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Cysteinyl leukotrienes as novel host factors facilitating *Cryptococcus neoformans* penetration into the brain

Longkun Zhu[1,2], **Ravi Maruvada**[1], **Adam Sapirstein**[3], **Marc Peters-Golden**[4], and **Kwang Sik Kim**[1]

[1]Department of Pediatrics, Division of Pediatric Infectious Diseases, Johns Hopkins University School of Medicine, 200 North Wolfe Street, Room 3157, Baltimore, MD 21287

[2]Department of Cell Biology and Medical Genetics/Center for Cell and Developmental Biology, School of Basic Medical Sciences Fujian Medical University, No.1 Xue Yuan Lu, Shangjie Town, Minho County, Fuzhou 350108, Fujian, China

[3]Department of Anesthesiology and Critical Care medicine, 1800 Orleans St. Zayed 9127, Baltimore, MD 21287

[4]Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Michigan, 6301 MSRB III, 1150 W. Medical Center Dr., Ann Arbor, MI 48109

Summary

Cryptococcus neoformas infection of the central nervous system (CNS) continues to be an important cause of mortality and morbidity, and a major contributing factor is our incomplete knowledge of the pathogenesis of this disease. Here we provide the first direct evidence that *C. neoformans* exploits host cysteinyl leukotrienes (LTs), formed via LT biosynthetic pathways involving cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) and 5-lipoxygenase (5-LO) and acting via cysteinyl leukotriene type 1 receptor (CysLT1), for penetration of the blood-brain barrier. Gene deletion of $cPLA_2\alpha$ and 5-LO as well as pharmacological inhibition of $cPLA_2\alpha$, 5-LO and CysLT1 were effective in preventing *C. neoformans* penetration of the blood-brain barrier *in vitro* and *in vivo*. A CysLT1 antagonist enhanced the efficacy of an anti-fungal agent in therapy of *C. neoformans* CNS infection in mice. These findings demonstrate that host cysteinyl LTs, dependent on the actions of $cPLA_2\alpha$ and 5-LO, promote *C. neoformans* penetration of the blood-brain barrier and represent novel targets for elucidating the pathogenesis and therapeutic development of *C. neoformans* CNS infection.

Introduction

C. neoformans is responsible for life-threatening CNS infection in immunocompromised patients, especially those infected with HIV-1 (Day *et al.*, 2013; Eisenman *et al.*, 2007;

Correspondence should be addressed to KSK (kwangkim@jhmi.edu).

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Competing Interests Statement

The authors declare that they have no competing financial interest.

Hakim *et al.*, 2000; Jarvis *et al.*, 2013; Mitchell *et al.*, 1995; Mwaba *et al.*, 2001; Park *et al.*, 2009; Perfect *et al.*, 2002; Powderly *et al.*, 1993; Saag *et al.*, 2000; Warkentien *et al.*, 2010). One million cases of cryptococcal meningoencephalitis are estimated to occur globally per year, with >60% mortality within 3 months of infection (Day *et al.*, 2013; Mwaba *et al.*, 2001; Park *et al.*, 2009). Even when treatment with amphotericin B and flucytosine is available, mortality ranges between 15–30% and is much higher (41–70%) in low-income countries where such antifungal regimens are not readily accessible (Day *et al.*, 2013; Jarvis *et al.*, 2013; Mwaba *et al.*, 2001; Park *et al.*, 2009; Saag *et al.*, 2000). A recent study reported that cerebrospinal fluid (CSF) fungal burden predicts acute mortality in HIV-associated cryptococcal meningoencephalitis (Jarvis *et al.*, 2013). These findings illustrate the need for a novel strategy for decreasing fungal burden in improving outcomes in HIV-infected patients.

C. neoformans CNS infection is a leading contributor to mortality in HIV-infected individuals with CD4+ counts <100 cells/μl, the threshold at risk for cryptococcal CNS infection, and blood cryptococcal antigen (CRAG) screen and early institution of preemptive antifungal therapy is shown to be efficacious in improving survival (Boulware *et al.*, 2014; Jarvis *et al.*, 2009; Meya *et al.*, 2010). A 6-month survival in asymptomatic CRAG+ persons with CD4 <100/μl, however, is approximately 75% with the currently recommended WHO therapy, fluconazole (Boulware *et al.*, 2014; Jarvis *et al.*, 2009; Meya *et al.*, 2010). The timing of mortality, occurring several weeks later, suggests that better antifungal therapy may be able to improve outcomes. These findings indicate that new approaches are needed to investigate the pathogenesis, prevention and therapy of *C. neoformans* CNS infection. Development of such a strategy, however, has been hampered by our incomplete knowledge on how *C. neoformans* penetrates the blood-brain barrier (Kim, 2008), the essential step required for the development of CNS infection.

C. neoformans is commonly acquired by inhalation. Extrapulmonary dissemination to the bloodstream leads to infection of distant organs, resulting in meningoencephalitis (Day *et al.*, 2013; Eisenman *et al.*, 2007; Hakim *et al.*, 2000; Jarvis *et al.*, 2013; Mitchell *et al.*, 1995; Mwaba *et al.*, 2001; Park *et al.*, 2009; Perfect *et al.*, 2002; Powderly *et al.*, 1993; Saag *et al.*, 2000; Warkentien *et al.*, 2010). Several lines of evidence from human cases and experimental animal models of *C. neoformans* meningoencephalitis indicate that *C. neoformans* penetration into the brain follows fungemia, and cerebral capillaries are the portal of entry into the brain (Chang *et al.*, 2004; Charlier *et al.*, 2005; Chretien *et al.*, 2002; Lee *et al.*, 1996; Neuville *et al.*, 2002; Olszewski *et al.*, 2004; Shi *et al.*, 2010). Since the entry of *C. neoformans* into the brain occurred in the cerebral microvasculature, we developed an *in vitro* blood-brain barrier model with human brain microvascular endothelial cells (HBMEC) (Chang *et al.*, 2004; Kim, 2008, Kim *et al.*; 2004; Maruvada *et al.*, 2012; Rüffer *et al.*, 2004; Stins *et al.*, 1997) for investigating *C. neoformans* penetration of the blood-brain barrier.

C. neoformans penetration into the brain has been shown to involve mainly transcellular and Trojan-horse penetrations of the blood-brain barrier and has been examined in animal models of intravenous, intranasal and intratracheal inoculations (Chang *et al.*, 2004; Charlier *et al.*, 2005; Chretien *et al.*, 2002; Dromer *et al.*, 2011; Kim, 2008; Neuville *et al.*, 2002;

Olszewski *et al.*, 2004; Shi *et al.*, 2010). Transcellular and Trojan-horse penetrations may not be mutually exclusive and can function in parallel in these animal models (Dromer *et al.*, 2011). We showed that *C. neoformans* strains exhibit the ability to traverse the HBMEC monolayer without affecting HBMEC integrity, and HBMEC traversal is correlated with *C. neoformans* penetration into the brain (Chang *et al.*, 2004; Kim, 2008; Maruvada *et al.*, 2012; Shi *et al.*, 2010). The underlying mechanisms involved in *C. neoformans* penetration of the blood-brain barrier, however, remain incompletely understood.

Previous studies have identified several cryptococcal and host factors contributing to penetration into the brain, which include a cryptococcal metalloprotease as well as cps1-CD44 and plb1-Rac1 interactions (Vu et al, 2014; Chang et al, 2006; Jong et al, 2012; Maruvada et al, 2012). We identified the CysLT1 antagonist, montelukast from our chemical screen inhibiting *C. neoformans* traversal of HBMEC monolayer. CysLT1 has been previously shown to contribute to invasion of HBMEC monolayer and penetration into the brain by bacteria causing meningitis such as *E. coli* and group B *Streptococcus* (Zhu et al, 2010; Maruvada et al, 2012). Here we elucidated the mechanisms by which cysteinyl leukotrienes are generated and contribute to *C. neoformans* penetration of the blood-brain barrier *in vitro* and *in vivo*.

