Fungal derived 15-keto-prostaglandin E_2 and host proliferator-activated receptor gamma (PPAR-γ) promote *C. neoformans* growth during infection.

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Running Title: Fungal derived 15-keto-prostaglandin E_2 and PPAR-γ promotes *C. neoformans* infection

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Abstract

Cryptococcus neoformans is one of the leading causes of invasive fungal infection in humans worldwide. *C. neoformans* can use 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 a key role for eicosanoid lipid mediators produced by *C. neoformans* in regulating host responses. *C. neoformans* is known to secrete several eicosanoids that are highly similar to those found in vertebrate hosts. Using the eicosanoid deficient cryptococcal mutant *Δplb1*, we demonstrate that prostaglandin E_2 is required by *C. neoformans* for proliferation within macrophages and, using our zebrafish model of cryptococcosis, we confirm this role for PGE_2 *in vivo*. Furthermore, we show that PGE_2 must be dehydrogenated into 15-keto PGE_2 to promote fungal growth. We find that activation of the intracellular 15-keto PGE_2 receptor PPAR-γ promotes fungal burden in zebrafish suggesting that cryptococcal 15-keto-PGE_2 is a novel virulence factor that may act as an agonist for PPAR-γ.

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 a number of eicosanoids that are notable for their similarity to vertebrate eicosanoids, it is therefore possible that fungal-derived eicosanoids may mimic 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 growth during infection. We subsequently find that prostaglandin E_2 must be converted to 15-keto prostaglandin E_2 within the host before it has these effects. Furthermore, we provide evidence that the mechanism of prostaglandin E_2/15-keto prostaglandin E_2 mediated virulence is via activation of host PPAR-γ – an intracellular eicosanoid receptor known to interact with 15-keto PGE_2.

Introduction

Cryptococcus neoformans is an opportunistic pathogen and that infects individuals who have severe immunodeficiencies such as late-stage HIV infection. *C. neoformans* is estimated to infect up to 1 million individuals each year globally and causes hundreds of thousands of deaths (1,2). *C. neoformans* infection begins as a respiratory infection but in the absence of an effective immune response the fungus disseminates to the central nervous system causing meningitis, and eventually death (3,4). *C. neoformans* initially grows within the alveolar spaces of the lungs as budding yeast before it is phagocytosed by macrophages. In normal immunity, macrophages must become activated by further inflammatory signals from the host immune system before they can effectively kill *C. neoformans* (5,6). When this does not occur macrophages cannot kill *C. neoformans;* instead the fungus is able to proliferate rapidly intracellularly and may use macrophages to disseminate to the central nervous system where it causes fatal meningitis (7-9).

An important group of inflammatory mediators produced during the initial stages of microbial infection by macrophages are eicosanoids. Eicosanoids are a diverse group of potent lipid signalling molecules that have a short range of action and communicate through autocrine or paracrine routes. During infection macrophages produce large amounts of a particular group of eicosanoids called prostaglandins (10,11). Prostaglandins have a number of physiological effects throughout the body, but in the context of immunity they are known to strongly influence the inflammatory state(12). Two particular prostaglandins - PGE_2 and PGD_2 – are the best-studied eicosanoid inflammatory mediators. During infection macrophages produce both PGE_2 and PGD_2 and, via autocrine routes, macrophages are highly responsive to PGE_2 and PGD_2 (12). In vertebrate immunity, the synthesis of eicosanoids such as PGE_2 is carefully regulated by feedback loops to ensure that the potent effects of these molecules are properly constrained. Exogenous sources of eicosanoids within the body, such as from eicosanoid-producing parasites (13) or tumours that overproduce eicosanoids (14,15), disrupt host inflammatory signaling as they are not subject to the same regulation.

During infection, *C. neoformans* produces a range of eicosanoid species which are indistinguishable from those produced by their vertebrate hosts (16-18). Currently only two *C. neoformans* enzymes - phospholipase B1 and laccase - are known to be associated with cryptococcal eicosanoid synthesis (18,19).

Deletion of phospholipase B1 reduces secreted levels of all eicosanoids produced by *C. neoformans* suggesting that it has high level role in eicosanoid synthesis (19), perhaps fulfilling the role of phospholipase A_2 in higher organisms. In contrast, it is possible that the cryptococcal enzyme has putative PGE_2 synthase activity (18). The synthesis of eicosanoids by *C. neoformans* raises the possibility that the fungus is able to manipulate host inflammation by directly manipulating host eicosanoid signaling during establishment of infection *in vivo*. During respiratory infection of mice, the inhibition of prostaglandin E_2 receptors EP2 and EP4 leads to better host survival accompanied by a shift towards Th1/M1 macrophage activation (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 hypothesised that eicosanoids produced by *C. neoformans* were able to promote intracellular proliferation within macrophages and virulence in the host. We have previously shown that the phospholipase B1 deficient strain *Δplb1,* that lacks eicosanoid synthesis, cannot replicate or survive within macrophages (21). Thus, we sought to determine whether this deficiency is directly due to lower levels of eicosanoids produced by *C. neoformans*. To address this question we have combined *in vitro* macrophage infection assays with our previous published *in vivo* zebrafish model of cryptococcosis (22). Using this approach, we show that prostaglandin E_2 is sufficient to promote growth of the *Δplb1* during *in vitro* infection of macrophages. We find that this eicosanoid is also able to promote growth of *C. neoformans in vivo* but activity requires the dehydrogenation of PGE_2 into 15-keto PGE_2, and the activity of the 15-keto PGE_2 receptor PPAR-γ.

