9662 treatment significantly reduced the proliferation of H99, but not *Δplb1* (Fig

14

330    5B, p=0.026, 1.22-fold decrease vs. DMSO). Further supporting that PPAR-$\gamma$ activation is

331    necessary for the successful intracellular parasitism of host macrophages by *C. neoformans*.

332    Finally, to confirm that PPAR-$\gamma$ activation alone promoted *C. neoformans* infection we treated

333    2dpf infected zebrafish larvae with TLT at the same concentration known to activate PPAR-$\gamma$ in

334    PPAR-$\gamma$ reporter fish (Fig 5A ii and (36)). We found that TLT treatment significantly increased

335    the fungal burden of *Δplb1*-GFP (Fig 5E and, p = 0.0089, 1.68-fold increase vs. DMSO), H99-

336    GFP (Fig 5D , p = 0.0044, 1.46-fold increase vs. DMSO) and *Δlac1*-GFP (Fig 5F, p = 0.01, 1.94-

337    fold increase vs. DMSO) infected larvae similar to 15-keto-$PGE_2$ treatment. Thus, we could

338    show that host PPAR-$\gamma$ activation was sufficient to promote cryptococcal growth during infection

339    and was a consequence of fungal derived prostaglandins.

340

341

342

343 **Discussion**

344 We have shown for the first time that eicosanoids produced by *C. neoformans* promote fungal

345 virulence both *in vitro* and *in vivo*. In this respect we have shown that the intracellular growth

346 defects of two eicosanoid deficient *C. neoformans* strains *Δplb1* and *Δlac1* (21,23) can be

347 rescued with the addition of exogenous PGE$_2$. Furthermore, our *in vitro* co-infection assay, *in*

348 *vitro* infection assays with aspirin, and *in vivo* infection assays provide evidence that the source

349 of this eicosanoid during infection is from the pathogen, rather than the host. Using an *in vivo*

350 zebrafish larvae model of cryptococcosis we find that that PGE$_2$ must be dehydrogenated into

351 15-keto-PGE$_2$ before to influence fungal growth. Finally, we provide evidence that the

352 mechanism of PGE$_2$/15-keto-PGE$_2$ mediated growth promotion during larval infection is via the

353 activation of PPAR-$\gamma$ (33,34,40-42).

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355 In a previous study it was identified that the *C. neoformans* mutant *Δplb1* (that lacks the *PLB1*

356 gene coding for phospholipase B1) was deficient in replication and survival in macrophages

357 (21), a phenotype also observed by a number of studies using different *in vitro* infection assays

358 (19,23). In this study we demonstrate that supplementing *Δplb1* with exogenous prostaglandin

359 E$_2$ during *in vitro* macrophages infection is sufficient to restore the mutant's intracellular

360 proliferation defect. A key goal of this study was to investigate how eicosanoids produced by *C.*

361 *neoformans* modulate pathogenesis *in vivo* (22). To facilitate *in vivo* measurement of fungal

362 burden we created two GFP-tagged strains with constitutive GFP expression - *Δplb1*-GFP and

363 *Δlac1*-GFP - to use alongside the GFP-tagged H99 parental strain previously produced (31).

364 These two mutants are the only *C. neoformans* mutants known to have a deficiency in

365 eicosanoid synthesis. *Δplb1* cannot produce any eicosanoid species suggesting phospholipase

366 B1 is high in the eicosanoid synthesis pathway while *Δlac1* has a specific defect in PGE$_2$

367 suggesting it might be a prostaglandin E$_2$ synthase enzyme. To our knowledge these are the

368 first GFP tagged versions of *Δplb1* and *Δlac1* created. Characterisation of *Δplb1*-GFP and

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369    *Δlac1*-GFP *in vivo* revealed that both strains have significantly reduced fungal burdens

370    compared to H99-GFP. This is the first report of *Δlac1* in zebrafish but these observations do

371    confirm a previous zebrafish study showing that non-fluorescent *Δplb1* had attenuated infectious

372    burden in zebrafish larvae (43). To confirm that $PGE_2$ is also required for cryptococcal growth *in*

373    *vivo* we treated *Δplb1*-GFP and *Δlac1*-GFP infected zebrafish larvae with exogenous $PGE_2$ to

374    determine how it would affect fungal burden. In agreement with our *in vitro* findings we found

375    that $PGE_2$ significantly improved the growth of both of these strains within larvae. Interestingly

376    we also found that $PGE_2$ improved the growth of H99-GFP, perhaps representing a wider

377    manipulation of host immunity during *in vivo* infection.

378    In vertebrate cells, $PGE_2$ is converted into 15-keto-$PGE_2$ by the enzyme 15-prostaglandin

379    dehydrogenase (15PGDH), furthermore it has been reported that *C. neoformans* has enzymatic

380    activity analogous to 15PGDH (18). To investigate how the dehydrogenation of $PGE_2$ to 15-

381    keto-$PGE_2$ influenced fungal burden we treated infected larvae with 16,16-dm-$PGE_2$ – a

382    synthetic variant of $PGE_2$ which is resistant to dehydrogenation (25). Interestingly we found that

383    16,16-dm-$PGE_2$ was unable to promote the growth of *Δplb1*-GFP*,* H99-GFP or *Δlac1*-GFP within

384    infected larvae. These findings indicate that 15-keto-$PGE_2$, rather than $PGE_2$, promotes

385    cryptococcal virulence. We subsequently treated infected larvae with exogenous 15-keto-$PGE_2$

386    and confirmed that 15-keto-$PGE_2$ treatment was sufficient to promote the growth of both *Δplb1*-

387    GFP and H99-GFP, but not *Δlac1*-GFP (discussed below), without the need for $PGE_2$. We

388    therefore propose that $PGE_2$ produced by *C. neoformans* during infection must be enzymatically

389    dehydrogenated into 15-keto-$PGE_2$ to promote cryptococcal virulence. These findings represent

390    the identification of a new virulence factor (15-keto-$PGE_2$) produced by *C. neoformans,* as well

391    as the first-time identification of an eicosanoid other than $PGE_2$ with a role in promoting

392    cryptococcal growth. Furthermore, our findings suggest that previous studies which identify

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393    $PGE_2$ as a promoter of cryptococcal virulence (19,20,44) may have observed additive effects

394    from both $PGE_2$ and 15-keto-$PGE_2$ activity.

395    Our experiments with the *Δlac1*-GFP strain reveals that this strain appears to respond in a

396    similar way to $PGE_2$, 16,16-dm-$PGE_2$ and troglitazone as *Δplb1*-GFP but appears to be

397    unresponsive to 15-keto-$PGE_2$. At this time, we cannot fully explain this phenotype, *Δlac1*-GFP

398    was generally less responsive to $PGE_2$ and troglitazone treatments compared to *Δplb1*-GFP so

399    it is possible that that higher concentrations of 15-keto-$PGE_2$ would be needed to rescue its

400    growth defect, however experimentation with higher concentrations of 15-keto-$PGE_2$ led to

401    significant host toxicity. The unresponsiveness of *Δlac1* to eicosanoid treatment could be due to

402    unrelated virulence defects caused by laccase deficiency. Cryptococcal laccase expression is

403    required for the production of fungal melanin – a well characterized virulence factor produced by

404    *C. neoformans* (45,46). It is therefore likely that virulence defects unrelated to eicosanoid

405    synthesis are responsible for the differences between the two mutant phenotypes.

