Author summary

Cryptococcus neoformans is an opportunistic fungal pathogen that is responsible for significant numbers of deaths in the

neoformans is able to manipulate the host inflammatory state during infection by directly manipulating host eicosanoid signaling.

It has previously been reported that the inhibition of prostaglandin E_2 receptors EP2 and EP4 during murine pulmonary infection leads to better host survival accompanied by a shift towards Th1/M1 macrophage activation, however it was not determined if PGE_2 was derived from the host or the fungus [20]. Therefore, a key aspect of *C. neoformans* pathogenesis remains unanswered: do eicosanoids produced by *C. neoformans* manipulate host innate immune cells function during infection?

We have previously shown that the eicosanoid deficient strain *Δplb1* has reduced proliferation and survival within macrophages [21]. We hypothesised that eicosanoids produced by *C. neoformans* support intracellular proliferation within macrophages and subsequently promote pathogenesis. To address this hypothesis, we combined *in vitro* macrophage infection assays with our previous published *in vivo* zebrafish model of cryptococcosis [22]. We found that PGE_2 was sufficient to promote growth of *Δplb1* and *Δlac1* independent of host PGE_2 production, *in vitro* and *in vivo*. We show that the effects of PGE_2 in cryptococcal infection are mediated by its dehydrogenated form, 15-keto-PGE_2. Finally, we determine that 15-keto-PGE_2 promotes *C. neoformans* infection via the activation of the host nuclear transcription factor PPAR-γ, demonstrating that 15-keto-PGE_2 and PPAR-γ are new factors in cryptococcal infection.

Results

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

We have previously shown that the *C. neoformans* mutant strain *Δplb1* has impaired proliferation and survival within J774 murine macrophages *in vitro* [21]. The *Δplb1* strain has a deletion in the *PLB1* gene which codes for the secreted enzyme phospholipase B1 [23]. The *Δplb1* strain is known to produce lower levels of fungal eicosanoids indicating that phospholipase B1 is involved in fungal eicosanoid synthesis [19]. It has been proposed that the attenuation of this strain within macrophages could be because it cannot produce eicosanoids [19]. A previous study has identified PGE_2 as an eicosanoid that promotes cryptococcal virulence and manipulates macrophage activation, however this study did not determine if PGE_2 was produced by the host or *C. neoformans* [20]. We hypothesised that PGE_2Ð or other phospholipase B1 derived eicosanoid

actual number of CFUs produced±a difference between the expected CFU count (200 CFU) and the actual CFU count indicates a loss of *Cryptococcus* cell viability. In this case viability assays showed that exogenous PGE_2 produced no significant increase in the viability of *Δplb1* cells within the phagosome (S1A Fig).