Results and Discussion

Since *C. neoformans* traversal of the HBMEC monolayer is correlated with penetration into the brain, we used *C. neoformans* traversal of HBMEC monolayer as a relevant biological assay to screen a chemical library for discovery of targets affecting *C. neoformans* traversal of the blood-brain barrier. Our chemical screen identified montelukast as an inhibitor of *C. neoformans* traversal of HBMEC monolayer, yet montelukast did not affect the growth of *C. neoformans* strains B-3501A and H99 and also did not affect the HBMEC viability, as assessed by live/dead stain (Molecular probes) (Stins *et al.*, 2001). Montelukast is a selective antagonist of cysteinyl leukotriene type 1 receptor (CysLT1) and inhibits cysteinyl leukotriene (CysLT) binding to CysLT1 (Peters-Golden and Henderson, 2007). Since host CysLTs are formed via LT biosynthetic pathways involving cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) and 5-lipoxygenase (5-LO), we hypothesize that host $cPLA_2\alpha$ and 5-LO contribute to *C. neoformans* penetration of the blood-brain barrier.

We showed that *C. neoformans* strains exploit host $cPLA_2\alpha$ for their traversal of HBMEC monolayer, as shown by the time-dependent $cPLA_2\alpha$ phosphorylation in response to *C. neoformans* strains B-3501A and H99 (Fig. 1A) and the ability of arachidonoyl trifluoromethylketone (AACOCF3, $cPLA_2\alpha$ inhibitor) at 20 μM to significantly inhibit *C. neoformans* traversal of HBMEC monolayer (Fig. 1B). AACOCF3 at 20 μM was efficient in preventing $cPLA_2\alpha$ phosphorylation in response to *C. neoformans* strains H99 (serotype A) and B-3501A (serotype D), but did not affect the growth of *C. neoformans* strains (Fig 1. C & D) or the integrity of the HBMEC monolayer, as assessed by live/dead staining (Molecular probes).

We next examined whether pharmacologic inhibition and gene deletion of $cPLA_2\alpha$ affects *C. neoformans* penetration into the brain in BALB/c mice. Each animal received 1×10^5

colony forming units (CFUs) of *C. neoformans* strain B-3501A via the tail vein. 24 hours later, blood specimens were obtained for determination of CFUs, and the animals perfused with sterile Ringer's solution until the perfused solution became colorless. The brains, spleens, kidneys and lungs were removed, weighed, homogenized and cultured for determinations of CFUs, as described previously (Chang *et al.*, 2004; Maruvada *et al.*, 2012). *C. neoformans* strain B-3501A was completely cleared from the blood at 24 hours after intravenous injection and no viable yeasts were recovered from the blood. The CFUs in the brains were, therefore, most likely to represent the yeast cells that had penetrated into and survived in the brain.

For pharmacological inhibition, AACOCF3 (4 mM in 50 μl PBS, a dose which inhibits cPLA$_2\alpha$ activity in mice, Kalyvas *et al.*, 2004) was administered intravenously 30 mins before *C. neoformans* injection, and this resulted in significantly decreased penetration of *C. neoformans* into the brain of BALB/c mice (Fig. 1E). In contrast, AACOCF3 did not affect *C. neoformans* penetration into the spleen, kidney and lung, as shown by similar numbers of CFUs recovered from the animals receiving AACOCF3 or vehicle control (Fig. 1E).

The effect of gene deletion of host cPLA$_2\alpha$ in *C. neoformans* penetration into the brain was assessed in cPLA$_2\alpha^{-/-}$ mice compared to their littermate control cPLA$_2\alpha^{+/+}$ mice (Sapirstein *et al.*, 2005; Zhu *et al*, 2010). The yeast counts recovered from the brains (CFUs/gm) of cPLA$_2\alpha^{-/-}$ mice were significantly less than those of cPLA$_2\alpha^{+/+}$ animals (Fig. 1F), while the yeast counts from the kidneys did not differ between cPLA$_2\alpha^{-/-}$ and cPLA$_2\alpha^{+/+}$ mice. These findings support that host cPLA$_2\alpha$ contributes to *C. neoformans* penetration into the brain *in vivo*.

cPLA$_2\alpha$ mediates arachidonic acid release from membrane phospholipids (Ghosh *et al.*, 2006), and we examined whether exogenous arachidonic acid affects *C. neoformans* penetration into the brain of cPLA$_2\alpha$−/− mice. Intravenous administration of arachidonic acid (1.2 μg/mouse in 50 μl PBS, a dose which restores cPLA2α-dependent vascular responses in cPLA2α$^{-/-}$ mice, Ichinose *et al.*, 2002; Zhu *et al*, 2010) 30 min before *C. neoformans* injection significantly enhanced *C. neoformans* penetration into the brain to the level observed in the wild type mice, while arachidonic acid failed to enhance *C. neoformans* penetration into the kidney (Fig. 1F). The enhancement of *C. neoformans* penetration into the brain by exogenous arachidonic acid in cPLA$_2\alpha^{-/-}$ mice was not accompanied by any changes in the blood-brain barrier permeability, as shown by a lack of significantly increased extravasation of intravenously administered Evans blue dye into the brain of infected animals receiving arachidonic acid compared to those without arachidonic acid administration. These findings are consistent with those of our previous studies, where *C. neoformans* penetration of the blood-brain barrier was not accompanied by any changes in the blood-brain barrier integrity (Chang *et al.*, 2004). These *in vitro* and *in vivo* findings using pharmacological inhibition and gene deletion demonstrate that *C. neoformans* exploits host cPLA$_2\alpha$ for traversal of the blood-brain barrier and penetration into the brain, while host cPLA$_2\alpha$ does not affect *C. neoformans* penetration into non-brain organs.

Leukotrienes (LTs) are synthesized from arachidonate by 5-lipoxygenase (5-LO) and 5-LO activating protein (FLAP) (Peters-Golden and Henderson, 2007), and we next determined

whether *C. neoformans* exploitation of host cPLA$_2\alpha$ for penetration of the blood-brain barrier depends on 5-LO products of arachidonic acid. This issue was examined initially using zileuton (a selective inhibitor of 5-LO) and MK886 (an inhibitor of FLAP) for assessing their effect on *C. neoformans* traversal of HBMEC monolayer. Zileuton exhibited a dose-dependent inhibition of *C. neoformans* strains H99 and B-3501A traversal of HBMEC, and MK886 at 50 μM inhibited strain H99 traversal of HBMEC (Fig. 2A). Zileuton at 400 μM and MK886 at 50 μM did not affect the growth of *C. neoformans* strains and also did not affect the integrity of HBMEC monolayer, as assessed by transendothelial electrical resistance (TEER) before and after traversal assays. The inhibition of HBMEC traversal by pharmacological inhibition of 5-LO and FLAP indicate that endogenous LT biosynthesis via 5-LO and FLAP is likely to play an important role in *C. neoformans* penetration of the blood-brain barrier.