406    We have found that the phospholipase B1 dependent attenuation of *Δplb1* can be rescued with

407    the addition of exogenous $PGE_2$. This indicates that synthesis and secretion of $PGE_2$ by *C.*

408    *neoformans* is a virulence factor. Although our data indicated that *C. neoformans* was the

409    source of $PGE_2$ we wanted exclude the possibility that host-derived $PGE_2$ was also contributing

410    to virulence. To explore this possibility, we blocked host prostaglandin synthesis - we reasoned

411    that if host $PGE_2$ was not required that blocking its production would not affect the growth of *C.*

412    *neoformans.* To do this we first treated J774 macrophages infected with H99-GFP and *Δplb1*-

413    GFP with aspirin – a cyclooxygenase inhibitor that blocks both COX-1 and COX-2 activity – and

414    found that this had no effect on intracellular proliferation. Subsequently we attempted to block

415    cyclooxygenase activity in zebrafish but found aspirin was lethal at the zebrafish larvae's

416    currently stage of development, instead we used individual inhibitors specific for COX-1 (NS-

417    398) and COX-2 (SC-560). We found that each inhibitor decreases fungal burden of H99-GFP

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418   infected larvae but not $\Delta plb1$-GFP infected larvae. Due to the phospholipase B1 dependence of

419   this phenotype we think that these inhibitors might be affecting eicosanoid production by the *C.*

420   *neoformans* itself. The ability of broad COX inhibitors like aspirin/indomethacin to inhibit

421   eicosanoid production by *C. neoformans* is controversial (17,28) however our study is the first to

422   use such selective COX-1 and COX-2 inhibitors on *C. neoformans*. This experiment remained

423   inconclusive as to whether host $PGE_2$ synthesis promotes virulence so to block $PGE_2$ in

424   zebrafish larvae without potential off target effects we used CRISPR Cas9 technology to

425   knockdown expression of the prostaglandin $E_2$ synthase gene *ptges* in zebrafish larvae.

426   Ablating zebrafish *ptges* with this approach did not affect the fungal burden of $\Delta plb1$-GFP

427   infected larvae but it did cause increased burden in H99-GFP infected zebrafish. This

428   phenotype is interesting because it suggests host $PGE_2$ might actually be inhibitory to

429   cryptococcal virulence, furthermore this phenotype was phospholipase B1 dependent which

430   suggests host-derived $PGE_2$ might interact in some way with *Cryptococcus*-derived eicosanoids.

431   This phenotype was not seen *in vitro* with aspirin so it is possible that the inhibitory effects of

432   host-derived $PGE_2$ influence a non-macrophage cell type in zebrafish larvae.

433   To confirm our observations that *C. neoformans* was the source of $PGE_2$ during infection we

434   performed co-infection assays with H99 wild type cryptococci (eicosanoid producing) and $\Delta plb1$

435   (eicosanoid deficient) within the same macrophage and found that co-infection was sufficient to

436   promote the intracellular growth of $\Delta plb1$. We also observed similar interactions during $\Delta lac1$

437   co-infection (a second eicosanoid deficient *C. neoformans* mutant). These observations agree

438   with previous studies that suggest eicosanoids are virulence factors produced by *C. neoformans*

439   during macrophage infection (19,28). To identify if the secreted factor produced by *C.*

440   *neoformans* was $PGE_2$, we measured the levels of $PGE_2$ from *Cryptococcus* infected

441   macrophages to see if there was an observable increase in this eicosanoid during infection.

442   Although $PGE_2$ was detected, we did not see any significant difference between infected and

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443    uninfected macrophages, an observation confirmed using two different detection techniques –

444    ELISA and LC MS/MS. These data suggest that $PGE_2$ produced by *C. neoformans* during

445    macrophage infection is contained within the macrophage, likely in close proximity to the fungus

446    and that the host receptor targeted by these eicosanoids is therefore likely to be intracellular.

447    Our *in vitro* co-infection experiments indicate that *C. neoformans* secretes virulence enhancing

448    eicosanoids during infection.

449    The biological activity of 15-keto-$PGE_2$ is far less studied than $PGE_2$. It is known that 15-keto-

450    $PGE_2$ cannot bind to prostaglandin $E_2$ EP receptors, this means it can act as a negative

451    regulator of $PGE_2$ activity i.e. cells up-regulate 15PGDH activity to lower $PGE_2$ levels (47). Our

452    findings however suggested that 15-keto-$PGE_2$ did have a biological activity independent of

453    $PGE_2$ synthesis, possibly via a distinct eicosanoid receptor. It has been demonstrated that 15-

454    keto-$PGE_2$ can activate the intracellular eicosanoid receptor peroxisome proliferator associated

455    receptor gamma (PPAR- $\gamma$) (32).  Activation of PPAR-$\gamma$ by *C. neoformans* has not been

456    described previously but it is compatible with what we know of cryptococcal pathogenesis.

457    PPAR-$\gamma$ is a nuclear receptor normally found within the cytosol. Upon ligand binding PPAR-$\gamma$

458    forms a heterodimer with Retinoid X receptor (RXR) and translocates to the nucleus where it

459    influences the expression of target genes which possess a peroxisome proliferation hormone

460    response element (PPRE) (48). If eicosanoids are produced by *C. neoformans* during

461    intracellular infection, it is likely that they bind to an intracellular eicosanoid receptor.

462    Additionally, activation of PPAR-$\gamma$ within macrophages is known to promote the expression of

463    anti-inflammatory genes which could make the macrophage more amenable to parasitism by

464    the fungus.

465    To investigate whether PPAR- $\gamma$ activation within macrophages occurs during *C. neoformans*

466    infection we performed immunofluorescent staining of H99 and *Δplb1* infected J774

467    macrophages. We found that infection with *C. neoformans* led to increased nuclear localization

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468    of PPAR- $\gamma$ indicating that the fungus was activating endogenous PPAR- $\gamma$ during infection. We

469    also found that macrophages infected with H99 had higher levels of PPAR- $\gamma$ activation than

470    *Δplb1* infected macrophages. This strongly suggests that eicosanoids produced by *C.*

471    *neoformans* are responsible for activating PPAR- $\gamma$.

472    To confirm that 15-keto-PGE$_2$ is an agonist to PPAR-$\gamma$ we performed experiments with zebrafish

473    larvae from PPAR-$\gamma$ GFP reporter fish (36) and demonstrated that 15-keto-PGE$_2$ binds to

474    zebrafish PPAR- $\gamma$. To determine if 15-keto-PGE$_2$ is an agonist to PPAR- $\gamma$ we found that

475    treating *Δplb1*-GFP infected zebrafish larvae with GW9662 at the same time as 15-keto-PGE$_2$

476    blocked the virulence enhancing effects of the eicosanoid. These data indicate that 15-keto-

477    PGE$_2$ is a partial agonist to PPAR-$\gamma$ (in zebrafish at least). Partial agonists are weak agonists

478    that bind to and activate receptors, but not at the same efficacy as a full agonist. Partial agonists

479    to PPAR-$\gamma$ have been reported previously, partial PPAR agonists bind to the ligand binding

480    domain of PPAR-$\gamma$ with a lower affinity than full PPAR agonists and as a result activate smaller

481    subsets of PPAR-$\gamma$ controlled genes (38,39,49-52).

482    We also found that activation of PPAR-$\gamma$ alone was sufficient to mediate cryptococcal virulence.

483    In this respect, we found that the *in vitro* intracellular proliferation of the wild type H99

484    cryptococcal strain within J774 macrophages could be suppressed using a PPAR-$\gamma$ antagonist

485    GW9662. We could also block the rescuing effect of 15-keto-PGE$_2$ on *Δplb1*-GFP during

486    zebrafish infection using GW9662. Finally we found that *Cryptococcus* infected zebrafish treated

487    with troglitazone at a concentration that is known to activate PPAR-$\gamma$ (53) had increased fungal

488    burdens when infected with *Δplb1*-GFP*, Δlac1*-GFP and H99-GFP strains. Taken together these

489    experiments provide convincing evidence that a novel cryptococcal virulence factor - 15-keto-

490    PGE$_2$ – enhances the virulence of *C. neoformans* by activation of host PPAR-$\gamma$ and that

491    macrophages are one of the key targets of this eicosanoid during infection.

492   In this study, we have shown for the first time that eicosanoids produced by *C. neoformans* can

493   promote virulence in an *in vivo* host. Furthermore, we have provided evidence that this virulence

494   occurs via eicosanoid mediated manipulation of host macrophages. We have identified that the

495   eicosanoid responsible for these effects is 15-keto-PGE$_2$ which is derived from the

496   dehydrogenation of PGE$_2$ produced by *C. neoformans*. We have subsequently demonstrated

497   that 15-keto-PGE$_2$ mediates its effects via activation of PPAR- $\gamma$, an intracellular eicosanoid

498   receptor known to promote anti-inflammatory immune pathways within macrophages.   We

499   provide compelling evidence that eicosanoids produced by *C. neoformans* enhance virulence,

500   identifies a novel virulence factor – 15-keto-PGE$_2$ – and describes a novel mechanism of host

501   manipulation by *C. neoformans* - activation of PPAR- $\gamma$. Most importantly this study provides a

502   potential new therapeutic pathway for treatment of cryptococcal infection, as several eicosanoid

503   modulating drugs are approved for patient treatment (54) .