Exogenous prostaglandin E_2 rescues in vivo growth

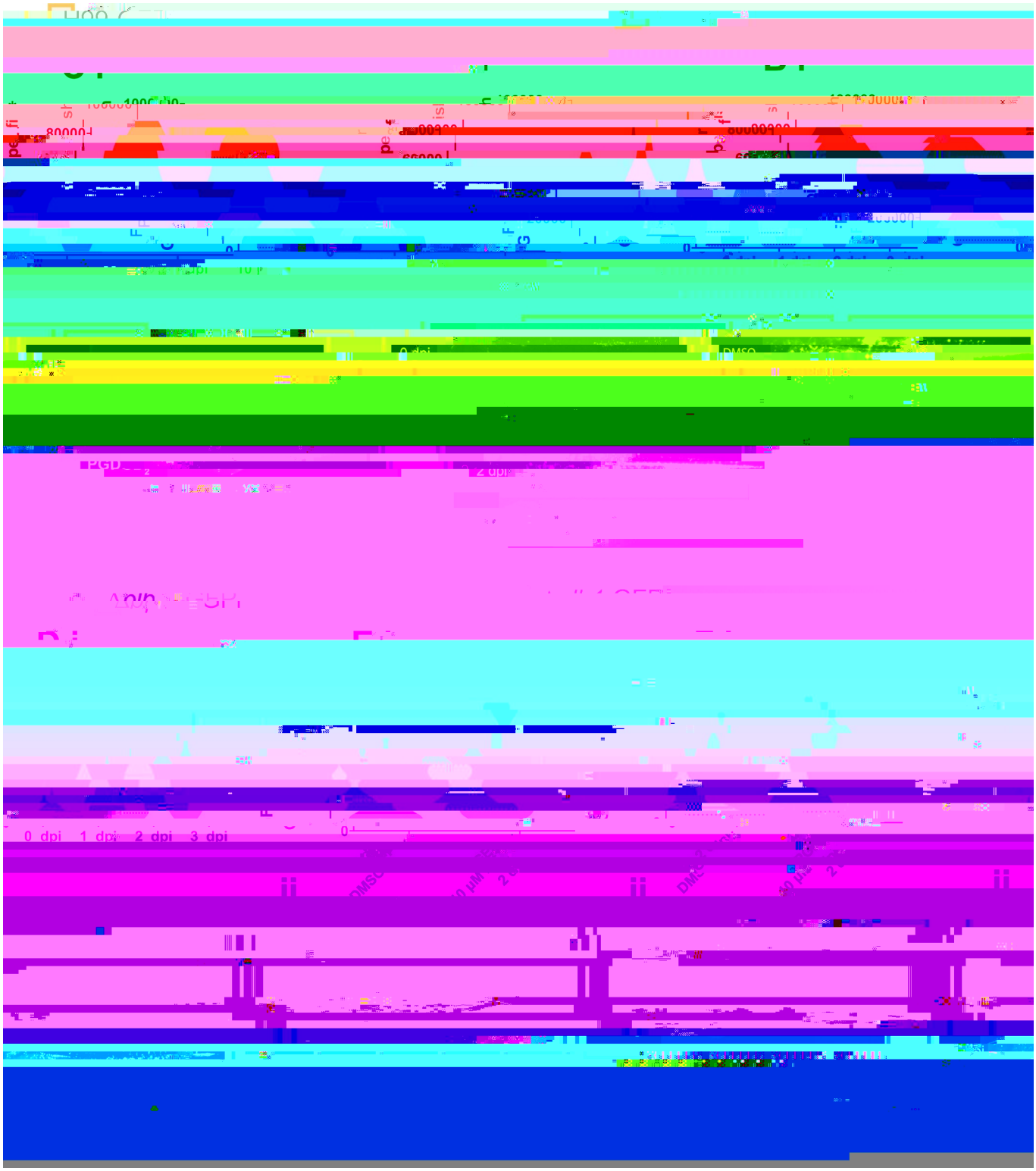
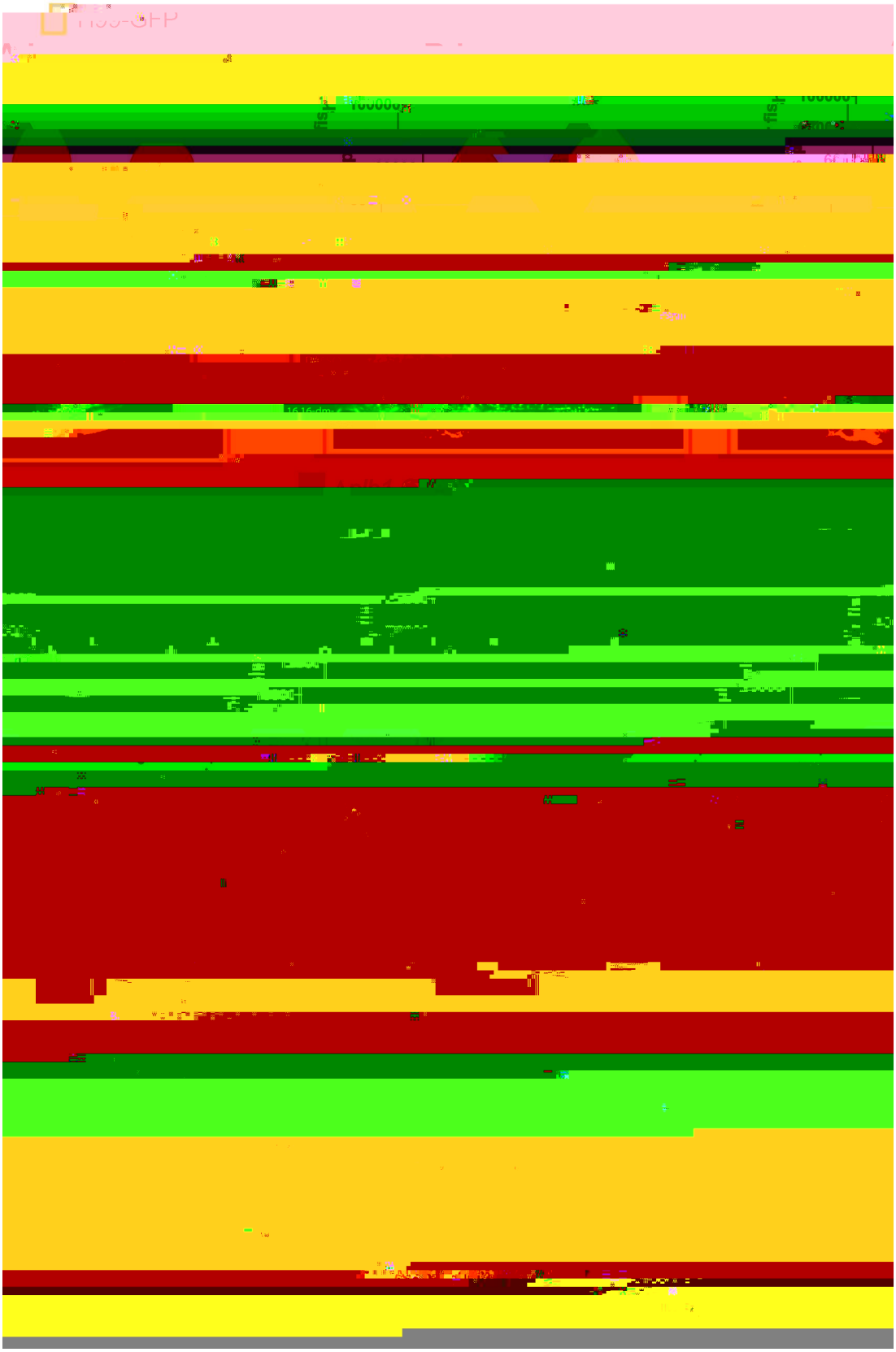