The role of 5-LO in *C. neoformans* penetration into the brain was examined using 5-LO$^{-/-}$ mice as compared to their strain-matched wild type mice (Serezani *et al.*, 2005; Zhu *et al.*, 2010). In these animal studies, *C. neoformans* strain H99 was administered via intravenous inoculation as described above, as well as via intratracheal inoculation to mimic the natural route of infection (acquisition via inhalation). The intravenous inoculation model measures the ability of *C. neoformans* to penetrate into the brain from systemic dissemination, while the intratracheal inoculation model measures the ability of extrapulmonary dissemination to the brain from the lungs, and both models have been used for assessing transcellular and Trojan-horse penetrations of the blood-brain barrier (Chang *et al.*, 2004; Charlier *et al.*, 2005; Chretien *et al.*, 2002; Dromer *et al.*, 2011; Neuville *et al.*, 2002; Olszewski *et al.*, 2004; Shi *et al.*, 2010). *C. neoformans* strain H99 penetration into the brain following intravenous as well as intratracheal inoculations was significantly less in 5-LO$^{-/-}$ mice than in their wild type animals, as shown by significantly less yeast counts recovered from the brains of 5-LO $^{-/-}$ mice compared to those of their wild type animals (Fig. 2, B & C). In contrast, the yeast counts recovered from the non-brain sites (e.g., spleen, kidney and lung) were similar between 5-LO$^{-/-}$ and wild type mice (Fig. 2, B &C). These findings demonstrate that *C. neoformans* exploits host 5-LO for penetration into the brain following intravenous and intratracheal administrations.

Arachidonic acid is metabolized to LTB4 or cysteinyl leukotrienes (LTC4, LTD4 and LTE4) and these terminal LTs exhibit their biological actions via interaction with their respective G-protein coupled receptors, BLT1 and CysLT1 (Murphy *et al.*, 2007; Peters-Golden *et al.*, 2007). The role of individual classes of LTs in *C. neoformans* penetration of the blood-brain barrier was next determined by examining the effects of the CysLT1 antagonist (montelukast) and the BLT1 antagonist (CP105696) (Peters-Golden and Henderson, 2007) in cryptococcal traversal of the HBMEC monolayer. Pretreatment of HBMEC with montelukast significantly inhibited *C. neoformans* traversal in a dose-dependent manner (Fig. 3, A & B), while CP105696 did not exhibit any inhibition (Fig. 3C). Montelukast at 50 μM did not affect the growth of *C. neoformans* strains and also did not affect the integrity of HBMEC monolayer, as assessed by TEER before and after traversal assays.

We next examined the effect of montelukast in *C. neoformans* penetration into the brain following intravenous inoculation. Administration of montelukast (1.6 mM in 100 μl PBS, a

dose which exhibits CysLT1 antagonist activity in mice, Genovese *et al.*, 2008) intravenously 15 mins before and 6 hours after *C. neoformans* injection significantly decreased *C. neoformans* penetration of the brain of BALB/c mice, but did not affect *C. neoformans* penetration into the spleen, kidney and lung (Fig. 3D). These *in vitro* and *in vivo* findings with montelukast demonstrate for the first time that cysteinyl LTs contribute to *C. neoformans* penetration of the blood-brain barrier.

CysLT1 antagonists have been developed for allergic airway disease and shown to be safe and well tolerated in clinical trials (Barnes *et al.*, 1997; Capra *et al.*, 2007; Montuschi *et al.*, 2007). Since CysLT1 antagonist (montelukast) was effective in preventing *C. neoformans* penetration of the blood-brain barrier, we next examined its efficacy in the treatment of *C. neoformans* CNS infection, alone and in combination with anti-fungal drug (fluconazole). The animals received *C. neoformans* via intratracheal inoculation, and then montelukast via intraperitonal administration, followed by daily administration of montelukast and fluconazole, alone or in combination, for 6 and 13 days to mimic a likely application of these drugs for therapy of *C. neoformans* CNS infection. As expected, administration of fluconazole was effective in significantly inhibiting *C. neoformans* CFUs recovered from the brain compared to vehicle control (Fig. 3,E&F). The combination of montelukast and fluconazole, however, was significantly more effective than individual drugs alone in reducing *C. neoformans* CFUs recovered from the brain (Fig. 3, E & F).

In addition, our immunofluorescence studies revealed that the expression of CysLT1 was evident in the brain capillaries of animals with *C. neoformans* penetration into the brain following intratracheal inoculation, while CysLT1 expression was not discernible in the brain capillaries of uninfected animals (Fig 4), suggesting that the CysLT1 expression in the brain capillaries is likely to be up-regulated with *C. neoformans* infection of the brain.

Taken together, these findings suggest that counteracting a host factor involved in *C. neoformans* penetration of the blood-brain barrier (e.g., cysteinyl LTs) is beneficial in prevention and therapy of *C. neoformans* CNS infection, and also suggest that a combination of montelukast and fluconazole may represent an attractive preemptive therapeutic regimen for asymptomatic CRAG+ persons with CD4<100/μl.

It has been documented that eicosanoids can be produced not only by the host, but also by *Cryptococcus* phospholipase (Noverr *et al.*, 2003; Noverr *et al.*, 2001). We next examined whether prevention of *C. neoformans* penetration into the brain by the $cPLA_2\alpha$ inhibitor and the CysLT1 antagonist was the result of their inhibitory effects on cysteinyl LTs arising from arachidonate liberated by *Cryptococcus* or host phospholipase. This issue was examined by using the phospholipase B1 (*plb1*) mutant derived from *C. neoformans* strain H99 compared to its reconstituted strain along with wild type strain H99 (Noverr *et al.*, 2003). As shown previously (Maruvada et al, 2012), the penetration of the *plb1* mutant across the HBMEC monolayer was significantly decreased compared to that of the reconstituted strain and strain H99 (Fig 5A). AACOCF3 and montelukast were effective in significant inhibition of HBMEC traversal by the *plb1* mutant, the reconstituted and wild type strains (Fig 5A). More importantly, the penetration of the *plb1* mutant into the brain was significantly defective in $cPLA_2\alpha^{-/-}$ mice compared to wild type mice (Fig. 5B). In contrast, the yeast counts

recovered from non-brain sites (kidney, spleen and lung) did not differ between cPLA$_2\alpha^{-/-}$ and wild type mice. We also showed that montelukast significantly inhibited the *plb1* mutant's penetration into the brain compared to vehicle control (Fig. 5C). Our findings with pharmacological inhibition of cPLA$_2\alpha$ and CysLT1 as well as genetic deletion of cPLA$_2\alpha$ are, therefore, likely to stem from their effect on host cPLA$_2\alpha$ and CysLT1, not on fungal *plb1*, supporting that host cPLA$_2\alpha$ and cysteinyl LTs contribute to *C. neoformans* traversal of HBMEC and penetration into the brain, independent of *Cryptococcus* phospholipase. These findings are also consistent with those of pharmacological inhibition studies (Fig 3D, E & F), where *C. neoformans* penetration into the brain was prevented by administration of montelukast alone and in combination with fluconazole, indicating that host-derived, rather than cryptococcal-derived, eicosanoid production is responsible for *C. neoformans* penetration of the blood-brain barrier. In addition, we showed that *C. gattii* strains exhibited the ability to traverse the HBMEC monolayer and their traversal frequency (1.3 % to 2.4 %) was similar to that of *C. neoformans*. Of interest, *C. gattii* traversal was inhibited by AACOCF3 and montelukast (Fig 5D). Taken together, the above findings demonstrate that host cPLA$_2\alpha$, 5-LO and cysteinyl LTs are exploited by *C. neoformas var. neoformans* (strain 3501A), *C. neofromans var. grubii* (strain H99) as well as *C. gattii* strains for penetration of the blood-brain barrier.