105 **Results**

106 *C. neoformans prostaglandin E_2 is required for growth in macrophages*

107 We have shown previously that the *C. neoformans PLB1* gene (coding for the secreted enzyme
108 phospholipase B1) deletion mutant *Δplb1* (23) has impaired proliferation and survival during infection of
109 J774 murine macrophages *in vitro* (21). It has been proposed that the attenuation of *Δplb1* could be because
110 this strain cannot produce eicosanoids such as PGE_2 which could be used by *C. neoformans* to interfere
111 with macrophage activation (19).
112
113 To establish if *C. neoformans* requires PGE_2 synthesis for growth within macrophages we investigated
114 whether the intracellular proliferation defect of *Δplb1* could be rescued with the addition of exogenous PGE_2
115 to infected cells. We found that the addition of exogenous PGE_2 to *Δplb1* infected J774 macrophages was
116 sufficient to partially recover the intracellular proliferation rate (IPR) of *Δplb1* compared to its parental strain
117 H99 (Fig 1A). These findings support our hypothesis that eicosanoid synthesis by *C. neoformans* is required
118 to promote proliferation of the fungus during macrophage infection, furthermore they identify PGE_2 as a
119 mediator of cryptococcal virulence during macrophage infection.
120
121 *Phospholipase B1 dependent secreted factors are produced by C. neoformans to promote macrophage*
122 *infection*
123
124 After finding that PGE_2 is required by *C. neoformans* to proliferate within macrophages, we next wanted to
125 determine whether *C. neoformans* is the source of this growth promoting factor during macrophage
126 infection. To test if *C. neoformans* produces phospholipase B1 dependent secretary factors, such as PGE_2,
127 to promote intacellular growth we used a co-infection' assay which has previously been used to investigate
128 the interaction of different *C. gattii* strains within the same macrophage (24). If the parental strain H99
129 produces growth promoting factors that *Δplb1* cannot, we reasoned that *Δplb1* cells would display improved
130 intracellular replication when H99 was also present within the same macrophage.
131

To produce co-infection, we infected macrophages with a 50:50 mixture of *Δplb1* and H99-GFP (25) (Fig 2B i; as described previously for *C. gattii* (24)). The intracellular proliferation of *Δplb1* was calculated by counting the change in number of non-fluorescent *Δplb1* cells over an 18hr period from time-lapse movies of infected cells. Although we infected our macrophages with a 50:50 mixture of *Δplb1* and H99-GFP we found that co-infected macrophages did not always contain the same ratio of each strain. To control for differences in burden between macrophages, we only scored macrophages that had an initial burden of two *Δplb1* yeast cells to one H99-GFP yeast cell or vice versa. Interestingly we found that *Δplb1* (Fig 1B) proliferated better when accompanied by two H99-GFP yeast cells in the same macrophage (Fig 1 B ii, 1:2) as opposed to when two *Δplb1* yeast cells were accompanied by one H99-GFP yeast cell (Fig 1 B ii, 2:1). This suggests that there is a secreted factor produced by H99, but absent in *Δplb1* that can promote intracellular proliferation within macrophages.

PGE_2 synthesis by C. neoformans is required for growth in vivo.

After showing that PGE_2 produced by *C. neoformans* promotes *in vitro* fungal growth within macrophages we wanted to see if these interactions could be reproduced *in vivo*. Zebrafish have been proven to be an excellent model for understanding vertebrate eicosanoid biology (26,27) so we chose to investigate these interactions using our zebrafish model of cryptococcosis (22). One of the advantages of this infection model is that because we use fluorescently tagged *C. neoformans* strains, the fungal burden within infected larvae can be non-invasively imaged throughout infection. To use *Δplb1* with our zebrafish larvae infection model we generated a constitutively expressed GFP tagged version of the *Δplb1* (*Δplb1*-GFP). We next evaluated the growth of *Δplb1*-GFP by infecting zebrafish larval with *Δplb1*—GFP and measuring the growth of the fungus at 1,2 and 3 days post infection (dpi). Analysis of fungal burden revealed that *Δplb1*-GFP had a lower fungal burden at 1, 2 and 3 days post infection compared to the parental strain H99-GFP (Fig 2A, C). These data show that the *Δplb1* mutant has a similar growth deficiency in our *in* vivo model to our *in vitro* data and previous studies (21,23,28).

After confirming that fungal burden is reduced *in vivo,* we next wanted to investigate if this phenotype was due to an inability to generate PGE_2. We treated *Δplb1* and H99 infections with PGE_2 and PGD_2 (PGD_2 tends to produce context-dependent opposing immune signalling to PGE_2 (15)). PGE_2, but not PGD_2, increased fungal burden of both the parental H99 strain (Fig 2B, p = 0.0137, 1.35-fold increase vs. DMSO) and the *Δplb1-GFP* mutant (Fig 2F, p= 0.0001, 2.15-fold increase vs. DMSO). Taken together these data show that PGE_2 is required for growth *in vivo*; this is in agreement with our *in vitro* findings suggesting that growth *in vivo* may rely on eicosanoid production during macrophage infection.

Prostaglandin E_2 must be dehydrogenated into 15-keto-PGE_2 in order to facilitate C. neoformans growth.

PGE_2 produced by *C. neoformans* can be further converted into 15-keto-PGE_2 (18). Due to the possibility that some of the PGE_2 added to zebrafish might be converted to 15-keto-PGE_2 we wanted to determine if the effects we observed were due to the activity of PGE_2 or 15-keto-PGE_2. To test this, we treated infected larvae with 16,16-dimethyl PGE_2, which cannot be dehydrogenated into 15-keto-PGE_2, but otherwise has comparable activity to PGE_2 (29). Interestingly, in contrast to PGE_2, we found that 16,16-dimethyl PGE_2 treatment did not increase the fungal burden of *Δplb1-GFP* (Fig 4E and 4G, p= 0.9782) *or* H99-GFP infected larvae (Fig 4A and 4C, p= 0.9954). To confirm that *C. neoformans* growth rate is facilitated by 15-keto-PGE_2 we treated infected larvae with exogenous 15-keto-PGE_2 and found that this was sufficient to significantly increase the fungal burden of both *Δplb1-GFP* (Fig 4F and 4H, p = 0.0119, 1.56-fold increase vs. DMSO) and H99-GFP infections (Fig 4B and 4C, p = 0.0048, 1.36-fold increase vs. DMSO). Taken together, these results demonstrated that the increase in *C. neoformans* burden we observed with PGE_2 treatment for both *Δplb1-GFP* and H99-GFP were due to dehydrogenation of PGE_2 into 15-keto-PGE_2.

15-keto-PGE_2 promotes C. neoformans growth by activating host PPAR-γ

We next wanted to determine if 15-keto-PGE_2 is able to promote cryptococcal growth by interfering with host eicosanoid signalling pathways. 16,16-dimethyl PGE_2 signals through the same host receptors as

PGE_2 so its lack of activity in this system strongly suggests different molecular pathways for 15-keto PGE_2 and PGE_2. With 15-keto PGE_2 has been reported as an agonist at the peroxisome proliferation associated receptor gamma (PPAR-γ) (30); a transcription factor that controls expression of many inflammation related. To determine if 15-keto PGE_2 promotes the growth of *C. neoformans* during host infection by activating host PPAR-γ we treated infected larvae with a specific agonist of PPAR-γ - Troglitazone (TLT) - at a concentration (0.55 µM) shown to strongly activate PPAR-γ in zebrafish larvae (31). We found that TLT treatment produced a significantly increased the fungal burden of *Δplb1-GFP* (Fig 5B and 5D, $p = 0.0089$, 1.68-fold increase vs. DMSO) and H99-GFP (Fig 5A and 5C, $p = 0.0044$, 1.46-fold increase vs. DMSO) infected larvae similar to 15-keto PGE_2 treatment. These findings suggest that 15-keto PGE_2 promotes fungal growth by activating PPAR-γ.