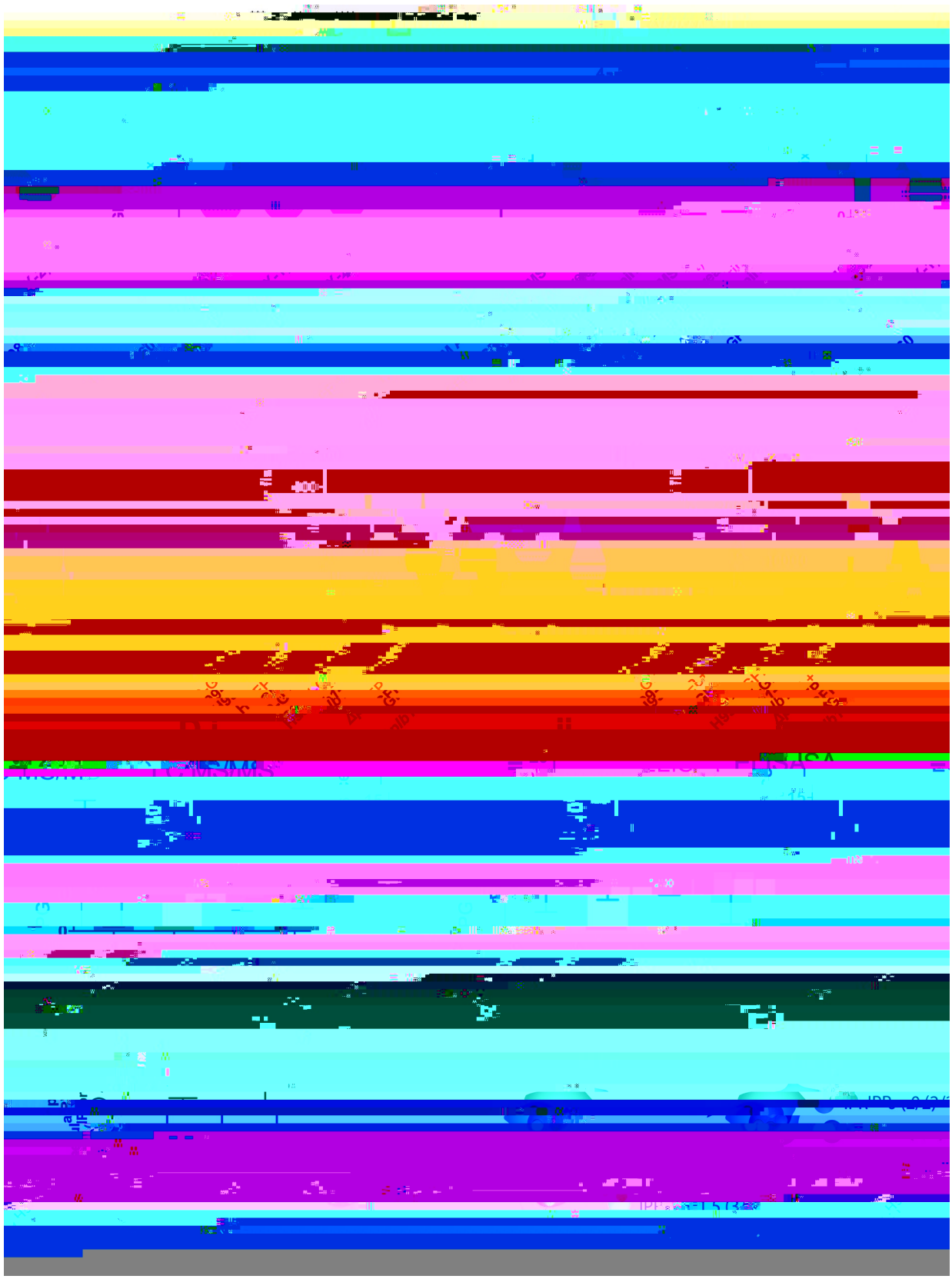