Protein kinase C (PKC) is a family of at least 10 serine/threonine kinases that transduce multiple signals in the regulation of a variety of cellular functions, which include cytoskeleton rearrangements (Hryciw *et al.*, 2005; Larsson *et al.*, 2006). We have previously shown that *C. neoformans* exploits host cell cytoskeleton rearrangements for penetration of HBMEC monolayer, as documented by microvilli formation at the entry sites of HBMEC (Chang *et al.*, 2004). We, therefore, hypothesized that *C. neoformans* exploits PKCα for traversal of HBMEC monolayer. The role of PKCα in *C. neoformans* traversal of HBMEC monolayer was shown by our demonstrations that PKCα activation occurs in response to *C. neoformans* in a time-dependent manner in HBMEC (Fig. 6A), and that *C. neoformans* traversal was significantly decreased in HBMEC expressing dominant-negative PKCα compared to the control vector-transfected HBMEC (Fig. 6, B & C).

More importantly, PKCα activation in response to *C. neoformans* was inhibited by cPLA$_2\alpha$ inhibitor (AACOCF3) and CysLT1 antagonist (montelukast), but not by BLT1 antagonist (CP105696) (Fig. 6A). These findings indicate that host cPLA$_2\alpha$, 5-LO and cysteinyl LTs, but not LTB4, contribute to *C. neoformans* traversal of HBMEC monolayer, most likely via PKCα activation. This concept was further supported by our demonstration that exogenous cysteinyl LT (LTD4, 1 μM) significantly enhanced *C. neoformans* traversal of control vector-transfected HBMEC compared to vehicle control (0.5% ethanol), while it failed to exhibit such an enhancement in HBMEC expressing dominant-negative PKCα (Fig. 6, B & C). In addition, PKCα activation was shown to occur in response to LTD4, but not to LTB4, in HBMEC (Fig 6D). Taken together, these findings demonstrate for the first time that PKCα is downstream of cPLA$_2\alpha$, 5-LO and cysteinyl LTs in *C. neoformans* penetration of the blood-brain barrier.

cPLA$_2\alpha$ has been shown to be involved in the development of arthritis, bone resorption and pulmonary fibrosis (Hegen *et al.*, 2003; Miyaura *et al.*, 2003; Nagase *et al.*, 2002), while LTs

have been involved in respiratory diseases, allergic diseases and cardiovascular diseases (Evans *et al.*, 2008; Funk *et al.*, 2005; Montuschi *et al.*, 2007; Peters-Golden and Henderson, 2007), but the roles of cPLA$_2\alpha$, 5-LO and LTs in *C. neoformans* penetration of the blood-brain barrier have not been explored. Our findings reported here demonstrate for the first time that (a) *C. neoformans* exploits host cPLA$_2\alpha$ and 5-LO for the generation of cysteinyl LTs responsible for penetration of the blood-brain barrier *in vitro* and *in viv*o, (b) the actions of cysteinyl LTs occur via CysLT1 and PKCα, and (c) the contribution of host cPLA$_2\alpha$ and CysLT1 to *C. neoformans* penetration of the blood-brain barrier was independent of cryptococcal *plb1*. Our findings also demonstrate that inhibition of host molecules exploited by *C. neoformans* and *C. gattii* for penetration of the blood-brain barrier, as shown here with cPLA$_2\alpha$ inhibitor and CysLT1 antagonist, is likely to provide a novel approach for prevention of *C. neoformans* and *C. gattii* penetration into the brain, the essential step required for development of meningoencephalitis. We also show that the CysLT1 antagonist (montelukast) in combination with anti-fungal drug (fluconazole) was significantly more effective than single agents alone in therapy of *C. neoformans* CNS infection. These findings suggest that pharmacologic inhibition of host factors involved in *C. neoformans* penetration of the blood-brain barrier is a useful adjunct to anti-fungal drugs in prevention and therapy of *C. neoformans* meningoencephalitis.

Experimental procedures

Reagents

Arachidonic acid (AA) was purchased from Cayman Chemical Company (Ann Arbor, MI). Evans Blue was purchased from Sigma (St Louis, NO). Arachidonyltrifluoromethyl ketone (AACOCF3; cPLA$_2$ inhibitor) was purchased from Biomol Laboratories. (Plymouth Meeting, PA). Leukotriene D4 (LTD4), Leukotriene B4 (LTB4), and montelukast (cysteinyl-leukotriene type 1 receptor antagonist) were purchased from Cayman Chemical Company (Ann Arbor, MI). CP105696 (LTB4 receptor antagonist) was a gift from Pfizer. cPLA$_2$, phospho-cPLA$_2\alpha$ and phospho-PKCα antibodies were purchased from Cell Signaling Technologies (Danvers, MA), and PKC antibodies, PECAM-1 antibodies, and cysteinyl-leukotriene type 1 receptor (CysLT1) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA)

Mice

cPLA$_2\alpha^{-/-}$ and littermate control cPLA$_2\alpha^{+/+}$, either male or female, between 10~13w and 18~26g, that had been backcrossed on the BALB/C strain for >10 generations (Sapirstein *et al.*, 2005; Zhu et al, 2010), and female 5-LO$^{-/-}$ (129-Alox5$^{tm1Fun/J}$) and strain-matched wild-type mice (129SvEv), 9~12w and 18~26g (Serezani *et al.*, 2005; Zhu et al, 2010), were used. All procedures and handling techniques were approved by The Johns Hopkins Animal Care and Use Committee and Fujian Medical University Animal Care and Use Committee, Fuzhou, China.

Yeast strains

C. neoformans strains H99 and B-3501A represent encapsulated serotype A and D strains, respectively, that have been used for genomic sequencing (Loftus *et al.*, 2003). *plb1* isogenic

strain of H99 was generated as previously described (Cox *et al.*, 2001). GFP-tagged H99 (H99-GFP) was provided by Robin C. May, University of Birmingham, UK (Voelz *et al.*, 2010). *C. gattii* strains were provided by S. Zhang, Johns Hopkins Hopsital Microbiology Laboratory. Yeast cells were grown aerobically at 37 °C in 1 % yeast extract, 2 % peptone and 2 % glucose (YPD) broth. Cells were harvested at early exponential phase, washed with PBS and resuspended in Hams-F12/M199 (1: 1, v/v), 5 % fresh human serum (experimental medium). The numbers of *Cryptococcus* cells were determined by direct counting from a haemocytometer, which was verified by determinations of colony forming units (CFUs) on YPD agar after 2 days of incubation at 30° C.

Characterization and culture of HBMEC

HBMEC were isolated and characterized as described previously (Stins et al., 1997). Briefly, brain specimens were cut into small pieces and homogenized in DMEM containing 2 % FBS (DMEM-S) using a Dounce homogenizer with a loose fitting. The homogenate was centrifuged in 15 % dextran in DMEM-S for 10 min at 10 000 g. The pellet containing crude microvessels was further digested in a solution containing 1 mg/ml collagenase/dispase in DMEM-S for 1 h at 37 °C. Microvascular capillaries were isolated by adsorption to a column of glass beads (0.25–0.3 mm) and washing off from the beads. HBMEC were plated on rat tail collagen/fibronectin-coated dishes or glass coverslips and cultured in RPMI 1640-based medium with growth factors, 10 % heat-inactivated FBS, 10 % NuSerum, 5 U heparin ml-1, 2 mM L-glutamine, 1 mM sodium pyruvate, non-essential amino acids, vitamins and 100 U penicillin and streptomycin ml-1. Viability of HBMEC was assessed by examining morphology and by trypan blue exclusion. HBMEC were positive for factor VIII-Rag, took up fluorescently labeled acetylated low-density lipoprotein and expressed γ-glutamyl transpeptidase. HBMEC were maintained in RPMI-based medium, including 10 % FBS and 10 % NuSerum (BD Biosciences), at 37 °C in a humid atmosphere of 5 % CO2.