504

**Materials and methods**

506   (all reagents are from Sigma-Aldrich, UK unless otherwise stated)

**Ethics statement**

508   Animal work was performed following UK law: Animal (Scientific Procedures) Act 1986, under

509   Project License PPL 40/3574 and P1A4A7A5E. Ethical approval was granted by the University

510   of Sheffield Local Ethical Review Panel. Experiments using the PPAR-γ reporter fish line (53)

511   were conducted at the University of Toronto following approved animal protocols (# 00000698

512   "A live zebrafish-based screening system for human nuclear receptor ligand and cofactor

513   discovery") under a OMAFRA certificate.

**Zebrafish**

515   The following zebrafish strains were used for this study: *Nacre* wild type strain,

516   *Tg(mpeg1:mCherryCAAX)sh378)* transgenic strain and the double mutant *casper*, for PPARγ

517   reporter experiments (53), which lacks all melanophores and iridophores (55). Zebrafish were

518   maintained according to standard protocols. Adult fish were maintained on a 14:10 – hour light /

519   dark cycle at 28 °C in UK Home Office approved facilities in the Bateson Centre aquaria at the

520   University of Sheffield.

### *C. neoformans*

522   The H99-GFP strain has been previously described (31). The Δ*plb1*-GFP and Δ*lac1*-GFP

523   stains was generated for this study by transforming existing deletion mutant strains (23,56) with

524   a GFP expression construct (see below for transformation protocol). All strains used are in the

525   *C. neoformans* variety *grubii* H99 genetic background.

526   *Cryptococcus* strains were grown for 18 hours at 28 °C, rotating horizontally at 20 rpm.

527   *Cryptococcus* cultures were pelleted at 3300g for 1 minute, washed twice with PBS (Oxoidm

528   Basingstoke, UK) and re-suspended in 1ml PBS. Washed cells were then counted with a

529   haemocytometer and used as described below.

### *C. neoformans* transformation

531   *C. neoformans* strains Δ*plb1* and Δ*lac1* were biolistically transformed using the pAG32_GFP

532   transformation construct as previously described for H99-GFP (31). Stable transformants were

533   identified by passaging positive GFP fluorescent colonies for at least 3 passages on YPD agar

534   supplemented with 250 μg/ml Hygromycin B.

### Zebrafish CRISPR

536   CRISPR generation was performed as previously described (29). Briefly gRNA spanning the

537   ATG start codon of zebrafish *ptges* or *tyr* was injected along with Cas9 protein and tracrRNA

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538  into zebrafish embryos at the single cell stage. Crispant larvae were infected with *C.*

539  *neoformans* as described above at 2 dpf. The genotype of each larvae was confirmed post

540  assay – genomic DNA was extracted from each larvae and the ATG was PCR amplified with

541  primers spanning the ATG site of *ptges* (Forward primer gccaagtataatgaggaatggg, Reverse

542  primer aatgtttggattaaacgcgact) producing a 345-bp product. This product was digest with MwoI

543  – wildtype digests produced bands at 184, 109 and 52 bp while mutant digests produced bands

544  at 293 and 52 bp (Supplementary Figure 4).

**J774 Macrophage infection – with exogenous PGE$_2$ treatment**

546  J774 macrophage (J774 cells were obtained from the ATCC, American Type Culture Collection)

547  infection was performed as previously described (21) with the following alterations. J774 murine

548  macrophage-like cells were cultured for a minimum of 4 passages in T75 tissue culture flasks at

549  37$^o$C 5% $CO_2$ in DMEM (High glucose, Sigma) supplemented with 10% Fetal Bovine Calf

550  Serum (Invitrogen), 1% 10,000 units Penicillin / 10 mg streptomycin and 1 % 200 mM L –

551  glutamine, fully confluent cells were used for each experiment. Macrophages were counted by

552  haemocytometer and diluted to a concentration of $1x10^5$ cells per ml in DMEM supplemented

553  with 1 µg/ml lipopolysaccharide (LPS from *E. coli,* Sigma L2630) before being plated into 24 well

554  microplates (Greiner) and incubated for 24 hours (37 $^o$C 5% $CO_2$).

555  Following 24-hour incubation, medium was removed and replaced with 1 ml DMEM

556  supplemented with 2 nM prostaglandin E$_2$ (CAY14010, 1mg/ml stock in 100% ethanol).

557  Macrophage wells were then infected with 100 µl $1x10^6$ yeast/ml *Cryptococcus* cells (from

558  overnight culture, washed. See above) opsonized with anti-capsular IgG monoclonal antibody

559  (18b7, a kind gift from Arturo Casadevall). Cells were incubated for 2 hours (37 $^o$C 5% $CO_2$) and

560  then washed with 37 $^o$C PBS until extracellular yeast were removed. After washing, infected

561  cells were treated with 1ml DMEM supplemented with PGE$_2$.

562    To calculate IPR, replicate wells for each treatment/strain were counted at 0 and 18 hours. Each

563    well was washed once with 1ml 37 $^o$C PBS prior to counting to remove any *Cryptococcus* cells

564    released by macrophage death or vomocytosis. Intra-macrophage Cryptococci were released

565    by lysis with 200 µl dH$_2$O for 20 minutes (lysis confirmed under microscope). Lysate was

566    removed to a clean microcentrifuge tube and an additional 200 µl was used to wash the well to

567    make a total lysate volume of 400 µl. *Cryptocccoccus* cells within lysates were counted by

568    haemocytometer. IPR was calculated by dividing the total number of counted yeast at 18hr by

569    the total at 0hr.

570    To assess the viability of *C. neoformans* cells recovered from macrophages we used our

571    previously published colony forming unit (CFU) viability assay (21). Lysates from *C. neoformans*

572    infected J774 cells were prepared from cells at 0hr and 18hr time points. The concentration of

573    *C. neoformans* cells in the lysate was calculated by haemocytomter counting, the lysates were

574    then diluted to give an expected concentration of $2 \times 10^3$ yeast cells per ml. 100 µl of this diluted

575    lysate was spread onto a YPD agar plate and incubated for 48 hr at 25$^o$C prior to colony

576    counting.

577    **J774 Macrophage co-infection.**

578    J774 cells were prepared and seeded at a concentration of $1 \times 10^5$ per ml as above in 24 well

579    microplates and incubated for 24 hours (37 $^o$C 5% CO$_2$), 45 minutes prior to infection J774 cells

580    were activated with 150 ng/ml phorbol 12-myristate 13-acetate in DMSO added to 1 ml serum

581    free DMEM. Following activation J774 cells were washed and infected with 100 µl / $1 \times 10^6$

582    yeast/ml 50:50 mix of Δ*plb1* (non-fluorescent) and H99-GFP (e.g. $5 \times 10^5$ Δ*plb1* and $5 \times 10^5$ H99-

583    GFP) or Δ*lac1-GFP* and H99 (non-fluorescent). Infected cells were incubated for 2 hours (37 $^o$C

584    5% CO$_2$) to allow for phagocytosis of *Cryptococcus* and then washed multiple times with 37 $^o$C

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585    PBS to remove unphagocytosed yeast, each well was observed between washes to ensure that

586    macrophages were not being washed away. After washing 1 ml DMEM was added to each well.

587    Co-infected cells were imaged over 20 hours using a Nikon TE2000 microscope fitted with a

588    climate controlled incubation chamber (37 $^{\circ}$C 5% $CO_2$) using a Digital Sight DS-QiMC camera

589    and a Plan APO Ph1 20x objective lens (Nikon). GFP and bright field images were captured

590    every 4 minutes for 20 hours. Co-infection movies were scored manually. For example co-

591    infected macrophages that contained 2 Δ*plb1* (non-fluorescent) and 1 H99-GFP (GFP positive)

592    yeast cells at 0 hr were tracked for 18 hours and before the burden of each strain within the

593    macrophage was counted again. The IPR for Δ*plb1* within co-infected macrophages was

594    calculated by dividing the number of Δ*plb1* cells within a macrophage at 18 hr by the number at

595    0 hr.