In conclusion, we have shown for the first time that eicosanoids produced by *C. neoformans* are required for virulence both *in vitro* and *in vivo*. We have shown that the intracellular growth defect of *Δplb1 (21,23)* can be rescued with the addition of exogenous PGE_2 (Fig 1A). Furthermore, with our co-infection experiments we provide evidence that the source of this eicosanoid during infection is from the pathogen, rather than the host (Fig 1 B). Using a zebrafish larvae *in vivo* model of cryptococcosis we have gone on to show that a recently described fungal burden defect during larval infection can also be rescued with the addition of PGE2 (Fig 2). Additionally we find that that PGE_2 appears to require dehydrogenated into 15-keto PGE2 before it has a biological effect (Fig 2 and 3). Finally, we provide evidence that the mechanism of PGE2/15-keto PGE2 mediated growth promotion during larval infection may be via the activation of PPAR-γ – an intracellular receptor within macrophages that 15-keto PGE_2 is thought to activate.

Discussion

We have identified an unknown mechanism used by *C. neoformans* to manipulate host innate immunity whereby eicosanoids secreted by the fungus are able to manipulate the function of macrophages and promote fungal growth in the host through 15-keto PGE2 and the host receptor PPAR-γ.

We have previously identified that the *PLB1* gene deletion mutant *Δplb1* was deficient in replication and survival in macrophages (21), an observation also supported by a number of studies using different *in vitro* infection assays (19,23). In this study we have shown that the treating *Δplb1* infected macrophages with exogenous prostaglandin E_2 is sufficient to restore intracellular proliferation of *Δplb1*. During cryptococcal respiratory infection it has been observed that PGE_2 levels increase significantly in the lung (20). Our study identifies cryptococci as the source of PGE_2 and additionally it identifies the direct effect of PGE_2 on *Cryptococcus* intracellular proliferation, confirming PGE_2 as virulence factor required for growth in macrophages. As a PGE_2 is a phospholipase B1 dependent factor we hypothesised that co-infection would also be sufficient to recover intracellular growth in macrophages. Analysis of complementary ratios of mutant to parental strain numbers in co-infected macrophages demonstrated rescue, supporting our conclusion that cryptococcal derived PGE_2 is required for virulence.

A key goal of this study was to investigate how eicosanoids produced by *C. neoformans* may affect pathogenesis within a living host. We chose to use a zebrafish larvae model of cryptococcosis that our group has recently developed (22). To facilitate non-invasive fungal burden measurement within live larvae we created a GFP-tagged *Δplb1* strain with constitutive expression (*Δplb1-GFP)* to use alongside the GFP-tagged H99 parental strain we have previously produced (25). To our knowledge this is the first GFP tagged version of *Δplb1* created, and it also represents one of the only examples of a fluorescently tagged mutant *C. neoformans* strain. Characterisation of *Δplb1-GFP* using our zebrafish model of cryptococcosis revealed that this strain also has significantly reduced growth *in vivo* compared to H99-GFP, these findings confirm findings from a similar zebrafish cryptococcosis model which also found that *Δplb1* (non-fluorescent) had attenuated burden during larval infection (32).

To ascertain whether PGE_2 is also required for cryptococcal growth *in vivo* we treated zebrafish larvae infected with *Δplb1-GFP* or H99-GFP with exogenous PGE_2. In agreement with our *in vitro* findings we

found that PGE_2 significantly improved the growth of *Δplb1-GFP* within larvae. Interestingly we also found that PGE_2 improved the growth of our H99-GFP parental control perhaps representing a wider manipulation of host immunity in *in vivo* infection. During larval infection, *C. neoformans* interacts closely with macrophages, it is therefore likely that the PGE_2 dependent growth observed in our *in vivo* model is due to host macrophage manipulation facilitated by *Cryptococcus* derived eicosanoids.

In vertebrate cells, PGE_2 is converted into 15-keto PGE_2 by the dehydrogenase enzyme 15-prostaglandin dehydrogenase (15PGDH). To determine whether the biological effect we observed with PGE_2 were affected by the conversion of to PGE_2 or 15-keto PGE_2 we treated infected larvae with 16,16 dm-PGE_2 – a synthetic variant of PGE_2 which is resistant to dehydrogenation (29). Interestingly we found that 16,16 dm-PGE_2 was not able to promote the growth of *Δplb1-GFP* or H99-GFP within infected larvae. These findings indicated that 15-keto PGE_2, rather than PGE_2, was the bioactive molecule during cryptococcal infection. To confirm these findings, we treated infected larvae with exogenous 15-keto-PGE_2 and found that 15-keto-PGE_2 treatment was sufficient to promote the growth of both *Δplb1-GFP* and H99-GFP without the need for PGE_2. We propose that PGE_2 produced by *C. neoformans* is enzymatically dehyodrogenated into 15-keto PGE_2, as previously mentioned the vertebrate enzyme 15PGDH can convert PGE_2 into 15-keto PGE_2, additionally it has been reported that *C. neoformans* itself has enzymatic activity analogous to 15PGDH (18), therefore it is possible that *C. neoformans* may produce both PGE_2 and 15-keto-PGE_2. These findings represent the identification of a new virulence factor produced by *C. neoformans* as well as the first time that an eicosanoid other than PGE_2 has been identified as promoting cryptococcal growth. Furthermore, our findings suggest that previous studies which identify PGE_2 as a promoter of cryptococcal virulence (19,20,33) may have observed multiplicative effects from both PGE_2 and 15-keto PGE_2 activity.

The biological activity of 15-keto PGE_2 is far less studied than its parent species PGE_2. 15-keto PGE_2 is unable to bind to the prostaglandin E_2 EP receptors which means that one of its physiological functions is to act as a negative regulator of PGE_2 activity i.e. cells up-regulate 15PGDH activity to lower PGE_2 levels (34). Asour findings showed that 15-keto PGE_2 does have an effect during *C. neoformans* infection, we next sought the cellular receptor for 15-keto PGE_2 . With this in mind it has been demonstrated that 15-keto

266 PGE_2 is an agonist for the intracellular eicosanoid receptor peroxisome proliferator associated receptor
267 gamma (PPAR- γ) (30). PPAR- γ is a nuclear receptor normally found within the cytosol, upon ligand
268 binding PPAR- γ forms a heterodimer with Retinoid X receptor (RXR) and translocates to the nucleus where
269 it influences the expression of target genes which possess a peroxisome proliferation hormone response
270 element (PPRE) (35).