Identification of montelukast affecting *C. neoformans* traversal of primary HBMEC monolayer

We used *C. neoformans* traversal of the primary HBMEC monolayer as a biologically relevant assay for screen of the Johns Hopkins Drug Library (JHDL) (Chong *et al.*, 2006) for identification of targets affecting *C. neoformans* traversal of the blood-brain barrier, as follows. Primary HBMEC grown in 96-well Transwell inserts (with a pore size of 8 μm) were incubated with the JHDL (at a final concentration of 10 μM) for 60 min at room temperature, and then examined for *C. neoformans* traversal, as previously described (Chang *et al.*, 2004; Maruvada *et al.*, 2012). This screening assay included strain H99 in vehicle (DMSO)-treated HBMEC as a positive control for transcytosis, while the wells without HBMEC were used as control for any inhibitory effect of the drugs on growth of *C. neoformans*. Since this JHDL contains antifungal drugs, those wells exposed to antifungals were used as a positive control for determination of drugs that affect *C. neoformans* growth. The assay was highly reproducible, and the coefficient of correlation from at least two separate experiments was r = 0.98 (P<0.0001). From this screen, we identified montelukast, an antagonist of cysteinyl leukotriene type 1 receptor (CysLT1) (Peters-Golden *et al.*, 2007), inhibited *C. neoformans* traversal of HBMEC, without affecting HBMEC integrity, as assessed by live/dead stain (Molecular Probes) and *C. neoformans* growth. CysLT1 has not

been previously appreciated for its involvement in *C. neoformans* penetration of the blood-brain barrier.

Infection of HBMEC with adenovirus

HBMEC (~70% confluency) were infected (at MOI of 50) with dialyzed adenovirus of Ad5CA dominant negative-PKC-α and vector control, as described previously (Gorshkova *et al.*, 2008)

Traversal of C. neoformans across the HBMEC monolayer

HBMEC were cultured on Transwell polycarbonate tissue-culture inserts with a pore diameter of 8 μm (Corning Costar) for 5 days (Chang *et al.,* 2004). On the morning of the assay, HBMEC monolayer was washed with experimental medium and 10^7 *C. neoformans* cells were added to the upper chamber. At 9 h of incubation at 37 °C, sample was taken from the lower chamber and plated for determinations of CFUs. Colonies were counted after 2 days of incubation at 30° C. The integrity of the HBMEC monolayer was assessed by measurements of the transendothelial electrical resistance (TEER) before and after assays.

Experimental hematogenous C. neoformans CNS infection in mice

Mice were anesthetized with pentobarbital sodium given subcutaneously at 50 mg/kg. Montelukast 5 mg/kg in 100μl PBS was injected through tail vein 15 min before and 6h after tail vein injection of *C. neoformans* (1×10^5 cells in 100 μl PBS). Control mice received 3.3% ethanol in 100 μl PBS. 24h after *C. neoformans* injection, mouse chest was cut open, and blood from right ventricle was collected and plated for bacterial counts (CFUs). Then mouse was perfused with a mammalian Ringer's solution by transcardiac perfusion through a 23-gauge needle inserted into the left ventricle of the heart under the perfusion pressure of about 100 mmHg. The perfusate exited through a cut in the right atrium. The composition of the mammalian Ringer solution was: (in mM) 132 NaCl, 4.6 KCl, 2 CaCl2, 1.2 MgSO4, 5.5 glucose, 5.0 NaHCO3, and 20 HEPES and Na-HEPES, containing 10 mg/ml BSA; pH of the Ringer solution was maintained at 7.40–7.45 by adjustment of the ratio of Na-HEPES to HEPES. Ringer solution used in this study has been shown not to change the microvessel permeability (Zhu *et al*, 2004). 30 min after perfusion of Ringer solution, mice were decapitated. The brains were removed, weighed and homogenized in 2ml RPMI followed by plating for brain Cryptococcus counts in YPD agar plates. Kidneys, lungs and spleens were also dissected out for determinations of bacterial counts (CFUs/gm). The brains were removed, weighed and homogenized in 2ml RPMI followed by plating for brain Cryptococcus counts in YPD agar plates.

Intratracheal inoculation

Mice were anesthetized with pentobarbital sodium given subcutaneously at 50 mg/kg. A small incision was made in the skin over the trachea. A 30-gauge needle (Becton Dickinson, Franklin Lakes, NJ) was attached to a tuberculin syringe (Becton Dickinson). The needle was bent and inserted into the trachea, and (containing 10^5 CFU Cryptococcus) was delivered. For control (sham) group mice, a 30-μl PBS was delivered. The skin was sutured

with a cyanoacrylate adhesive, and the mice recovered with no visible trauma (Noverr *et al.*, 2003).

Immunofluorescence

After 7 days of intratracheal inoculation with GFP-tagged *C. neoformans* (H99-GFP), mouse chest was cut open, and mouse was perfused with a mammalian Ringer's solution by transcardiac perfusion through a 23-gauge needle inserted into the left ventricle of the heart under the perfusion pressure of about 100 mmHg for 20 min. The brains were removed and put in liquid nitrogen. Serial cryosections (10 μm) were incubated overnight with a monoclonal rat anti-PECAM-1 primary antibody (Santa Cruz, CA) and a polyclonal rabbit anti-CysLT1primary antibody (Santa Cruz, CA). Afterward, sections were incubated with Dylight 488 goat Anti-Rat IgG secondary antibody (EarthOx Life Sciences, Millbrae, CA) and Cy3 goat anti-Rabbit IgG secondary antibody (Beyotime Biotechnology, Shanghai, China). Slides were imaged through fluorescence microscopy with a Nikon Eclipse Ti-S and DS-Ri2 digital camera (Nikon)

Immunoblotting and immunoprecipitation

The lysates of HBMEC incubated with *C. neoformans* were prepared for Western blotting and immunoprecipitation as described previously (Reddy *et al.*, 2000).

Statistical Analysis

Data are expressed as mean ± SEM. Differences of Cryptococcus counts in the brain, spleen, kidney and lung (CFUs/gm of organs) between different groups of mice were determined by Wilcoxon rank sum test or Student's t test. Differences of Cryptococcus traversal across HBMEC monolayer were determined by Student's t test. $P<0.05$ was considered significant.

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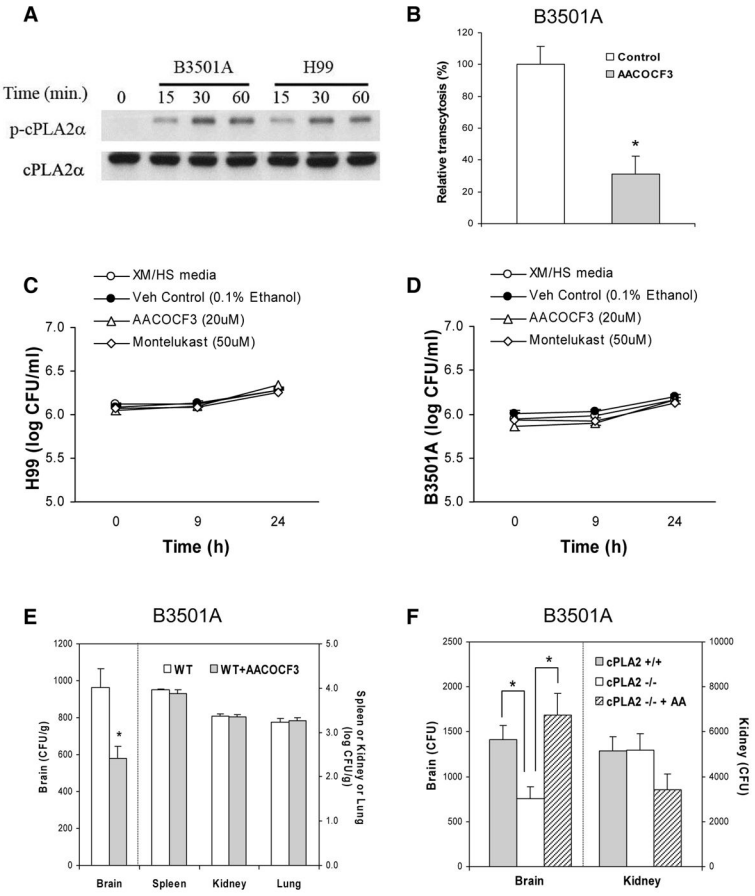