**Immunofluorescence**

597    J774 cells were cultured to confluency as discussed above and seeded onto sterile 13 mm

598    glass coverslips at a density of $10^5$ cells per ml without activation by phorbol 12-myristate 13-

599    acetate. H99-GFP and Δ*plb1*-GFP were opsonised with 18B7 for one hour. 18B7 was then

600    removed by centrifugation and fungal cells were suspended in 1 ml of PBS with 1:200 FITC for

601    one hour. Supernatant was removed again, cells were resuspended in PBS, and J774 were

602    infected with $10^6$ of either H99-GFP or Δ*pbl1*-GFP in serum free DMEM. After two hours of

603    infection media was removed from J774s and the J774s were washed three times with PBS. 10

604    μm TLT or DMSO was added to uninfected cells to act as controls. Cells were then left for 18

605    hours at 37 $^{\circ}$C 5 % $CO_2$.

606    After 18 hours supernatants were removed and J774s were fixed with cold methanol for 5

607    minutes at -20 $^{\circ}$C before washing with PBS three times, leaving PBS for five minutes at room

608    temperature between washes.  Coverslips were blocked with 5% sheep serum in 0.1% triton

609    (block solution) for 20 minutes before being transferred into the primary PPAR-γ antibody (1:50,

610    Santa Cruz Biotechnology sc-7273 lot #B1417) with 1:10 human IgG in block solution for one

611    hour. Coverslips were washed 3 times in PBS and incubated with 1:200 anti-mouse TRITC,

612    1:40 anti-human IgG, and 0.41 µl/ml DAPI in block solution for one hour. Coverslips were then

613    washed three times with PBS, three times with water, and fixed to slides using MOWIOL. Slides

614    were left in the dark overnight and imaged the following day. Imaging was performed on a Nikon

615    Eclispe Ti microscope with a x60 DIC objective. Cells were imaged with filter sets for Cy3

616    (PPAR-γ , 500ms exposure) GFP (*Cryptococcus*, 35 ms exposure) and DAPI (Nuclei, 5ms)

617    dyes in addition to DIC.

618    The intensity of nuclear staining was analysed for at least 30 cells per coverslip, using ImageJ

619    2.0.0 a line ROI was drawn from the outside of cell, through the nucleus measuring the mean

620    grey value along the line. For *Cryptococcus* infected conditions uninfected and infected cells

621    were measured separately upon the same coverslip using the GFP channel to distinguish

622    between infected and uninfected cells.

623    **J774 aspirin timelapse**

624    Macrophages were seeded at $10^5$ per ml into 24 well plates as described above. After two hours

625    cells requiring aspirin were treated with 1 mM aspirin in DMSO in fresh DMEM. Cells were then

626    incubated overnight for 18 hours at 37 $^o$C 5% $CO_2$. H99-GFP and $\Delta plb1$-GFP were prepared at

627    $10^6$ cells per ml as described above, and opsonised with 18B7 for one hour. J774s were then

628    infected with the fungal cells in fresh serum free DMEM for two hours before removing the

629    supernatant, washing three times in PBS, and adding fresh serum free DMEM. Cells were

630    imaged for 18 hours on a Nikon Eclispe Ti equipped with  a climate controlled stage

631    (Temperature - 37 $^o$C, Atmosphere -  5% $CO_2$ / 95% air) with a x20 Lambda Apo NA 0.75 phase

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632    contrast objective brightfield images were taken at an interval of 2 minutes, 50 ms exposure.

633    Analysis was performed by manual counts of intracellular and extracellular cryptococci.

**Zebrafish infection**

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635    Washed and counted *Cryptococcus* cells from overnight culture were pelleted at 3300g for 1

636    minute and re-suspended in 10% Polyvinylpyrrolidinone (PVP), 0.5% Phenol Red in PBS to give

637    the required inoculum in 1 nl. This injection fluid was loaded into glass capillaries shaped with a

638    needle puller for microinjection. Zebrafish larvae were injected at 2-days post fertilisation; the

639    embryos were anesthetised by immersion in 0.168 mg/ml tricaine in E3 before being transferred

640    onto microscope slides coated with 3% methyl cellulose in E3 for injection. Prepared larvae

641    were injected with two 0.5 nl boluses of injection fluid by compressed air into the yolk sac

642    circulation valley. Following injection, larvae were removed from the glass slide and transferred

643    to E3 to recover from anaesthetic and then transferred to fresh E3 to remove residual methyl

644    cellulose. Successfully infected larvae (displaying systemic infection throughout the body and no

645    visible signs of damage resulting from injection) were sorted using a fluorescent

646    stereomicroscope. Infected larvae were maintained at 28 $^{o}$C.

**Eicosanoid / receptor agonist treatment of infected zebrafish larvae**

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648    All compounds were purchased from Cayman Chemical. Compounds were resuspended in

649    DMSO and stored at -20 $^{o}$C until used. Prostaglandin $E_2$ (CAY14010, 10mg/ml stock),

650    Prostaglandin $D_2$ (CAY12010, 10mg/ml stock), 16,16-dimethyl-$PGE_2$ (CAY14750, 10mg/ml

651    stock), 15-keto-$PGE_2$ (CAY14720, 10mg/ml stock), troglitazone (CAY14720, 10mg/ml stock),

652    GW9662 (CAY70785,1mg/ml stock), AH6809 (CAY14050, 1mg/ml stock), GW627368X

653    (CAY10009162, 10mg/ml stock), NS-398 (CAY70590, 9.4mg/ml stock), SC-560 (CAY70340,

654    5.3mg/ml stock).

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655 Treatment with exogenous compounds during larval infected was performed by adding

656 compounds (or equivalent solvent) to fish water (E3) to achieve the desired concentration. Fish

657 were immersed in compound supplemented E3 throughout the experiment from the time of

658 injection.

659 **Zebrafish fungal burden measurement**

660 Individual infected zebrafish embryos were placed into single wells of a 96 well plate (VWR) with

661 200 ul or E3 (unsupplemented E3, or E3 supplemented with eicosanoids / drugs depending on

662 the assay). Infected embryos were imaged at 0 days post infection (dpi), 1 dpi, 2 dpi and 3 dpi in

663 their 96 well plates using a Nikon Ti-E with a CFI Plan Achromat UW 2X N.A 0.06 objective

664 lens. Images were captured with a Neo sCMOS (Andor, Belfast, UK) and NIS Elements (Nikon,

665 Richmond, UK). Images were exported from NIS Elements into Image J FIJI as monochrome tif

666 files. Images were threshholded in FIJI using the 'moments' threshold preset and converted to

667 binary images to remove all pixels in the image that did not correspond to the intensity of the

668 fluorescently tagged *C. neoformans.* The outline of the embryo was traced using the 'polygon'

669 ROI tool, avoiding autofluorescence from the yolk sac. The total number of pixels in the

670 threshholded image were counted using the FIJI 'analyse particles' function, the 'total area'

671 measurement from the 'summary' readout was used for the total number of GFP$^+$ pixels in each

672 embryo.

673 **PPAR-$\gamma$ GFP reporter fish treatment**

674 PPAR$\gamma$ embryos were collected from homozygous ligand trap fish (F18). Embryos were raised

675 in a temperature-controlled water system under LD cycle at 28.5° C. in 0.5×E2 media in petri-

676 dishes till 1 dpf. Any developmentally delayed (dead or unfertilized) embryos were removed.

677 Chorions were removed enzymatically with Pronase (1mg/ml) and specimens were dispensed

678 into 24 well plates (10 per well) in 0.5×E2 media. For embryos 1% DMSO was used as a vehicle

29

679    control. Chemicals were stored in DMSO, diluted appropriately and added individually to 400 ul

680    of 0.5×E2 media with 0.05 U/ml penicillin and 50 ng/ml streptomycin and vortexed intensively for

681    1 min. 0.5×E2 media was removed from all wells with embryos and the 400 ul chemical

682    solutions were administered to different wells to embryos. For drug treatment embryos were

683    pre-incubated for 1 hour with compounds and then heat induced (28→37° C.) for 30 min in a

684    water bath. Embryos were incubated at 28°C for 18 h and then monitored using a fluorescent

685    dissection scope (SteREO Lumar.V12 Carl Zeiss) at 2 dpf. For analyzing GFP fluorescent

686    pattern, embryos were anesthetized with Tricaine (Sigma, Cat.# A-5040) and mounted in 2 %

687    methyl cellulose.