Fig 2. The prostaglandin E_2 dependent growth defect of ***plb1*** is also present ***in vivo***. A i H99-GFP infected larvae imaged at 0, 1, 2 and 3 dpi. At least 50 larvae measured per time point across 3 biological repeats. Box and whiskers show median, 5th percentile and 95th percentile. Unpaired Mann-Whitney U tests used to compare the burden between each strain for every time point, for p values see (S1Bii Fig). B i± H99-GFP Infected larvae treated with 10 μM prostaglandin E_2 or equivalent solvent (DMSO) control. At least 60 larvae measured per treatment group from 3 biological repeats. Box and whiskers show median, 5th percentile and 95th percentile. Unpaired Mann-Whitney U tests used to compare between treatments DMSO vs. 10 μM PGE_2 p = 0.0137 (threshold for

cryptococcal infection *in vivo*



This could affect macrophage number and subsequently fungal burden. We have previously observed that a large depletion of macrophages can lead to increased fungal burden in zebrafish larvae [22]. We performed whole body macrophage counts on 2 dpf uninfected larvae treated with PGE_2 or 15-keto-PGE_2 2 days post treatment (the same time points used in our infection assay).

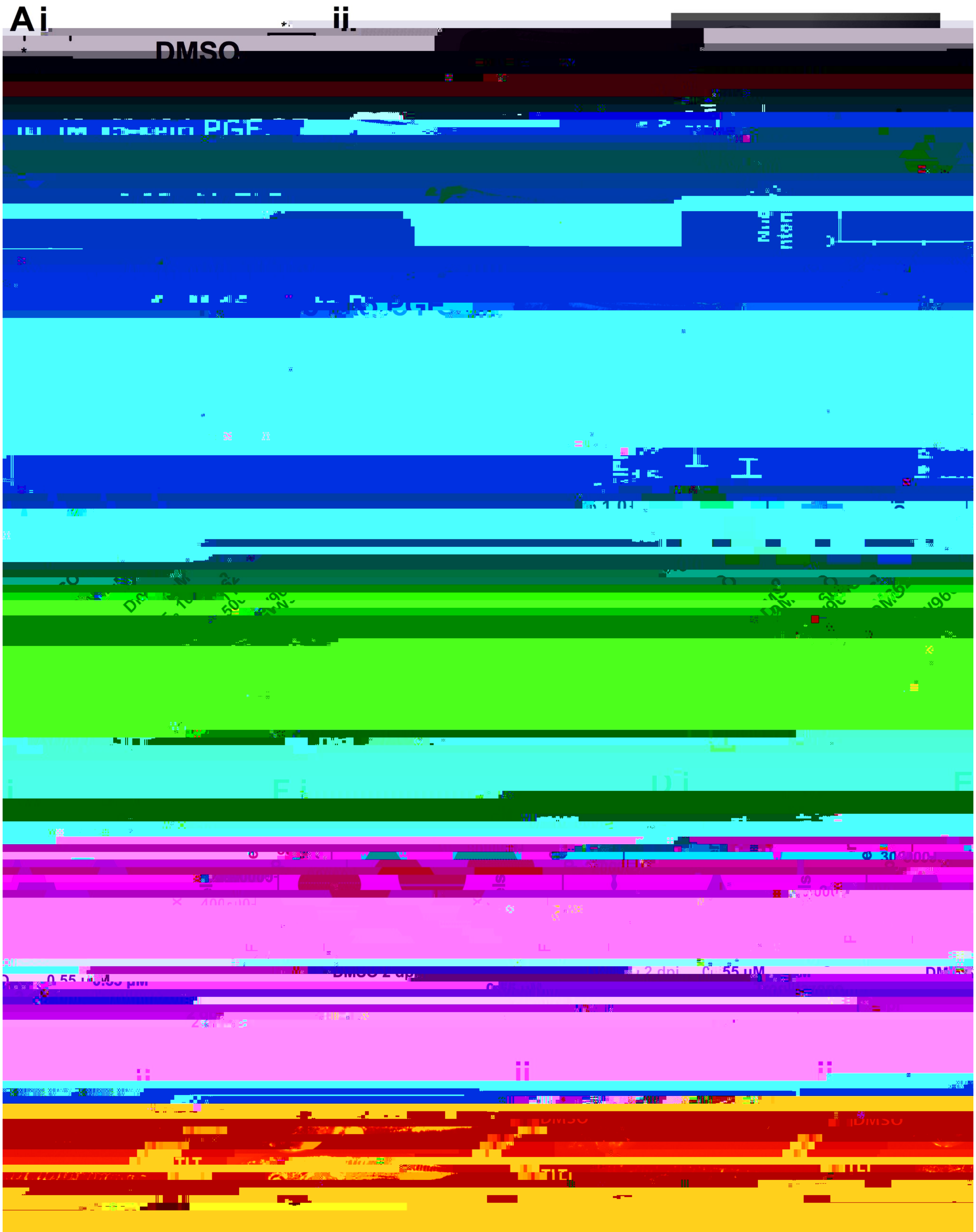


aspirin because we found that aspirin treatment led to lethal developmental defects in zebrafish larvae (unpublished observation). We infected 2 dpf zebrafish larvae with H99-GFP and *Δplb1*-GFP and treated with inhibitors for COX-1 (NS-398, 15 μM) and COX-2 (SC-560, 15 μM). We found that both inhibitors decreased the fungal burden of H99-GFP, but not *Δplb1*-GFP infected zebrafish larvae (Fig 4Bi and 4Bii, H99-GFP Ð NS-398, p = 0.0002, 1.85-fold decrease vs. DMSO. SC-560 p = <0.0001, 3.14-fold decrease vs DMSO). These findings were different to what we had observed *in vitro* but because this phenotype was phospholipase B1 dependent we reasoned that these inhibitors could be having off target effects on *C. neoformans*.*C. neoformans* does not have a homolog to cyclooxygenase however other studies have tried to inhibit eicosanoid production in *Cryptococcus* using cyclooxygenase inhibitors but their efficacy and target remain uncertain [17, 28]. To support our pharmacological evidence, we used a CRISPR/Cas9-mediated knockdown of the prostaglandin E_2 synthase gene (*ptges)* [29]. We used a knockdown of tyrosinase (*tyr*)±a gene