Figure 1. Host cPLA$_2$α is involved in *C. neoformans* traversal of the HBMEC monolayer and penetration into the brain

(A) Serine phosphorylation of cPLA$_2$α occurs in response to *C. neoformans* strains B-3501A and H99 in HBMEC in a time-dependent manner. The lysates of HBMEC incubated with *C. neoformans* were examined for phospho-cPLA$_2$α and cPLA$_2$α in Western blot analysis using specific antibodies.

(B) AACOCF3 significantly inhibited penetration of *C. neoformans* strain B-3501A across the HBMEC monolayer. Penetration of B-3501A across the HBMEC monolayer was examined in Transwell filters (8 μm pore size). HBMECs were pretreated with 20 μM AACOCF3 for 60 min and then 20 μM AACOCF3 solution were added to every 2 hr (0, 2, 4, 6, and 8 hr, respectively) after addition of 1×10^6 CFU of strain B-3501A to the upper chamber. Our previous studies revealed that traversal of *C. neoformans* across the HBMEC monolayer was evident over a 9 hr incubation period (Chang et al., 2004). At 9 hr of incubation at 37 °C, sample was taken from the lower chamber and plated for determinations of CFUs. Colonies were counted after 2 days of incubation at 30° C. Transcytosis frequency (%) was determined by (total CFUs recovered from the lower chamber/total number of cryptococcal cells added to the upper chamber) × 100, and expressed as relative frequency compared to transcytosis frequency with vehicle control (0.125% ethanol). Transcytosis frequency of *C. neoformans* strain B-3501A across HBMEC monolayer at 9 hr of incubation at 37 °C ranged between 1.4 and 8.9 %. Data shown are means ± SEM of triplicates. * $P<0.05$, unpaired t-test, compared to vehicle control.

(C and D) AACOCF3, a cPLA2α inhibitor, and Montelukast, a CysLT1 receptor antagonist, show no significant effects on growth of *C. neoformans* strains H99 (C) or B3501A (D). *C. neoformans* strains H99 (C) or B3501A (D) were growing in Hams-F12/M199 (1: 1, v/v), 5 % heat inactivated fetal bovine serum (FBS) (experimental medium, XM) and 5 % fresh human serum (HS) at 37°C, 5% CO_2 incubator. Cryptococcus counts were performed at 0, 9, and 24h incubation time. Data shown are mean ± SEM. Each experiment was performed in triplicate.

(E) AACOCF3 decreases *C. neoformans* B-3501A penetration into the brain of BALB/c mice (WT). 4 mM AACOCF3 in 50μl PBS was injected through tail vein 30 min before, 3 hr and 6 hr after tail vein injection of *C. neoformans* B-3501A (1×10^5 cells in 100μl PBS). Control mice were injected with 7% ethanol in100 μl PBS. *Cryptococcus* counts were determined 24 hr after injection of B-3501A. Data shown are mean ± SEM. $*P<0.05$, Student's t-test between WT (n=5), and WT + AACOCF3 (n=5).

(F) *C. neoformans* penetration into the brain of $cPLA_2\alpha-/-$ and their wild type mice. *Cryptococcus* counts were determined 24 hr after injection of B-3501A (1×10^5 cells) in 100 μl PBS through tail vein. 40 μM arachidonic acid (AA) in 100 μl PBS (1.2 μg/mouse) was injected through tail vein 30 min before *C. neoformans* injection. Data shown are mean ± SEM. $*P<0.05$, Student's t-test between $cPLA_{2\alpha}$ +/+ (n=5), $cPLA_{2\alpha}-/-$ (n=5), and $cPLA_{2\alpha}-/-$ + AA (n=3).

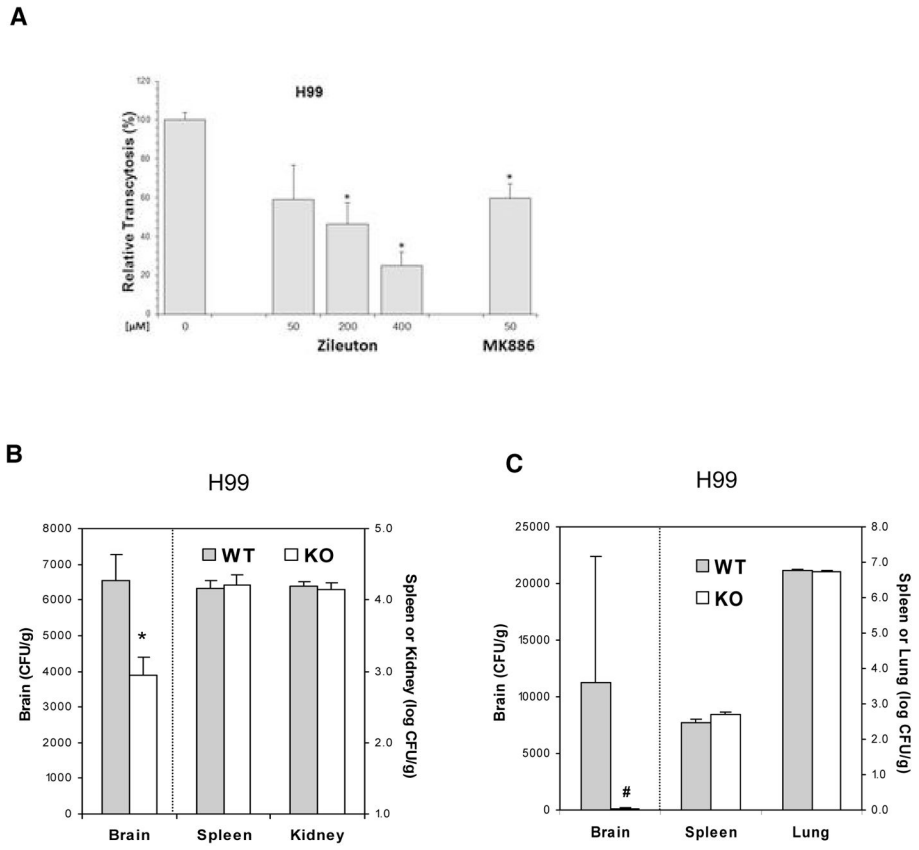

Figure 2. 5-LO is involved in *C. neoformans* traversal across the HBMEC monolayer and penetration into the brain

(A) Zileuton (5-LO inhibitor) and MK886 (FLAP inhibitor) inhibited *C. neoformans* strain H99 traversal of the HBMEC monolayer in a dose-dependent manner. Data shown are means ± SEM of triplicates. * $P<0.05$, unpaired t-test, compared to vehicle control.