688    **Whole body macrophage counts**

689    2 dpf transgenic zebrafish larvae which have fluorescently tagged macrophages due to an

690    mCherry fluorescent protein driven by the macrophage specific gene marker mpeg1 (57)

691    *Tg(mpeg1:mCherryCAAX)sh378)* (22) were treated with 10 µM $PGE_2$, 10 µM 15-keto-$PGE_2$ or

692    an equivalent DMSO control for 2 days. Larvae were then anesthetized by immersion in 0.168

693    mg/ml tricaine in E3 and imaged using a Nikon Ti-E with a Nikon Plan APO 20x/ 0.75 DIC N2

694    objective lens, taking z stacks of the entire body with 15 µM z steps. Macrophage counts were

695    made manually using ImageJ from maximum projections.

696    **Eicosanoid measurement (ELISA)**

697    J774 macrophages were seeded into 24 well plates at a concentration of $1x10^5$ per well and

698    incubated for 24 hours at 37 $^o$C 5 % $CO_2$. J774 cells were infected with *C. neoformans* as

699    described above, at the same MOI 1:10 and incubated for 18 hours with 1ml serum free DMEM.

700    At 18 hours post infection the supernatant was removed for ELISA analysis.

701    For ELISA analysis with aspirin treatment, wells requiring aspirin had supernatants removed

702    and replaced with fresh DMEM containing 1mM aspirin in 1% DMSO. Cells were left for 24

703    hours total. At 24 hours all wells received fresh serum free media. Wells requiring aspirin for the

704    duration received 1mM aspirin in DMSO. Aspirin treated cells requiring arachidonic acid were

705    treated with 30 μg per ml arachidonic acid in ethanol. Control wells received the following: either

706    1% DMSO, 30 μg per ml arachidonic acid, or ethanol. Cells were again left at 37 $^{o}$C 5 % $CO_2$ for

707    18 hours.

708    Supernatants were then removed and frozen at -80 $^{o}$C until use. Supernatants were analysed

709    as per the $PGE_2$ EIA ELISA kit instructions (Cayman Chemical).

**Eicosanoid measurement (Mass spectrometry)**

711    J774 macrophages were seeded into T25 tissue culture flasks at a concentration of $1.3x10^6$

712    cells per flask and incubated for 24 hours at 37 $^{o}$C 5 % $CO_2$. J774 cells were infected with *C.*

713    *neoformans* as described above, at the same MOI 1:10 and incubated for 18 hours with 2 ml

714    serum free DMEM. At 18 hours post infection infected cells were scraped from the flask with a

715    cell scraper into the existing supernatant and immediately snap frozen in ethanol / dry ice slurry.

716    All samples were stored at -80 $^{o}$C before analysis.

717    Lipids and lipid standards were purchased from Cayman Chemical (Ann Arbor, Michigan).

718    Deuterated standard Prostaglandin $E_2$-$d_4$ ($PGE_2$-$d_4$), ≥98% deuterated form. HPLC grade

719    solvents were from Thermo Fisher Scientific (Hemel Hempstead, Hertfordshire UK).

720    Lipids were extracted by adding a solvent mixture (1 mol/L acetic acid, isopropyl alcohol,

721    hexane (2:20:30, v/v/v)) to the sample at a ratio of 2.5 –1 ml sample, vortexing, and then adding

722    2.5 ml of hexane (58). Where quantitation was required, 2 ng $PGE_2$-$d_4$, was added to samples

723    before extraction, as internal standard. After vortexing and centrifugation, lipids were recovered

724    in the upper hexane layer. The samples were then re-extracted by addition of an equal volume

725    of hexane. The combined hexane layers were dried and analyzed for Prostaglandin $E_2$ ($PGE_2$)

726    using LC-MS/MS as below.

31

727    Lipid extracts were separated by reverse-phase HPLC using a ZORBAX RRHD Eclipse Plus

728    95Å C18, 2.1 x 150 mm, 1.8 µm column (Agilent Technologies, Cheshire, UK), kept in a column

729    oven maintained at 45°C. Lipids were eluted with a mobile phase consisting of A, water-B-acetic

730    acid of 95:5:0.01 (vol/vol/vol), and B, acetonitrile-methanol-acetic acid of 80:15:0.01 (vol/vol/vol),

731    in a gradient starting at 30% B. After 1 min that was ramped to 35% over 3 min, 67.5% over 8.5

732    min and to 100% over 5 min. This was subsequently maintained at 100% B for 3.5 min and then

733    at 30% B for 1.5 min, with a flow rate of 0.5 ml/min. Products were monitored by LC/MS/MS in

734    negative ion mode, on a 6500 Q-Trap (Sciex, Cheshire, United Kingdom) using parent-to-

735    daughter transitions of $m/z$ 351.2 → 271.2 ($PGE_2$), and $m/z$ 355.2 → 275.2 for $PGE_2$-$d_4$. ESI-

736    MS/MS conditions were: TEM 475 °C, GS1 60, GS2 60, CUR 35, IS -4500 V, dwell time 75 s,

737    DP -60 V, EP -10 V, CE -25 V and CXP at -10 V. $PGE_2$ was quantified using standard curves

738    generated by varying $PGE_2$ with a fixed amount of $PGE_2$-$d_4$.

739    **Acknowledgments**

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911

912

913 **Fig1**

914 **The intracellular proliferation defect of the *C.* neoformans mutant *Δplb1,* can be reversed**

915 **with the addition of exogenous prostaglandin E$_2$.** J774 murine macrophages were infected

916 with *Δplb1,* the parental strain H99 or a genetic reconstitute strain *Δplb1:PLB1* . Infected cells

917 were left untreated or treated with 2 nM PGE$_2$ or an equivalent solvent (ethanol) control. Mean

918 IPR from 5 biological repeats shown with error bars representing standard deviation. An

919 unpaired two tailed Student's t-test was performed to compare each treatment group. H99 etoh

920 vs H99 2 nM PGE$_2$ ns p = 0.7212, *Δplb1* etoh vs. *Δplb1* 2 nM PGE$_2$ * p = 0.0376, *Δplb1:PLB1*

921 etoh vs. *Δplb1:PLB1* 2 nM PGE$_2$ ns p=0.723.

922

923 **Fig2**

924 **The prostaglandin E$_2$ dependent growth defect of *Δplb1* is also present *in vivo*: A i** H99-

925 GFP infected larvae imaged at 0, 1, 2 and 3 dpi. At least 50 larvae measured per time point

926 across 3 biological repeats. Box and whiskers show median, 5$^{th}$ percentile and 95$^{th}$ percentile.

927 Unpaired Mann-Whitney U tests used to compare the burden between each strain for every time

928 point, for p values see (Supplementary Fig 1B ii). **B i** – H99-GFP Infected larvae treated with 10

929 μM prostaglandin E$_2$ or equivalent solvent (DMSO) control. At least 60 larvae measured per

930 treatment group from 3 biological repeats. Box and whiskers show median, 5$^{th}$ percentile and

931 95$^{th}$ percentile. Unpaired Mann-Whitney U tests used to compare between treatments DMSO

932 vs. 10 μM PGE$_2$ * p = 0.0137 (threshold for significance 0.017, corrected for multiple

933 comparisons). **C i,** H99-GFP Infected larvae treated with 10 μM prostaglandin D$_2$ or equivalent

934 solvent (DMSO) control. At least 60 larvae measured per treatment group from 4 biological

935 repeats. Box and whiskers show median, 5$^{th}$ percentile and 95$^{th}$ percentile. Unpaired Mann-

936 Whitney U tests used to compare between treatments DMSO vs. 10 μM PGD$_2$, ns p= 0.8 **D i**

937 *Δplb1*-GFP infected larvae (500 cell inoculum injected at 2 dpf) imaged at 0, 1, 2 and 3 dpi. N =

938 3. Box and whiskers show median, 5$^{th}$ percentile and 95$^{th}$ percentile. At least 87 larvae

38

939    measured for each time point from 3 biological repeats. Unpaired Mann-Whitney U tests used to

940    compare the burden between each strain for every time point, for p values see (Supplementary

941    Fig 1 A i + A ii). **E i** *Δplb1*-GFP Infected larvae treated with 10 µM prostaglandin $E_2$ or equivalent

942    solvent (DMSO) control. At least 35 larvae measured per treatment group from 2 biological

943    repeats. Box and whiskers show median, $5^{th}$ percentile and $95^{th}$ percentile. Unpaired Mann-