involved in the conversion of tyrosine into melanin as a control because *tyr*$^{-/-}$ crispants are easy to identify because they do not produce any pigment. We infected 2 dpf *ptges*$^{-/-}$ and *tyr*$^{-/-}$ zebrafish larvae with H99-GFP or *Δplb1*-GFP and measured the fungal burden at 3 dpi. We found that *ptges*$^{-/-}$ zebrafish infected with H99-GFP had a higher fungal burden at 3 dpi compared to *tyr*$^{-/-}$ zebrafish infected with H99-GFP whereas there was no difference between *ptges*$^{-/-}$ and *tyr*$^{-/-}$ zebrafish larvae infected with *Δplb1*-GFP (Fig 4C). Thus, both pharmacological and genetic inhibitions of host prostaglandin synthesis were not determinants of C. neoformans growth.

Phospholipase B1 dependent factors are sufficient to support ***plb1*** growth in macrophages

To further evidence that *C.*

to the nucleus upon activation, therefore cells where PPAR-γ is activated should have increased nuclear staining for PPAR-γ. We found that J774 macrophages



To confirm our observations that *C. neoformans* was the source of PGE_2 during infection we performed co-infection assays with H99 wild type cryptococci (eicosanoid producing) and *Δplb1* (eicosanoid deficient) within the same macrophage and found that co-infection was sufficient to promote the intracellular growth of *Δplb1*. We also observed similar interactions during *Δlac1* co-infection (a second eicosanoid deficient *C. neoformans* mutant). These observations agree with previous studies that suggest eicosanoids are virulence factors produced by *C. neoformans* during macrophage infection.

We also found that activation of PPAR-γ alone was sufficient to mediate cryptococcal virulence. In this respect, we

Cryptococcus strains were grown for 18 hours at 28 ^{O}C, rotating horizontally at 20 rpm. *Cryptococcus* cultures were pelleted at 3300g for 1 minute, washed twice with PBS (Oxoidm Basingstoke, UK) and re-suspended in 1ml PBS. Washed cells were then counted with a haemocytometer and used as described below.

C. neoformans transformation

C. neoformans strains Δ*plb1* and Δ*lac1* were biolistically transformed using the pAG32_GFP transformation construct as previously described for H99-GFP [42]. Stable transformants were identified by passaging positive GFP fluorescent colonies for at least 3 passages on YPD agar supplemented with 250 μg/ml Hygromycin B.