271 Activation of PPAR- γ by *C. neoformans* has not been described previously but agrees with what we know
272 of cryptococcal pathogenesis. PPAR- γ is an intracellular receptor found in many cell types including
273 macrophages, if eicosanoids are being produced by *C. neoformans* during intracellular infection it is also
274 likelier that they have bind to an eicosanoid receptor within the macrophage. Finally, activation of PPAR- γ
275 promotes the expression of genes that are anti-inflammatory, and in macrophages PPAR- γ activation can
276 lead to alternative activation. The growth promoting effects of 15-keto PGE_2 and the identification of PPAR-
277 γ as a receptor for PPAR- γ led us to hypothesise that the effect of 15-keto PGE_2 could be mediated by
278 PPAR- γ mediated activation of anti-inflammatory target genes. To establish whether PPAR- γ activation
279 could promote the growth of *C. neoformans* in our zebrafish model we treated infected larvae with the drug
280 troglitazone - a specific PPAR- γ agonist – at a concentration previously shown to activate PPAR- γ in
281 zebrafish larvae. At this concentration we found that troglitazone was able to increase the growth of *Δplb1-*
282 *GFP* and H99-GFP during infection. Although these findings strongly suggest that the cryptococcal growth
283 promoting effects of 15-keto PGE_2 may mediated via activation of PPAR- γ, further work will be needed to
284 prove that the effects we observe when PPAR- γ is activated by troglitazone are due to the activity 15-keto
285 PGE_2.

286 In conclusion we have shown for the first time that synthesis of eicosanoids by *C. neoformans* is able to
287 promote intracellular growth within macrophages and within an *in vivo* host. Additionally, we have
288 uncovered a new virulence factor – 15-keto-PGE_2 – that is produced from PGE_2 during infection. We provide
289 evidence for a possible mechanism for 15-keto PGE_2 via activation of PPAR- γ which is known to promote
290 anti-inflammatory immune pathways. Finally, we have provided a potential new therapeutic pathway for
291 treatment of cryptococcal infection as several eicosanoid modulating drugs are approved for patient
292 treatment (36) .

Materials and methods

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

Ethics statement

Animal work was performed following UK law: Animal (Scientific Procedures) Act 1986, under Project License PPL 40/3574. Ethical approval was granted by the University of Sheffield Local Ethical Review Panel.

Zebrafish

All zebrafish used in this study were the *Nacre* wild type strain. Zebrafish were maintained according to standard protocols. Adult fish were maintained on a 14:10 – hour light / dark cycle at 28 ^{o}C in UK Home Office approved facilities in the Bateson Centre aquaria at the University of Sheffield.

C. neoformans

The H99-GFP has been previously described (25). The Δ*plb1*-GFP was generated for this study by transforming existing deletion mutant strains Δ*plb1* (23) with a GFP expression construct (see below for transformation protocol). All strains used are in the *C. neoformans* variety *grubii* H99 genetic background.

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.

315 ***C. neoformans* transformation**

316 *C. neoformans* strains Δ*plb1* was biolistically transformed using the pAG32_GFP transformation
317 construct as previously described for H99-GFP (25). Stable transformants were identified by
318 passaging positive GFP fluorescent colonies for at least 3 passages on YPD agar supplemented
319 with 250 µg/ml Hygromycin B. Two stable Δ*plb1*-GFP transformants were identified (#1-1 and #1-
320 2), this study uses #1-2 as it was determined to be have the strongest and most homogenous
321 GFP signal.

322 **J774 Macrophage infection – with exogenous PGE_2 treatment**

323 J774 macrophage infection was performed as previously described (21) with the following
324 alterations. J774 murine macrophage-like cells were cultured for a minimum of 4 passages in T75
325 tissue culture flasks at 37ºC 5% CO_2 in DMEM (High glucose, Sigma) supplemented with 10%
326 Fetal Bovine Calf Serum (Invitrogen), 1% 10,000 units Penicillin / 10 mg streptomycin and 1 %
327 200 mM L – glutamine, fully confluent cells were used for each experiment. Macrophages were
328 counted by haemocytometer and diluted to a concentration of $1x10^5$ cells per ml in DMEM
329 supplemented with 1 µg/ml lipopolysaccharide (LPS from *E. coli,* Sigma L2630) before being
330 plated into 24 well microplates (Greiner) and incubated for 24 hours (37 ºC 5% CO_2).

331 Following 24-hour incubation, medium was removed and replaced with 1 ml DMEM supplemented
332 with 2 nM prostaglandin E_2 (CAY14010, 1mg/ml stock in 100% ethanol). Macrophage wells were
333 then infected with 100 µl $1x10^6$ yeast/ml *Cryptococcus* cells (from overnight culture, washed. See
334 above) opsonized with anti-capsular IgG monoclonal antibody (18b7, a kind gift from Arturo
335 Casadevall). Cells were incubated for 2 hours (37 ºC 5% CO_2) and then washed with 37 ºC PBS
336 until extracellular yeast were removed. After washing, infected cells were treated with 1ml DMEM
337 supplemented with PGE_2.

To calculate IPR, replicate wells for each treatment/strain were counted at 0 and 18 hours. Each well was washed once with 1ml 37 ^{o}C PBS prior to counting to remove any *Cryptococcus* cells released by macrophage death or vomocytosis. Intramacrophage Cryptococci were released by lysis with 200 µl dH_2O for 20 minutes (lysis confirmed under microscope). Lysate was removed to a clean microcentrifuge tube and an additional 200 µl was used to wash the well to make a total lysate volume of 400 µl. *Cryptoccoccus* cells within lysates were counted by haemocytometer. IPR was calculated by dividing the total number of counted yeast at 18hr by the total at 0hr.

J774 Macrophage co-infection.