944    Whitney U tests used to compare between treatments *Δplb1*-GFP DMSO vs 10 µM $PGE_2$ *** p =

945    0.0001 (threshold for significance 0.017, corrected for multiple comparisons). **F i** *Δplb1*-GFP

946    Infected larvae treated with 10 µM prostaglandin $D_2$ or equivalent solvent (DMSO) control. At

947    least 45 larvae measured per treatment group from 3 biological repeats. Box and whiskers show

948    median, $5^{th}$ percentile and $95^{th}$ percentile. Unpaired Mann-Whitney U tests used to compare

949    between treatments DMSO vs. 10 µM $PGD_2$. Ns p = 0.1 **A ii** Representative GFP images

950    (representative = median value) H99-GFP infected larvae, untreated at 0,1,2,3 dpi. **B ii, C ii**

951    Representative GFP images (representative = median value) H99-GFP infected larvae, at 2 dpi

952    treated with 10 µM $PGE_2$ (**B ii**) or $PGD_2$ (**C ii**). **D ii** Representative GFP images (representative

953    = median value) *Δplb1*-GFP infected larvae, untreated at 0,1,2,3 dpi. **E ii, F ii** Representative

954    GFP images (representative = median value) *Δplb1*-GFP infected larvae, at 2 dpi treated with

955    10 µM $PGE_2$ (**E ii**) or $PGD_2$ (**F ii**).

956

957    **Fig 3**

958    **The observed activity of $PGE_2$ is due to its dehydrogenated derivative 15-keto-$PGE_2$:**

959    Fungal burden measured at 2 days post infection (2 dpi) by counting GFP positive pixels in each

960    larvae. **A i** H99-GFP Infected larvae treated with 10 µM 16,16-dimethyl-prostaglandin $E_2$ or

961    equivalent solvent (DMSO) control. At least 75 larvae measured per treatment group from 4

962    biological repeats. Box and whiskers show median, $5^{th}$ percentile and $95^{th}$ percentile. Unpaired

963    Mann-Whitney U test used to compare between treatments, DMSO vs. 10 µM 16, 16-dm $PGE_2$

964    ns p = 0.9954. **B i** H99-GFP Infected larvae treated with 10 µM 15-keto-prostaglandin $E_2$ or

965     equivalent solvent (DMSO) control. At least 55 larvae measured per treatment group from 3

966     biological repeats. Unpaired Mann-Whitney U test used to compare between treatments DMSO

967     vs. 10 µM 15-keto=PGE$_2$ ** p = 0.0048 (threshold for significance 0.017, corrected for multiple

968     comparisons). **C i** *Δplb1*-GFP Infected larvae treated with 10 µM 16, 16-dimethyl prostaglandin

969     E$_2$ or equivalent solvent (DMSO) control. At least 45 larvae per treatment group from 3

970     biological repeats. Unpaired Mann-Whitney U test used to compare between treatments *Δplb1*-

971     GFP DMSO vs 10 µM 16, 16-dm PGE$_2$ ns p = 0.98. **D i** *Δplb1*-GFP Infected larvae treated with

972     10 µM 15-keto-prostaglandin E$_2$ or equivalent solvent (DMSO) control. At least 35 larvae

973     measured per treatment group from 2 biological repeats. Unpaired Mann-Whitney U test used to

974     compare between treatments DMSO vs 10 µM 15-keto-PGE$_2$ * p = 0.0119 (threshold for

975     significance 0.017, corrected for multiple comparisons). **A ii, B ii** Representative GFP images

976     (representative = median value) H99-GFP infected larvae, at 2dpi treated with 10 µM 16,16-dm-

977     PGE$_2$ (**A ii**) or 15-keto-PGE$_2$ (**B ii**). **C ii, D ii** Representative GFP images (representative =

978     median value) *Δplb1*-GFP infected larvae, at 2dpi treated with 10 µM 16,16-dm-PGE$_2$ (**C ii**) or

979     15-keto-PGE$_2$ (**D ii**). **E** H99-GFP infected larvae treated with a combination of 3 µM AH6809

980     and 3 µM GW627368X or equivalent solvent (DMSO) control. Box and whiskers show median,

981     5$^{th}$ percentile and 95$^{th}$ percentile. At least 64 larvae measured per treatment group from 4

982     biological repeats. Mann-Whitney U test used to compare between treatments, no significance

983     found.

984

985     **Fig 4**

986     **Host derived prostaglandins are not required for growth of C. neoformans: A** Intracellular

987     proliferation quantified from timelaspe movies of J774 macrophages infected with H99-GFP or

988     *Δplb1*-GFP and treated with 1 mM Aspirin – either for 18 hours before infection (pretreatment)

989     or throughout the time course of infection. One-way ANOVA with Tukey post-test performed

990   comparing all conditions. H99-GFP DMSO vs. *Δplb1*-GFP DMSO \*\*\* p = 0.0002. H99-GFP

991   DMSO vs. *Δplb1*-GFP 1 mM (pretreat) \*\*\*\* p = <0.0001. H99-GFP DMSO vs. *Δplb1*-GFP 1 mM

992   aspirin \*\*\* p = 0.0001. H99-GFP + 1mM aspirin (pretreat) vs. *Δplb1*-GFP DMSO \*\*\*\* p <0.0001.

993   H99-GFP + 1mM aspirin (pretreat) vs. *Δplb1*-GFP 1 mM aspirin (pretreat) \*\*\*\* p < 0.0001. H99-

994   GFP + 1mM aspirin (pretreat) vs. *Δplb1*-GFP 1 mM aspirin \*\*\*\* p <0.0001. H99-GFP+ 1mM

995   aspirin vs. *Δplb1*-GFP DMSO \*\*\* p = 0.0001. H99-GFP + 1mM aspirin vs. *Δplb1*-GFP 1 mM

996   aspirin (pretreat) \*\*\*\* p <0.0001. H99-GFP+ 1mM aspirin vs. *Δplb1*-GFP 1 mM aspirin \*\*\*\* p <

997   0.0001. **B i** H99-GFP infected larvae treated with 15 µM NS-398, 15 µM SC-560 or equivalent

998   solvent (DMSO) control. Box and whiskers show median, $5^{th}$ percentile and $95^{th}$ percentile. At

999   least 25 larvae measured per treatment group from 2 biological repeats. Mann-Whitney U test

1000   used to compare between treatments, DMSO vs. 15 µM \*\*\* p = 0.0002. **B ii** *Δplb1*-GFP infected

1001   larvae treated with 15 µM NS-398, 15 µM SC-560 or equivalent solvent (DMSO) control. Box

1002   and whiskers show median, $5^{th}$ percentile and $95^{th}$ percentile. At least 34 larvae measured per

1003   treatment group from 2 biological repeats. Mann-Whitney U test used to compare between

1004   treatments, no significance found. **C** 2 dpi zebrafish larvae crispants with CRISPR knockout

1005   against Prostaglandin $E_2$ synthase (*ptges)* or a Tyrosinase control (*tyr)* infected with H99-GFP or

1006   *Δplb1*-GFP, fungal burden quantified at 0 dpi (**C i**) and 3 dpi (**C ii**) – data shown is a single

1007   experiment but is representative of N=3 experiments. One way ANOVA with Tukey post-test

1008   performed to compare each condition **C i** no significance found **C ii** H99-GFP + *tyr* [-/-] vs. H99-

1009   GFP + *ptges* [-/-] \* p = 0.0390. H99-GFP + *ptges* [-/-] vs. *Δplb1*-GFP + *tyr* [-/-] \* p = 0.0313. H99-GFP

1010   + *ptges* [-/-] vs. *Δplb1*-GFP + *ptges* [-/-] \* p = 0.0121. **D i** $PGE_2$ monoclonal EIA ELISA performed on

1011   supernatants from *C. neoformans* infected macrophages collected at 18 hr post infection. Mean

1012   concentration of $PGE_2$ (pg per $1x10^6$ cells) plotted with SD, n = 4. One-way ANOVA with Tukey

1013   post-test performed, no significance found. **D ii** LC MS/MS mass spectrometry analysis

1014   performed on cell suspensions (infected J774 cells and supernatants) collected at 18 hr post

1015   infection. Mean concentration of $PGE_2$ (pg per $1x10^6$ cells) plotted with SD, n = 3. One-way

1016    ANOVA with Tukey post-test performed, no significance found. **E** J774 cells co-infected with a

1017    50:50 mix of *Δplb1* and H99-GFP. **i** Diagrammatic representation of co-infection experiment.