(B) *C. neoformans* penetration into the brain following intravenous inoculation is significantly decreased in 5-LO−/− mice compared to their wild type animals. The yeast counts from the brain, kidney, and spleen were determined 24 hr after injection of H99 (1×10^5 cells) in 100μl PBS through tail vein. Data shown are mean ± SEM. * $P<0.05$ by Student's t-test between wild type (WT, n=4) and 5-LO−/− mice (KO, n=4).

(C) *C. neoformans* penetration into the brain following intratracheal inoculation is significantly decreased in 5-LO−/− mice compared to wild type mice. The yeast counts from the brain, spleen, and lung were determined 7d after intratracheal inoculation of H99 (1×10^5 cells). Data shown are mean ± SEM. # $P<0.05$ by Wilcoxon Rank Sum test between wild type (WT, n=6) and 5-LO−/− mice (KO, n=6).

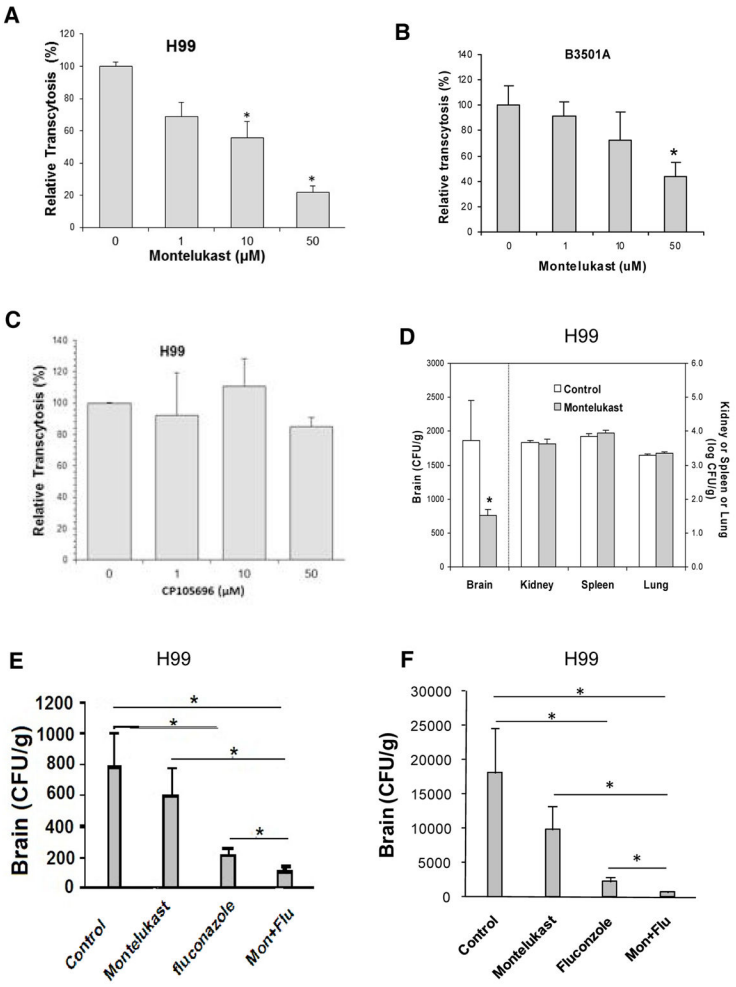

Figure 3. Cysteinyl LTs are involved in *C. neoformans* traversal across the HBMEC monolayer and penetration into the brain

(A and **B)** Montelukast (CysLT1 antagonist), but **(C)** not CP105696 (BLT1 antagonist) inhibited traversal of *C. neoformans* strains H99 (**A** and **C**) and B-3501A (**B**) across the HBMEC monolayer. Transcytosis of H99 or B-3501A was examined in Transwell filters (8 μm pore size). HBMECs were pretreated with montelukast at indicated concentrations (1, 10, 50 μM) for 60 min and then montelukast solution were added every 4 hr (0, 4, and 8 hr, respectively) after addition of 1×10^6 CFUs of strain H99 or B-3501A to the upper chamber. Data shown are mean ± SEM of triplicates. * $P<0.05$ by unpaired t-test, compared to vehicle control (0.1% ethanol).

(D) Montelukast decreases *C. neoformans* H99 penetration into the brain of BALB/c mice. 1.6 mM montelukast in 100μl PBS was injected through tail vein 15 min before, and 6 hr after tail vein injection of H99 (1×10^5 cells in 100μl PBS). Control mice received 4% DMSO in100 μl PBS. The yeast counts were determined after 24 hr injection of H99 (n=6 for each group). Data shown are mean ± SEM. * $P<0.05$ by two-tailed Wilcoxon Rank-Sum test.

(**E** and **F**) Effects of montelukast and fluoconzole on *C. neoformans* penetration into the brain of BALB/c mice (**E**) 7 and (**F**) 14 d after intratracheal inoculation of H99. On the day

of intratracheal inoculation of *C. neoformans*, montelukast (Mon) was given at dose of 5 mg/kg in 100 μl PBS via intraperitoneal (i.p.) injection 30 min before and 6 hr after inoculation of H99, followed by 5 mg/kg i.p. daily for 6 and 13 d. After intratracheal inoculation of *C. neoformans*, fluconazole (Flu) was suspended in sterile water and was given at dose of 15 mg/kg twice daily in a volume about 100 μl by gavage for 6 and 13 d. Control animals received 3.3% DMSO i.p. and sterile water via oral gavage. The yeast counts from the brain were determined 7 and 14 d after intratracheal inoculation of H99 (1×10^5 CFUs). Data represent mean ± SEM (n=10). * $P<0.05$ by Student's t-test.

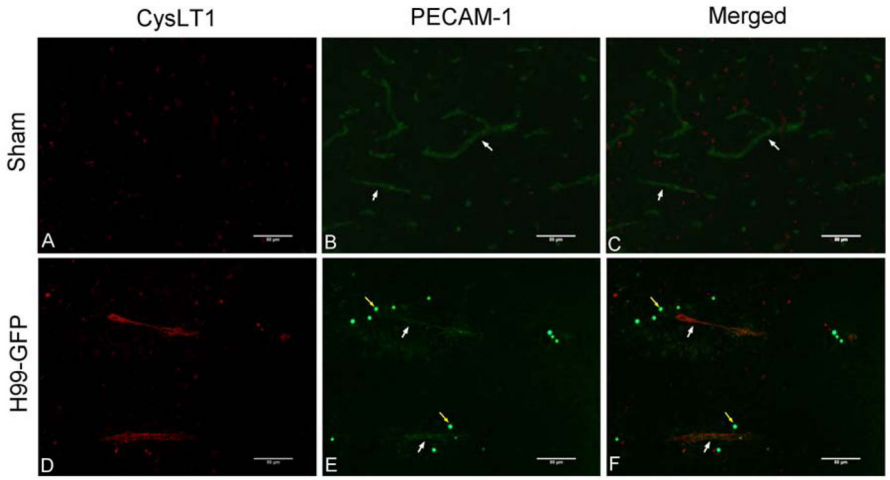

Figure 4. Immnofluorescence demonstration of CysLT1 in the mouse brain microvessels following intratracheal inoculation of GFP-tagged *Cryptococcus neoformans* (H99-GFP)

CysLT1 expression was not evident in the brain microvessels of uninfected mice (Sham), while CysLT1 expression was demonstrated in the brain microvessels (shown with PECAM-1) of mice infected with GFP-tagged *Cryptococcus neoformans* (H99-GFP). A and D, CysLT1 expression; B and E, microvessels shown with PECAM-1; C and F, merge of CysLT1 and PECAM-1 staining. Yellow arrows indicate H99-GFP, and white arrows indicate microvessels. Scale bar= 50 μm.