J774 cells were prepared and seeded at a concentration of $1x10^5$ per ml as above in 24 well microplates and incubated for 24 hours (37 ^{o}C 5% CO_2), 45 minutes prior to infection J774 cells were activated with 150 ng/ml phorbol 12-myristate 13-acetate in DMSO added to 1 ml serum free DMEM. Following activation J774 cells were washed and infected with 100 µl / $1x10^6$ yeast/ml 50:50 mix of Δ*plb1* (non-fluorescent) and H99-GFP (e.g. $5x10^5$ Δ*plb1* and $5x10^5$ H99-GFP). Infected cells were incubated for 2 hours (37 ^{o}C 5% CO_2) to allow for phagocytosis of *Cryptococcus* and then washed multiple times with 37 ^{o}C PBS to remove unphagocytosed yeast, each well was observed between washes to ensure that macrophages were not being washed away. After washing 1 ml DMEM was added to each well.

Co-infected cells were imaged over 20 hours using a Nikon TE2000 microscope fitted with a climate controlled incubation chamber (37 ^{o}C 5% CO_2) using a Digital Sight DS-QiMC camera and a Plan APO Ph1 20x objective lens (Nikon). GFP and bright field images were captured every 4 minutes for 20 hours. Co-infection movies were scored manually. Co-infected macrophages that contained 2 Δ*plb1* (non-fluorescent) and 1 H99-GFP (GFP positive) yeast cells at 0 hr were tracked for 18 hours and before the burden of each strain within the macrophage was counted

again. The IPR for Δ*plb1* within co-infected macrophages was calculated by dividing the number of Δ*plb1* cells within a macrophage at 18 hr by the number at 0 hr.

Zebrafish infection

Washed and counted *Cryptococcus* cells were pelleted at 3300g for 1 minute and re-suspended in 10% Polyvinylpyrrolidinone (PVP), 0.5% Phenol Red in PBS to give the required inoculum in 1 nl. This injection fluid was loaded into glass capillaries shaped with a needle puller for microinjection. Zebrafish larvae were injected at 2-days post fertilisation; the embryos were anesthetised by immersion in 0.168 mg/ml tricaine in E3 before being transferred onto microscope slides coated with 3% methyl cellulose in E3 for injection. Prepared larvae were injected with two 0.5 nl boluses of injection fluid by compressed air into the yolk sac circulation valley. Following injection, larvae were removed from the glass slide and transferred to E3 to recover from anaesthetic and then transferred to fresh E3 to remove residual methyl cellulose. Successfully infected larvae (displaying systemic infection throughout the body and no visible signs of damage resulting from injection) were sorted using a fluorescent stereomicroscope. Infected larvae were maintained at 28 ^{o}C.

Eicosanoid / receptor agonist treatment of infected zebrafish larvae

All compounds below were purchased from Cayman Chemical. Compounds were resuspended in DMSO and stored at -20 ^{o}C until used. Prostaglandin E_2 (CAY14010, 10mg/ml stock), Prostaglandin D_2 (CAY12010, 10mg/ml stock), 16,16-dimethyl PGE_2 (CAY14750, 10mg/ml stock), 15-keto PGE_2 (CAY14720, 10mg/ml stock) and troglitazone (CAY14720, 10mg/ml stock).

Treatment with exogenous compounds during larval infected was performed by adding compounds (or equivalent solvent) to fish water (E3) to achieve the desired concentration. Fish were immersed in compound supplemented E3 throughout the experiment from the time of injection.

386 **Zebrafish fungal burden measurement**

387 Individual infected zebrafish embryos were placed into single wells of a 96 well plate (VWR) with
388 200 ul or E3 (unsupplemented E3, or E3 supplemented with eicosanoids / receptor agonist
389 depending on the assay). Infected embryos were imaged at 0 days post infection (dpi), 1 dpi, 2
390 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
391 objective lens. Images were captured with a Neo sCMOS (Andor, Belfast, UK) and NIS Elements
392 (Nikon, Richmond, UK). Images were exported from NIS Elements into Image J FIJI as
393 monochrome tif files. Images were threshholded in FIJI using the 'moments' threshold preset and
394 converted to binary images to remove all pixels in the image that did not correspond to the
395 intensity of the fluorescently tagged *C. neoformans*. The outline of the embryo was traced using
396 the 'polygon' ROI tool, avoiding autofluorescence from the yolk sac. The total number of pixels in
397 the threshholded image were counted using the FIJI 'analyse particles' function, the 'total area'
398 measurement from the 'summary' readout was used for the total number of GFP + pixels in each
399 embryo.

400 **Acknowledgments**

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404 **Author Information**

405 The experiments for this study were conceived by RJE, RCM, SAR and SAJ.

406 The experiments for this study were performed by RJE and SN.

407 Analysis was performed by RJE, SN and SAJ.

408 Generation of GFP strains was performed by RJE and EB.

409 The manuscript was written by RJE, RCM, SAR and SAJ. With feedback from EB and SN.

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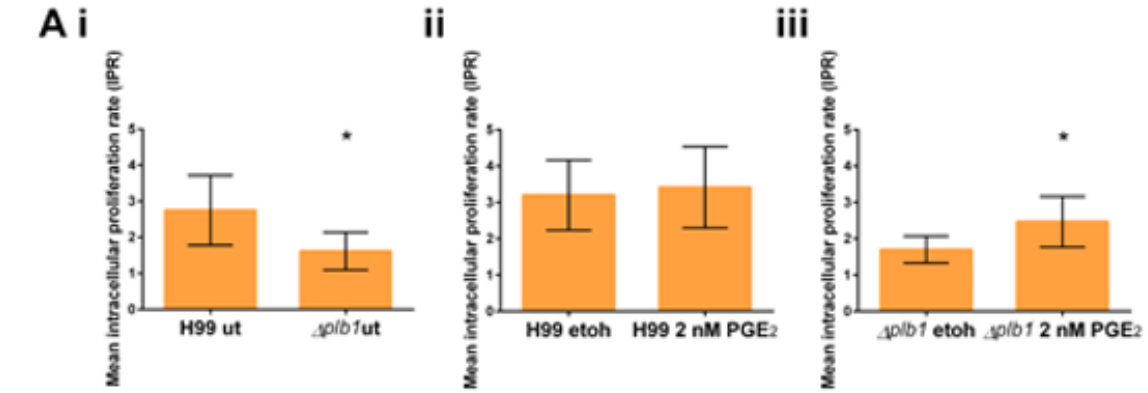