1018    GFP$^+$ (green) and GFP$^-$ (yellow) *C. neoformans* cells within the phagosome were quantified at 0

1019    hr, macrophages with a burden ratio of 1:2 or 2:1 were re-analysed at 18 hr, the IPR for *Δplb1*

1020    within 2:1 and 1:2 co-infected cells were calculated by dividing the burden at 18hr by burden at

1021    0 hr for GFP$^+$ (green) or GFP$^-$ (yellow) cells. **ii** Quantification of IPR for *Δplb1* cells within

1022    *Δplb1*:H99-GFP 2:1 or 1:2 co-infected macrophages. At least 35 co-infected macrophages were

1023    analysed for each condition over 4 experimental repeats. Student's T test performed to compare

1024    ratios – 2:1 vs 1:2 * p = 0.0137.

1025

1026    **Fig 5 15-keto-PGE$_2$ promotes fungal burden by activating host PPAR- $\gamma$.**

1027    **A i** J774 macrophages treated with Troglitazone (TLT - 0.25 μM), an equivalent DMSO control

1028    or infected with H99 or *Δplb1* fixed and stained at 18 hpi with Hoechst and antibody against

1029    PPAR-γ. Nuclear localization of PPAR-γ quantified by measuring nuclear grey value of at least

1030    30 cells per condition. A single experiment is shown that is representative of n = 2. One way

1031    ANOVA with Tukey post-test used to compare all conditions. DMSO vs. TLT ** p = 0.0049.

1032    DMSO vs H99 ** p = 0.0040. TLT vs *Δplb1* * p = 0.0212. H99 vs. *Δplb1* * p = 0.0187. **A ii**

1033    Transgenic zebrafish larvae with a GFP PPAR- γ reporter treated with DMSO, 250 nM

1034    Troglitazone or 10 μM 15-keto-PGE$_2$ + 250 nM Troglitazone overnight and imaged. Lateral

1035    views of 2 dpf embryos, anterior to the left, are shown. **B** J774 murine macrophages infected

1036    with *Δplb1* or the parental strain H99. Infected cells treated with 25 μM GW9662 (a PPAR-γ

1037    antagonist) or equivalent solvent (DMSO) control.  Mean IPR from 6 biological repeats shown

1038    with error bars representing standard deviation. An unpaired two tailed Student's t-test was

1039    performed to compare each treatment group. H99 DMSO vs. H99 25 μM GW9662 * p = 0.026.

1040    **C** *Δplb1*-GFP infected larvae treated with 10 µM 15-keto-PGE$_2$, 500 nM GW9662, 10 µM 15-

1041    keto-PGE$_2$ + 500 nM GW9662 or an equivalent solvent (DMSO) control. Box and whiskers show

1042    median, 5$^{th}$ percentile and 95$^{th}$ percentile. At least 35 larvae measured per treatment group from

1043    2 biological repeats. Mann-Whitney U test used to compare between treatments. DMSO vs. 15-

1044    keto-PGE$_2$ **** $p < 0.0001$ (threshold for significance 0.025, corrected for multiple comparisons),

1045    15-keto-PGE$_2$ vs. 15-keto-PGE$_2$ + 500 nm GW9662 ** $p = 0.005$ (threshold for significance

1046    0.025, corrected for multiple comparisons) **D-F** 2 day old (2 dpf) *Nacre* zebrafish larvae injected

1047    with 500 cell inoculum. Fungal burden measured at 2 days post infection (2 dpi) by counting

1048    GFP positive pixels within each larvae.  **D i** H99-GFP Infected larvae treated with 0.55 µM

1049    Troglitazone (TLT) equivalent solvent (DMSO) control. Box and whiskers show median, 5$^{th}$

1050    percentile and 95$^{th}$ percentile. At least 55 larvae measured per treatment group over 3 biological

1051    repeats. Mann-Whitney U test used to compare between treatments, DMSO vs. 0.55 µM

1052    Troglitazone ** $p = 0.0044$ (threshold for significance 0.025, corrected for multiple comparisons).

1053    **E i** *Δplb1*-GFP infected larvae treated with 0.55 µM Troglitazone (TLT) equivalent solvent

1054    (DMSO) control. Box and whiskers show median, 5$^{th}$ percentile and 95$^{th}$ percentile. At least 35

1055    larvae measured per treatment group from 2 biological repeats. Mann-Whitney U test used to

1056    compare between treatments, DMSO vs. 0.55 µM Troglitazone ** $p = 0.0089$ (threshold for

1057    significance 0.025, corrected for multiple comparisons). **F i** *Δlac1*-GFP infected larvae treated

1058    with 0.55 µM Troglitazone (TLT) equivalent solvent (DMSO) control. At least 60 larvae

1059    measured per treatment group from 3 biological repeats. Box and whiskers show median, 5$^{th}$

1060    percentile and 95$^{th}$ percentile, DMSO vs. 0.55 µM Troglitazone * $p = 0.01$ (threshold for

1061    significance 0.025, corrected for multiple comparisons). **D ii, E ii and F ii,** Representative GFP

1062    images (representative = median value) *C. neoformans* infected larvae, at 2 dpi treated with

1063    0.55 µM Troglitazone (TLT) **D ii -** H99-GFP, **E ii -** *Δplb1*-GFP and **F ii** *Δlac1*-GFP.

1064    **Supplementary Fig 1**

1065    **A** Quantification of the vialibility of *C. neoformans* retrieved from the phagosomes of J774

1066    macrophages at 2 hr and 20 hr post infection. Prior to, and during infection J774 macrophages

1067    were treated with 2 mM $PGE_2$ or the equivalent amount of solvent (Ethanol) *Cryptococcus* cells

1068    were counted with a hemocytometer / diluted and plated to give an expected number of 200

1069    CFU - dead *Cryptococcus* cells are indistinguishable from live cells when counting with a

1070    hemocytometer however a lower than expected CFU count would indicate that there is a

1071    decrease in viability. Data is displayed as the fold change between the CFU count at 2 hpi and

1072    20 hpi for each condition. A one-way ANOVA with Tukey post test was performed comparing all

1073    conditions. H99 ETOH vs. *Δplb1* ** p = 0.0052, H99 ETOH vs. *Δplb1* ETOH ** p = 0.0056, H99

1074    ETOH vs. *Δplb1* 2 mM $PGE_2$ * p = 0.029. **B i** Comparison of fungal burden between H99-GFP,

1075    *Δplb1*-GFP and *Δlac1*-GFP infected larvae (Data reproduced from Fig2 Ai, Di and

1076    Supplementary Fig 2 Bi for clarity) H99-GFP, *Δplb1*-GFP and *Δlac1*-GFP infected larvae imaged

1077    at 0, 1, 2 and 3 dpi. At least 50 larvae measured per time point from 3 biological repeats. Box

1078    and whiskers show median, 5th percentile and 95th percentile. Unpaired Mann-Whitney U tests

1079    used to compare the burden between each strain for every time point. **B ii** Table of Mann-

1080    Whitney U tests comparing burden for each strain between time points. **C** Whole body

1081    macrophage counts of zebrafish larvae treated at 2 dpf with 10 μM $PGE_2$, 10 μM 15-keto-$PGE_2$

1082    or an equivalent DMSO control for 2 days. Box and whiskers show median, 5th percentile and

1083    95th percentile. At least 12 larvae quantified per treatment group per biological repeat n = 4.