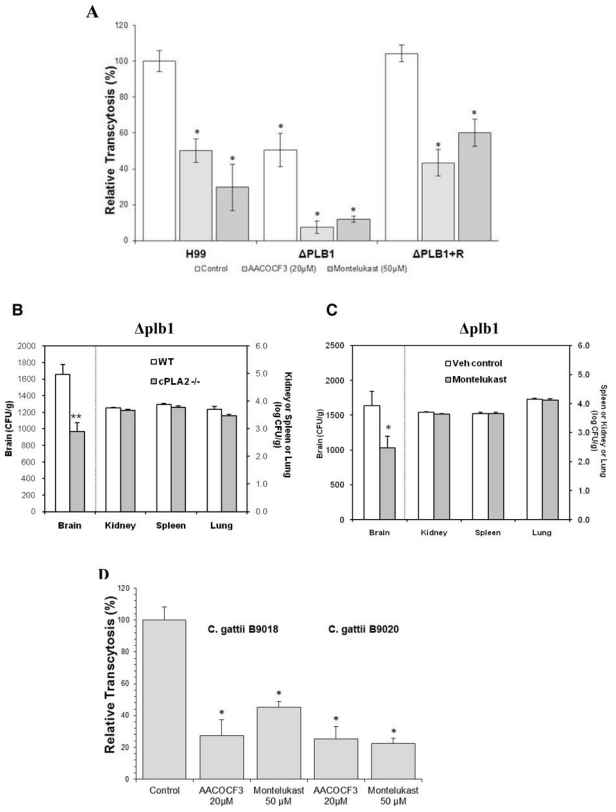

Figure 5. Roles of cryptococccal Plb1 in *C. neoformans* penetration of the blood-brain barrier

(A). The *plb1* mutant (ΔPLB1) was significantly defective in traversal of the HBMEC monolayer compared to its parent strain H99 and reconstituted strain (ΔPLB1 + R). AACOCF3, a cPLA2α inhibitor, and montelukast, the CysLT1 antagonist, significantly inhibited the HBMEC traversal of the *plb1* mutant, its parent strain and reconstituted strain. Traversal of the HBMEC monolayer was examined in Transwell filters (8 μm pore size). HBMEC were pretreated with 20 μM AACOCF3 for 60 min and then 20 μM AACOCF3 fresh solution were added to the transwell every 2 hr (0, 2, 4, 6, and 8 hr, respectively) or HBMECs were pretreated with 50 μM montelukast for 60 min and then montelukast solution were added every 4 hr (0, 4, and 8 hr, respectively) after addition of 1×10^6 CFUs of cryptococcal strains to the upper chamber. Data shown are means ± SEM of triplicates. * $P<0.05$, Student's t-test compared to vehicle control (n=3).

(B). The penetration of the *plb1* mutant into the brain is significantly decreased in $cPLA_2\alpha-/-$ mice compared to their wild type animals (WT). The yeast counts in the brain, kidney, spleen and lung were determined 24 hr after injection of the *plb1* mutant (1×10^5 cells) in 100 μl PBS through the tail vein. Data shown are mean ± SEM. ** $P<0.001$, Student's t-test between WT and cPLA2 −/− mice (n=7).

(C). Montelukast (the CysLT1 antagonist) significantly decreases the penetration of the *plb1* mutant into the brain of BALB/cJ mice. Montelukast 5 mg/kg in 100μl PBS was injected through tail vein 15 min before and 6 hr after tail vein injection of the *plb1* mutant (1×10^5 cells in 100μl PBS). Control mice received 3.3% ethanol in 100μl PBS. The yeast

counts were determined after 24 hr injection of the *plb1* mutant (n=6). Data shown are mean ± SEM. * $P<0.05$, Wilcoxon Rank-Sum test.

(D) *C. gattii* strains traversal of the HBMEC monolayer was inhibited by AACOCF3 and montelukast. Data shown are means ± SEM of triplicates. * $P<0.05$, Student's t-test compared to vehicle control (n=3).

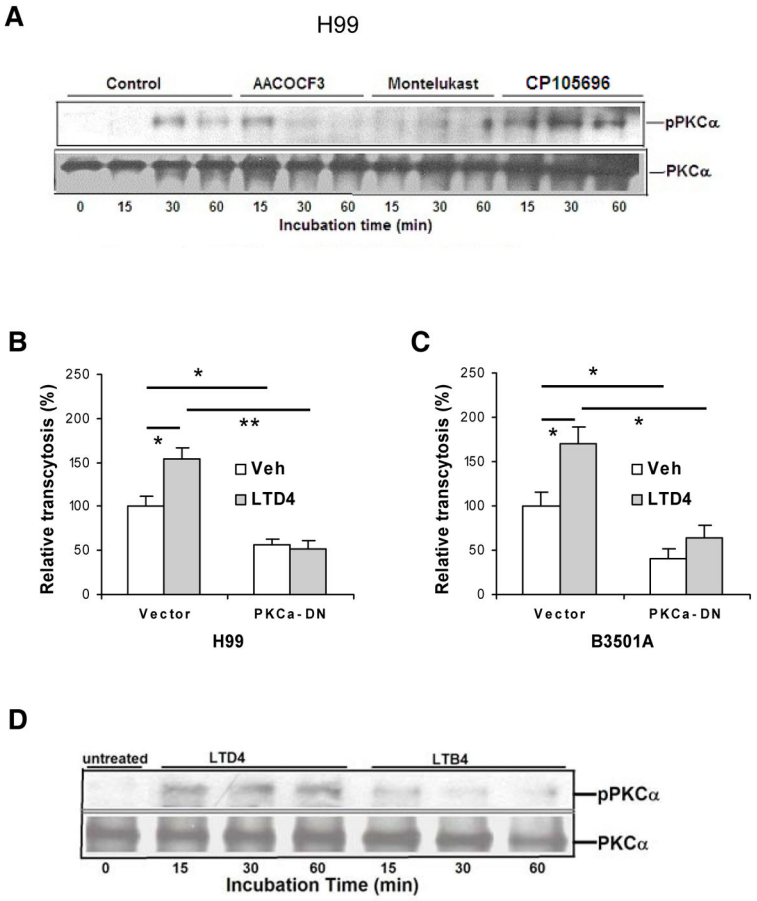

Figure 6. cPLA$_2$α and cysteinyl LTs contribute to *C. neoformans* traversal of the HBMEC monolayer via PKCα

(A) HBMEC incubated with *C. neoformans* strain H99 at 37°C for various times in the presence of inhibitors/antagonists or vehicle control were immunoprecipitated with PKCα antibody, and then assessed for phospho-PKC by Western blotting with phospho-PKC antibody. The HBMEC lysates were examined for the total amounts of PKC.

(B and **C)** Effect of LTD4 on penetration of *C. neoformans* strain H99 **(B)** or B-3501A **(C)** across monolayer of HBMEC transfected with dominant-negative PKCα construct. Subconfluent HBMEC in transwell insert were infected with adenovirus Ad5CA dominant negative-PKC-α (DN) or vector control (Vec) at MOI of 50. HBMEC were pretreated with 1 μM LTD4 for 30 min followed by transcytosis assays. 0.5% ethanol was used as vehicle control (Veh). Data shown are mean ± SEM. Each experiment was performed in triplicate. *P <0.05; **P <0.01, Student's t test.

(**D**) LTD4 is involved in PKCα activation in HBMEC. HBMEC incubated with LTD4 (1 μM) or LTB4 (1 μM) at 37°C for various times (min) were immunoprecipitated with PKCα antibody, and then assessed for phospho-PKC by Western blotting with phospho-PKC antibody. The HBMEC lysates were examined for the total amounts of PKC.