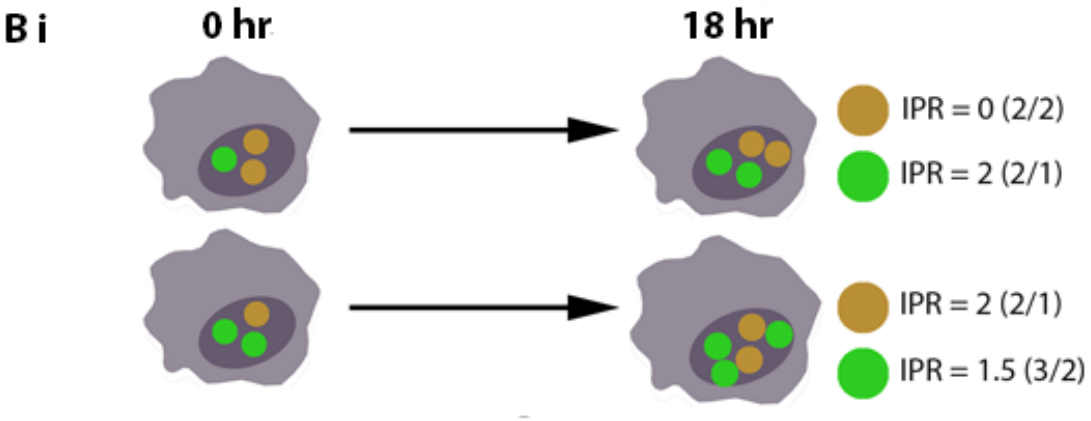

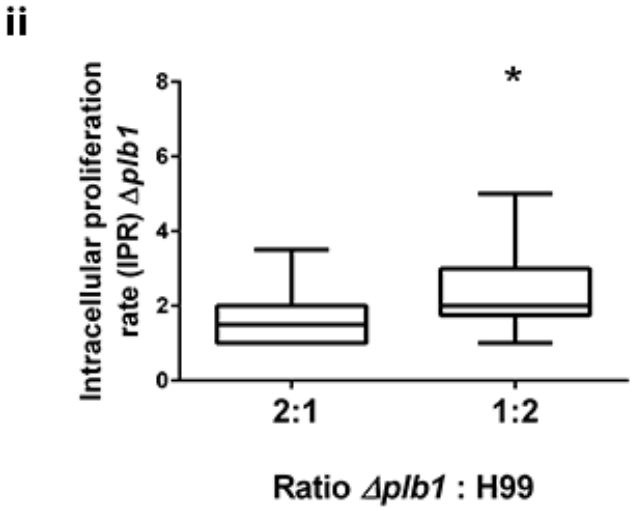

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Fig1

The intracellular proliferation defect of the *C.* neoformans mutant *Δplb1,* can be reversed with the addition of exogenous prostaglandin E_2. A J774 murine macrophages were infected with *Δplb1* or the parental strain H99. Infected cells were left untreated **(i)** or treated with 2 nM PGE_2 **(ii)** or an equivalent solvent (ethanol) control **(iii)**. Mean IPR from 5 biological repeats shown with error bars representing standard deviation. An unpaired two tailed Student's t-test was performed to compare each treatment group. **(i) *** p = 0.0303 **(ii)** ns p = 0.7212 **(iii)** * p = 0.0376. **B** J774 cells co-infected with a 50:50 mix of *Δplb1* and H99-GFP. **i** Diagrammatic representation of co-infection experiment. GFP^+ (green) and GFP^- (yellow) *C. neoformans* cells within the phagosome were quantified at 0 hr, macrophages with a burden ratio of 1:2 or 2:1 were reanalysed at 18 hr, the IPR for *Δplb1* within 2:1 and 1:2 co-infected cells was calculated by dividing the burden at 18hr by burden at 0 hr for GFP^+ (green) or GFP^- (yellow) cells. **ii** Quantification of IPR for *Δplb1* cells within *Δplb1*:H99-GFP 2:1 or 1:2 co-infected macrophages. At least 38 co-infected macrophages were analysed for each condition over 4 experimental repeats. Student's T test performed to compare ratios – 2:1 vs 1:2 * p = 0.0137.