1084    Mann-Whitney U test used to treatments to DMSO control ** p = 0.0025.

1085    **Supplementary Fig 2**

1086    **A** J774 cells co-infected with a 50:50 mix of *Δlac1*-GFP and H99. Quantification of IPR for

1087    *Δlac1*-GFP cells within *Δlac1*-GFP:H99 2:1 or 1:2 co-infected macrophages. At least 20 co-

1088    infected macrophages were analysed for each condition over 4 experimental repeats. Student's

1089    T test performed to compare ratios – 2:1 vs 1:2 * p = 0.012. **B i** *Δlac1*-GFP infected larvae

44

1090   imaged at 0, 1, 2 and 3 dpi. Fungal burden measured by counting GFP positive pixels in each

1091   larvae. At least 78 larvae measured per time point across 3 biological repeats. Box and

1092   whiskers show median, 5th percentile and 95th percentile. Unpaired Mann-Whitney U tests used

1093   to compare the burden between each strain for every time point, for p values see

1094   (Supplementary Fig2 A + B). **B ii** Representative GFP images (representative = median value)

1095   of 2dpi *Δlac1*-GFP infected larvae, untreated at 0,1,2,3 dpi **C i** *Δlac1*-GFP Infected larvae

1096   treated with 10 μM prostaglandin $E_2$ or equivalent solvent (DMSO) control. At least 70 larvae

1097   measured per treatment group across 4 biological repeats. Box and whiskers show median, 5th

1098   percentile and 95th percentile. Unpaired Mann-Whitney U test used to compare between

1099   treatments, DMSO vs. 10 μM $PGE_2$ * p = 0.035. **D i** *Δlac1*-GFP Infected larvae treated with 10

1100   μM 16,16-dimethyl prostaglandin $E_2$ or equivalent solvent (DMSO) control. At least 75 larvae

1101   measured per treatment group across 4 biological repeats. Box and whiskers show median, 5th

1102   percentile and 95th percentile. Unpaired Mann-Whitney U test used to compare between

1103   treatments, DMSO vs. 10 μM 16,16-dimethyl prostaglandin $E_2$ ns p = 0.062. **E i** *Δlac1*-GFP

1104   Infected larvae treated with 10 μM 15-keto-prostaglandin $E_2$ or equivalent solvent (DMSO)

1105   control. At least 58 larvae measured per treatment group across 3 biological repeats. Unpaired

1106   Mann-Whitney U test used to compare between treatments DMSO vs. 10 μM 15-keto-

1107   prostaglandin $E_2$ ns p= 0.50. **C ii, D ii, E ii** Representative GFP images (representative =

1108   median value) *Δlac1*-GFP infected larvae, at 2 dpi treated with 10 μM $PGE_2$ (**C ii**), 16,16-dm-

1109   $PGE_2$ (**D ii**) or 15-keto-$PGE_2$ (**E ii**).

1110

1111   **Supplementary Fig 3**

1112   **A** $PGE_2$ monoclonal EIA ELISA performed on supernatants from *C. neoformans* infected

1113   macrophages collected at 18 hr post infection. Mean concentration of $PGE_2$ (pg per $1x10^6$ cells)

1114   plotted with SD, n = 2**. B** Quantification of IPR for *Δplb1* cells within *Δplb1*:H99-GFP co-infected

1115    macrophages at initial burdens of 2:1, 3:1, 4:1 and vice versa. N=4. Student's T test performed

1116    to compare ratios – 2:1 vs 1:2 * p = 0.0137. **C** Example images of immunofluorescence

1117    experiments in J774 macrophages staining for PPAR-gamma nuclear localization (for

1118    quantification see Fig 5 Ai). J774 cells treated with DMSO, 0.25 µM Troglitazone, infected with

1119    H99-GFP or *Δplb1*-GFP at x60 magnification, scale bar = 10 µM. Images provided are from the

1120    Cy3 channel (PPAR-γ) and the corresponding cell in DIC. The area of the nuclei is marked with

1121    a white dotted line.

1122

1123    **Supplementary Fig 4**

1124

1125    Genotyping to confirm zebrafish *ptges* CRISPR. Zebrafish were genotyped post assay (5 dpf),

1126    an area of genomic DNA spanning the *ptges* gene ATG site (the CRISPR target) was amplified

1127    with PCR to produce a 345 bp product. This product was digested with MwoI to produce

1128    genotype specific banding patterns  **A.** Schematic of the banding patterns expected for each

1129    genotype following MwoI digestion – 1. Undigested product, a single 345 bp band 2. Wild type

1130    genotype, 184, 109 and 52 bp bands 3. Hetrozygous genotype (*ptges* $^{+/-}$) 293, 184, 109 and 52

1131    bp bands 4. Homozygous genotype (*ptges* $^{-/-}$) strong bands for 293 and 52 bp, weaker bands at

1132    184 and 109 bp can sometimes be seen indicating a small amount of wildtype *ptges* is still

1133    present (this is thought to be beneficial as low levels of *ptges* are required for larvae survival. **B**

1134    Genotyping for H99 infected larvae, L = DNA ladder (NEB 50 bp ladder), wt = undigested wild

1135    type control, wt dig = wild type digested control, numbers correspond to individual larvae

1136    genotyped. H99 + tyro = *tyr* $^{-/-}$ larvae infected with H99-GFP. H99 + ptges = *ptges* $^{-/-}$ larvae

1137    infected with H99-GFP **C** Genotyping for *Δplb1* infected larvae , L = DNA ladder (NEB 50 bp

1138    ladder), wt = undigested wild type control, wt dig = wild type digested control, numbers

1139    correspond to individual larvae genotyped. *Δplb1*+ tyro = *tyr* $^{-/-}$ larvae infected with *Δplb1*-GFP.

1140    *Δplb1*+ ptges = *ptges* $^{-/-}$ larvae infected with *Δplb1*-GFP.

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**H99-GFP**

**A i** — GFP+ pixels per fish vs 0 dpi, 1 dpi, 2 dpi, 3 dpi

**B i** — GFP+ pixels per fish; DMSO 2 dpi, 10 µM PGE₂ 2 dpi *

**C i** — GFP+ pixels per fish; DMSO 2 dpi, 10 µM PGD₂ 2 dpi

**i** — 0 dpi, 1 dpi, 2 dpi, 3 dpi

**ii** — DMSO, PGE₂

**ii** — DMSO, 10 µM PGD₂

**Δplb1-GFP**

**D i** — GFP+ pixels per fish vs 0 dpi, 1 dpi, 2 dpi, 3 dpi

**E i** — GFP+ pixels per fish; DMSO 2 dpi, 10 µM PGE₂ 2 dpi ***

**F i** — GFP+ pixels per fish; DMSO 2 dpi, 10 µM PGD₂ 2 dpi

**ii** — 0 dpi, 1 dpi, 2 dpi, 3 dpi

**ii** — DMSO, PGE₂

**ii** — DMSO, 10 µM PGD₂

**A** i — H99-GFP

GFP+ pixels per fish

DMSO 2 dpi | 10 µM 16,16-dm PGE₂ 2 dpi

ii

DMSO / 16,16-dm-PGE₂

**B** i

GFP+ pixels per fish

**

DMSO 2 dpi | 10 µM 15-keto-PGE₂ 2 dpi

ii

DMSO / 15-keto-PGE₂

Δplb1-GFP

**C** i

GFP+ pixels per fish

DMSO 2 dpi | 10 µM 16,16-dm PGE₂ 2 dpi

ii

DMSO / 16,16-dm-PGE₂ / H99-GFP

**D** i

GFP+ pixels per fish

*

DMSO 2 dpi | 10 µM 15-keto-PGE₂ 2 dpi

ii

DMSO / 15-keto-PGE₂

**E**

GFP+ pixels per fish

DMSO 2 dpi | 3 µM AH6809 + 3 µM GW627368X 2 dpi

**A i**

Nuclear plateau of intensity (grey value) vs Condition
- DMSO
- 0.25 μM TLT
- H99
- Δ*plb1*

** * ** * *

**A ii**

DMSO

10 μM 15-keto PGE$_2$

0.25 μM TLT

0.25 μM TLT +
10 μM 15-keto PGE$_2$

**B**

Mean IPR
- H99 DMSO
- H99 25 μM GW9662
- Δ*plb1* DMSO
- Δ*plb1* 25 μM GW9662

*

**C**

Δ*plb1*-GFP

GFP$^+$ pixels per fish
- DMSO
- 15-keto PGE$_2$ 10 μM
- GW9662 500 nM
- 15-keto PGE$_2$ + GW9662

*** *

H99-GFP    Δ*plb1*-GFP    Δ*lac1*-GFP

**D i**

GFP$^+$ pixels per fish
- DMSO 2 dpi
- 0.55 μM Troglitazone 2 dpi

**

**E i**

GFP$^+$ pixels per fish
- DMSO 2 dpi
- 0.55 μM Troglitazone 2 dpi

**

**F i**

GFP$^+$ pixels per fish
- DMSO 2 dpi
- 0.55 μM Troglitazone 2 dpi

*

**D ii**

DMSO

TLT

**E ii**

DMSO

TLT

**F ii**

DMSO

TLT