Zebrafish CRISPR

CRISPR generation was performed as previously described [29]. Briefly gRNA spanning the ATG start codon of zebrafish *ptges* or *tyr* was injected along with Cas9 protein and tracrRNA into zebrafish embryos at the single cell stage. Crispant larvae were infected with *C. neoformans* as described above at 2 dpf. The genotype of each larvae was confirmed post assay±genomic DNA was extracted from each larvae and the ATG was PCR amplified with primers spanning the ATG site of *ptges* (Forward primer gccaagtataatgaggaatggg, Reverse primer aatgtttggattaaacgcgact) producing a 345-bp product. This product was digest with MwoI±wild-type digests produced bands at 184, 109 and 52 bp while mutant digests produced bands at 293 and 52 bp (S4 Fig).

J774 Macrophage infection±with exogenous PGE_2 treatment

To assess the viability of *C. neoformans* cells recovered from macrophages we used our previously published colony forming unit (CFU) viability assay [21]. Lysates from *C. neoformans* infected J774 cells were prepared from cells at 0hr and 18hr time points. The concentration of *C. neoformans* cells in the lysate was calculated by haemocytomter counting, the lysates were then diluted to give an expected concentration of $2x10^3$

performed on a Nikon Eclispe Ti microscope with a x60 DIC objective. Cells were imaged with filter sets for Cy3 (PPAR-γ, 500ms exposure) GFP (*Cryptococcus*, 35 ms exposure) and DAPI (Nuclei, 5ms) dyes in addition to DIC.

The intensity of nuclear staining was analysed for at least 30 cells per coverslip, using ImageJ 2.0.0 a line ROI was drawn from the outside of cell, through the nucleus measuring the mean grey value along the line. For *Cryptococcus* infected conditions uninfected and infected cells were measured separately upon the same coverslip using the GFP channel to distinguish between infected and uninfected cells.

J774 aspirin timelapse

Macrophages were seeded at 10^5 per ml into 24 well plates as described above. After two hours cells requiring aspirin were treated with 1 mM aspirin in DMSO in fresh DMEM. Cells were then incubated overnight for 18 hours at 37 ^oC 5% CO_2. H99-GFP and Δ*plb1*-GFP were prepared at 10^6 cells per ml as described above, and opsonised with 18B7 for one hour. J774s were then infected with the fungal cells in fresh serum free DMEM for two hours before removing the supernatant, washing three times in PBS, and adding fresh serum free DMEM. Cells were imaged for 18 hours on a Nikon Eclispe Ti equipped with a climate controlled stage (Temperature– 37 ^oC, Atmosphere– 5% CO_2 / 95% air) with a x20 Lambda Apo NA 0.75 phase contrast objective brightfield images were taken at an interval of 2 minutes, 50 ms exposure. Analysis was performed by manual counts of intracellular and extracellular cryptococci.

Zebrafish infection

Zebrafish fungal burden measurement

Individual infected zebrafish embryos were placed into single wells of a 96 well plate (VWR) with 200 ul or E3 (unsupplemented E3, or E3 supplemented with eicosanoids / drugs depending on the assay). Infected embryos were imaged at 0-days post infection (dpi), 1 dpi, 2 dpi and 3 dpi in their 96 well plates using a Nikon Ti-E with a CFI Plan Achromat UW 2X N.A 0.06 objective lens. Images were captured with a Neo sCMOS (Andor, Belfast, UK) and NIS Elements (Nikon, Richmond, UK). Images were exported from NIS Elements into Image J FIJI as monochrome tif files. Images were threshholded in FIJI using the `moments' threshold preset and converted to binary images to remove all pixels in the image that did not correspond to the intensity of the fluorescently tagged *C. neoformans*. The outline of the embryo was traced using the `polygon' ROI tool, avoiding autofluorescence from the yolk sac. The total number of pixels in the threshholded image were counted using the FIJI `analyse particles' function, the `total area' measurement from the `summary' readout was used for the total number of GFP^+ pixels in each embryo.