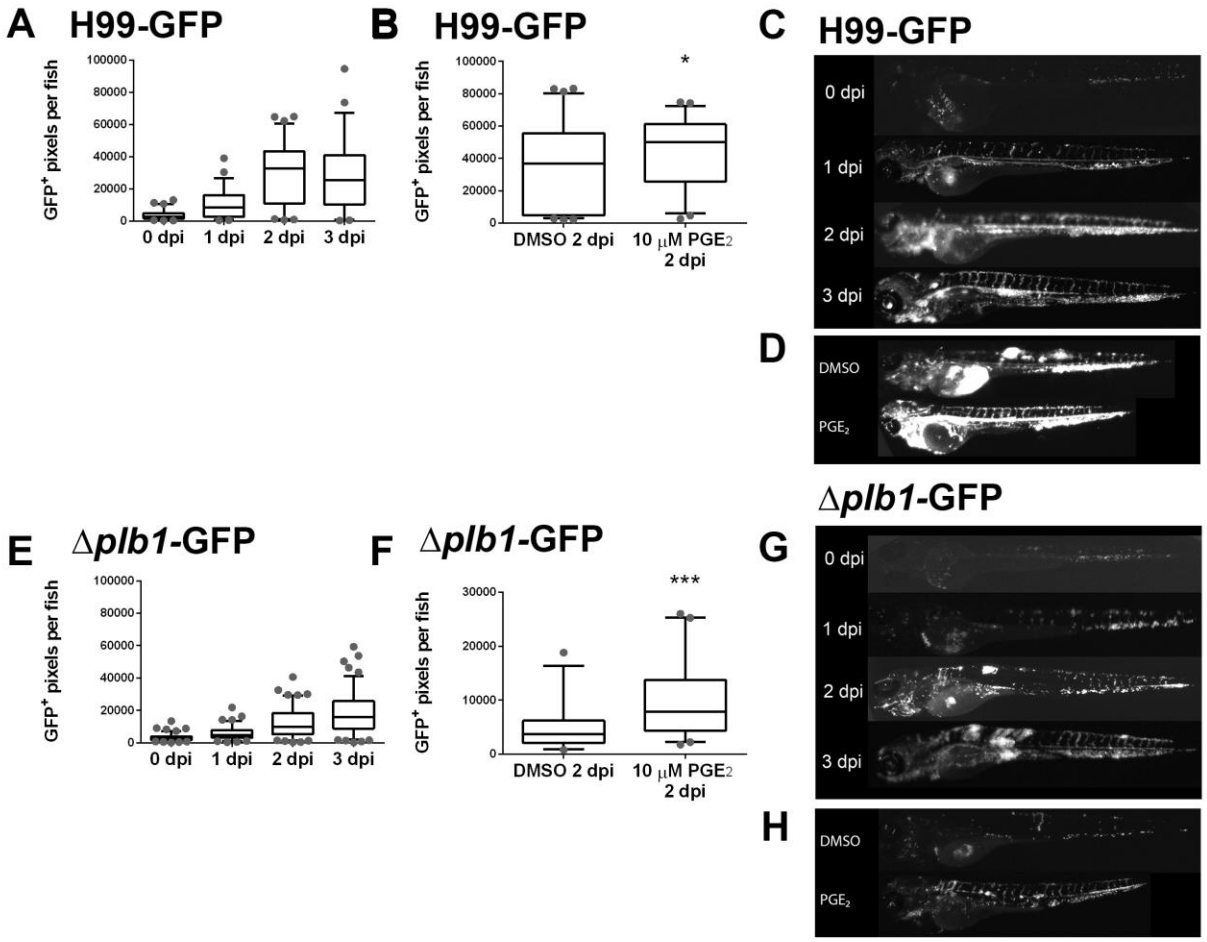

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Fig2

The prostaglandin E_2 dependent growth defect of *Δplb1* is also present *in vivo*: A H99-GFP infected larvae imaged at 0, 1, 2 and 3 dpi. At least 50 larvae measured per time point from 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 (Supplementary Fig1). **B** – 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 significance 0.017, corrected for multiple comparisons). **C,D** Representative GFP images (representative = median value) of 2dpi H99-GFP infected larvae, untreated at 0,1,2,3 dpi **(C)** or H99-GFP infected larvae with 10 µM PGE_2 at 2dpi **(D)**. **E** *Δplb1-GFP* infected larvae (500 cell inoculum injected at 2 dpf) imaged at 0, 1, 2 and 3 dpi. N = 3. Box and whiskers show median, 5th percentile and 95th percentile. At least 87 larvae measured for each timepoint from 3 biological repeats. Unpaired Mann-Whitney U tests used to compare the burden between each strain for every time point, for p values see (Supplementary table 1). **F** – *Δplb1-GFP* Infected larvae treated with 10 µM prostaglandin E_2 or equivalent solvent (DMSO) control. At least 38 larvae measured per treatment group from 2 biological repeats. Box and whiskers show median, 5th percentile and 95th percentile. Unpaired Mann-Whitney U tests used to compare between treatments *Δplb1-GFP* DMSO vs 10 µM PGE_2 *** p = 0.0001 (threshold for significance 0.017, corrected for multiple comparisons). **G,H** Representative GFP images (representative = median value) of 2dpi *Δplb1-GFP* infected larvae, untreated at 0,1,2,3 dpi **(G)** or *Δplb1-GFP* infected larvae with 10 µM PGE_2 at 2dpi **(H)**.

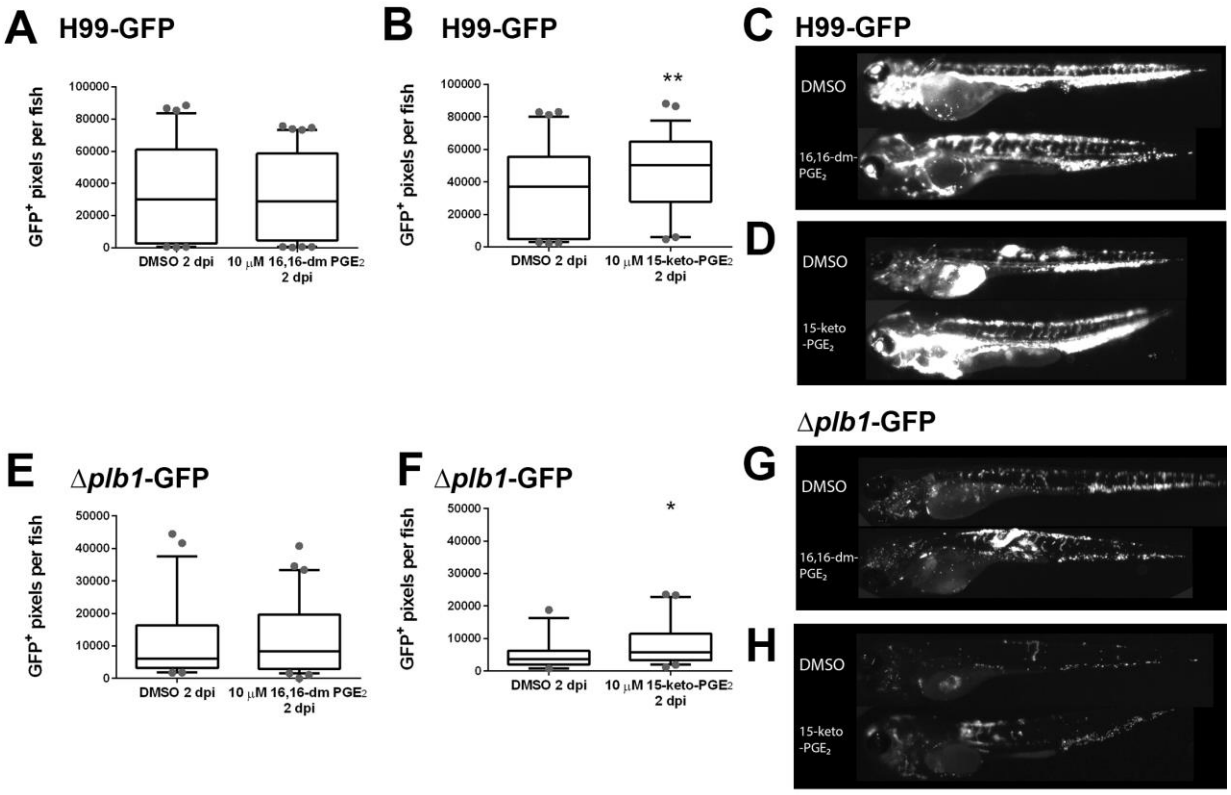

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Fig3

The observed activity of PGE_2 is due to its dehydrogenated derivative 15-keto PGE: Fungal burden measured at 2 days post infection (2 dpi) by counting GFP positive pixels in each larvae. **A** H99-GFP Infected larvae treated with 10 µM 16,16-dimethyl prostaglandin E_2 or equivalent solvent (DMSO) control. At least 75 larvae measured per treatment group from 4 biological repeats. Box and whiskers show median, 5^{th} percentile and 95^{th} percentile. Unpaired Mann-Whitney U test used to compare between treatments, DMSO vs. 10 µM 16, 16-dm PGE_2 ns p = 0.9954. **B** H99-GFP Infected larvae treated with 10 µM 15-keto prostaglandin E_2 or equivalent solvent (DMSO) control. At least 55 larvae measured per treatment group from 3 biological repeats. Unpaired Mann-Whitney U test used to compare between treatments DMSO vs. 10 µM 15-keto PGE_2 ** p = 0.0048 (threshold for significance 0.017, corrected for multiple comparisons). **C,D** Representative GFP images (representative = median value) of 2dpi H99-GFP infected larvae with 10 µM 16, 16-dimethyl prostaglandin E_2 (**C)** or 10 µM 15-keto prostaglandin E_2 **(D)**. **E** *Δplb1-GFP* Infected larvae treated with 10 µM 16, 16-dimethyl prostaglandin E_2 or equivalent solvent (DMSO) control. At least 45 larvae per treatment group from 3 biological repeats. Unpaired Mann-Whitney U test used to compare between treatments *Δplb1-GFP* DMSO vs 10 µM 16, 16-dm PGE_2 ns p = 0.9782. **F** *Δplb1-GFP* Infected larvae treated with 10 µM 15-keto prostaglandin E_2 or equivalent solvent (DMSO) control. At least 38 larvae measured per treatment group from 2 biological repeats. Unpaired Mann-Whitney U test used to compare between treatments DMSO vs 10 µM 15-keto PGE_2 * p = 0.0119 (threshold for significance 0.017, corrected for multiple comparisons).. **G,H** Representative GFP images (representative = median value) of 2dpi *Δplb1-GFP* infected larvae with 10 µM 16,16-dm PGE_2 **(G)** or 10 µM 15-keto PGE_2 **(H)**

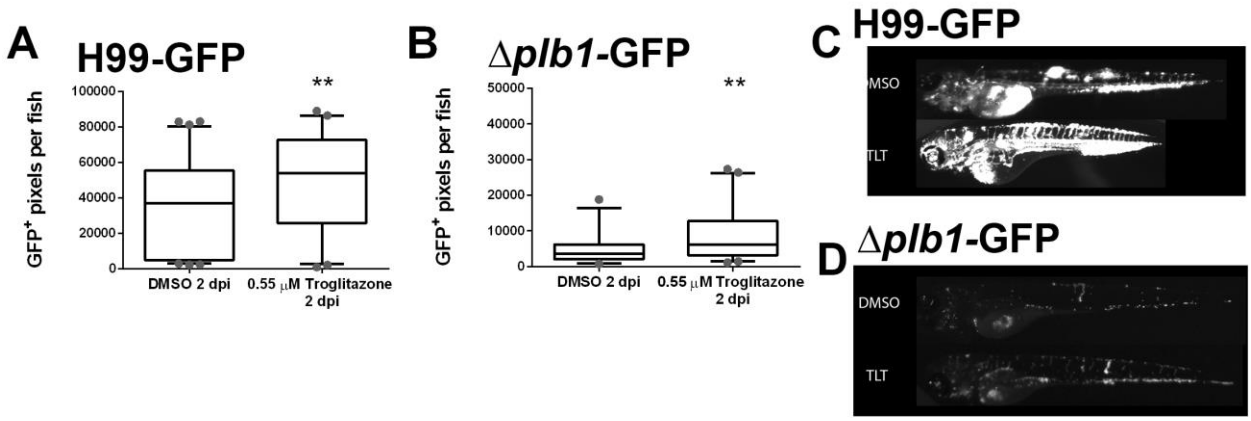

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Fig4

15-keto PGE_2 may promote fungal burden by activating host PPAR-γ. 2 day old (2 dpf) *Nacre* zebrafish larvae injected with 500 cell inoculum. Fungal burden measured at 2 days post infection (2 dpi) by counting GFP positive pixels within each larvae. **A** H99-GFP Infected larvae treated with 0.55 µM Troglitazone (TLT) equivalent solvent (DMSO) control. Box and whiskers show median, 5th percentile and 95th percentile. At least 55 larvae measured per treatment group over 3 biological repeats. Mann-Whitney U test used to compare between treatments, DMSO vs. 0.55 µM Troglitazone ** p = 0.0044. **B** *Δplb1-GFP* infected larvae treated with 0.55 µM Troglitazone (TLT) equivalent solvent (DMSO) control. Box and whiskers show median, 5th percentile and 95th percentile. At least 39 larvae measured per treatment group from 2 biological repeats. Mann-Whitney U test used to compare between treatments, DMSO vs. 0.55 µM Troglitazone ** p = 0.0089. **C,D** Representative GFP images (representative = median value) of 2dpi H99-GFP infected larvae with 0.55 µM TLT **(C)** or *Δplb1-GFP* infected larvae with 0.55 µM TLT **(D)**

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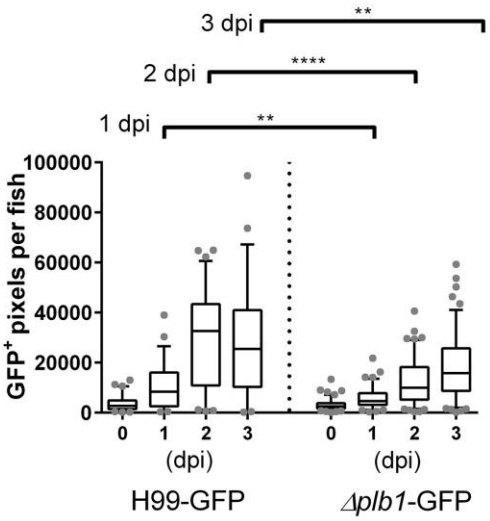

B

DPI (H99-GFP vs. *Δplb1*-GFP)	P value
0	ns p = 0.4980
1	** p = 0.0095
2	**** p = <0.0001
3	** p = 0.0013

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583 **Supplementary Fig 1**

584 **Comparison of fungal burden between H99-GFP and *Δplb1-GFP* infected larvae A** (Data reproduced
585 from Fig2 A and E for clarity) H99-GFP and *Δplb1-GFP* infected larvae imaged at 0, 1, 2 and 3 dpi. At
586 least 50 larvae measured per time point from 3 biological repeats. Box and whiskers show median, 5th
587 percentile and 95th percentile. Unpaired Mann-Whitney U tests used to compare the burden between
588 each strain for every time point. **B** Results of Mann-Whitney U tests comparing burden between

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