PPAR-**γ** GFP reporter fish treatment

PPARγ embryos [35,

left for 24 hours total. At 24 hours all wells received fresh serum free media. Wells requiring aspirin for the duration received 1 mM aspirin in DMSO. Aspirin treated cells requiring arachidonic acid were treated with 30 µg per ml arachidonic acid in ethanol. Control wells received the following: either 1% DMSO, 30 µg per ml arachidonic acid, or ethanol. Cells were again left at 37 ^{O}C 5% CO_2 for 18 hours.

Supernatants were then removed and frozen at -80 ^{O}C until use. Supernatants were analysed as per the PGE_2 EIA ELISA kit instructions (Cayman Chemical).

Eicosanoid measurement (mass spectrometry)

J774 macrophages were seeded into T25 tissue culture flasks at a concentration of $1.3x10^6$ cells per flask and incubated for 24 hours at 37 ^{O}C 5% CO_2. J774 cells were infected with *C. neoformans* as described above, at the same MOI 1:10 and incubated for 18 hours with 2 ml serum free DMEM. At 18 hours post infection infected cells were scraped from the flask with a cell scraper into the existing supernatant and immediately snap frozen in ethanol / dry ice slurry. All samples were stored at -80 ^{O}C before analysis.

Lipids and lipid standards were purchased from Cayman Chemical (Ann Arbor, Michigan). Deuterated standard Prostaglandin E_2-d_4 (PGE_2-d_4), 98% deuterated form. HPLC grade solvents were from Thermo Fisher Scientific (Hemel Hempstead, Hertfordshire UK).

Lipids were extracted by adding a solvent mixture (1 mol/L acetic acid, isopropyl alcohol, hexane (2:20:30, v/v/v)) to the sample at a ratio of 2.5±1 ml sample, vortexing, and then adding 2.5 ml of hexane [57]. Where quantitation was required, 2 ng PGE_2-d_4, was added to samples before extraction, as internal standard. After vortexing and centrifugation, lipids were recovered in the upper hexane layer. The samples were then re-extracted by addition of an equal volume of hexane. The combined hexane layers were dried and analyzed for Prostaglandin E_2 (PGE_2) using LC-MS/MS as below.

Lipid extracts were separated by reverse-phase HPLC using a ZORBAX RRHD Eclipse Plus 95Å C18, 2.1 x 150 mm, 1.8 µm column (Agilent Technologies, Cheshire, UK), kept in a column oven maintained at 45ÊC. Lipids were eluted with a mobile phase consisting of A, water-B-acetic acid of 95:5:0.01 (vol/vol/vol), and B, acetonitrile-methanol-acetic acid of 80:15:0.01 (vol/vol/vol), in a gradient starting at 30% B. After 1 min that was ramped to 35% over 3 min, 67.5% over 8.5 min and to 100% over 5 min. This was subsequently maintained at 100% B for 3.5 min and then at 30% B for 1.5 min, with a flow rate of 0.5 ml/min. Products were monitored by LC/MS/MS in negative ion mode, on a 6500 Q-Trap (Sciex, Cheshire, United Kingdom) using parent-to-daughter transitions of *m/z* 351.2 ! 271.2 (PGE_2), and *m/z* 355.2 ! 275.2 for PGE_2-d_4. ESI-MS/MS conditions were: TEM 475ÊC, GS1 60, GS2 60, CUR 35, IS -4500 V, dwell time 75 s, DP -60 V, EP -10 V, CE -25 V and CXP at -10 V. PGE_2 was quantified using standard curves generated by varying PGE_2 with a fixed amount of PGE_2-d_4.

Supporting

H99 ETOH vs. *Δplb1* 2 mM PGE_2 p = 0.029. B i Comparison of fungal burden between H99-GFP, *Δplb1*-GFP and *Δlac1*-GFP

S4 Fig. Genotyping to confirm zebrafish ***ptges*** CRISPR. Zebrafish were genotyped post assay (5 dpf), an area

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