Quantitative analysis reveals internalization of Cryptococcus neoformans by brain endothelial cells in vivo

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Abstract

Migration of *Cryptococcus neoformans* from the blood to the brain parenchyma is crucial to cause fatal meningoencephalitis. Although mechanisms involved in brain migration of *C. neoformans* have been widely studied in vitro, less is known about how the fungus crosses the blood-brain barrier (BBB) in vivo. This is in part because of the lack of an approach to quantitatively analyze the dynamics of fungal transmigration into the brain across the BBB in vivo. In this study, we report a novel approach to quantitatively analyze the interactions between *C. neoformans* and brain endothelial cells in a mouse model using flow cytometry. Using this system, we show that *C. neoformans* was internalized by brain endothelial cells in vivo and that mice infected with acapsular or heat-killed *C. neoformans* yeast cells displayed a lower frequency of endothelial cells containing the yeast cell compared to mice infected with wild-type or viable yeast cells, respectively. We further demonstrate that brain endothelial cells were invaded by serotype A strain (H99 strain) at a higher rate compared to serotype D strain (52D strain). Our experiments established that internalization of C. neoformans by brain endothelial cells occurred in vivo and offered a powerful approach to quantitatively analyze fungal migration into the brain.

Introduction

Cryptococcus neoformans (*C. neoformans*) is an encapsulated fungal yeast (FIG. 1) found worldwide. The original site of *C. neoformans* infection is in the lung, healthy individuals control fungal growth while fungus disseminate through blood stream and cross blood-brain-barrier causing fatal meningoencephalitis (FIG. 2). Cryptococcosis is caused by encapsulated fungal pathogen *Cryptococcus neoformans* (1). The fungal cells exist in the environment and when they are inhaled into the lung, they will initially induce lung infection. In immunocompetent individuals, the fungal cells are usually cleared by the immune cells or establish a latent infection in the lung. However, in immunocompromised individuals including AIDS patients, the organisms can disseminate from the lung to the brain, causing meningoencephalitis (1, 2). Cryptococcal meningoencephalitis is often fatal without treatment, and even with treatment two-thirds of patients die within a few months of diagnosis (3, 4). Worldwide, fatalities due to cryptococcal meningoencephalitis were recently estimated at more than 181,100 cases each year (5). It is believed that crossing of the blood-brain barrier (BBB) by *C. neoformans* is a critical step to cause meningoencephalitis (6). As such, the mechanism(s) of BBB crossing by *C. neoformans* is fundamental for understanding cryptococcal pathogenesis. There are several well-established pathways contributing to *C. neoformans* invasion to the brain, including transcytosis, paracellular pathway, causing tissue damages and trojan horse. Previous studies regarding the fungal invasion were mainly performed in vitro using endothelial cell lines mimicking the in vivo condition. However, relatively less is known about how *C. neoformans* transmigrates into the brain across the BBB in vivo, mainly because of technical challenges characterizing dynamic events of fungal BBB crossing in vivo. In vivo studies are urgently needed for solve the brain invasion problems.

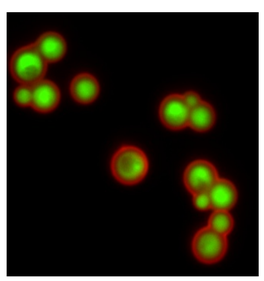

FIG. 1 *C. neoformans*
GFP-tag *C. neoformans* H99 strain with fluorescently labelled capsule(red)

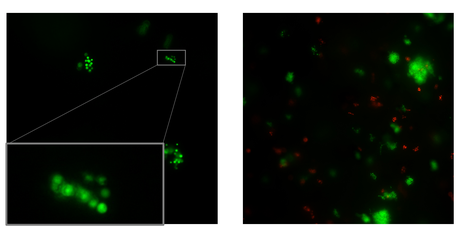

FIG. 2 *C. neoformans* growth in the brain
Intravital microscopy shows mouse brain with fluorescently labelled *C. neoformans* H99 infection 3dpi A. GFP-tagged *C. neoformans* grows inside the blood vessels form a lineal colony. B. GFP-tagged H99 (Green) and Td-tomato H99 (Red) 1:1 ratio infection to mouse shows fungus cross blood-brain-barrier and form fungal cluster in brain parenchyma.

Project Goal

To establish the novel strategy using flow cytometry to quantitatively analyze *Cryptococcus neoformans* transcytosis rate to brain endothelial cells in vivo.

Internalization of C. neoformans by brain endothelial cells occurs in vivo

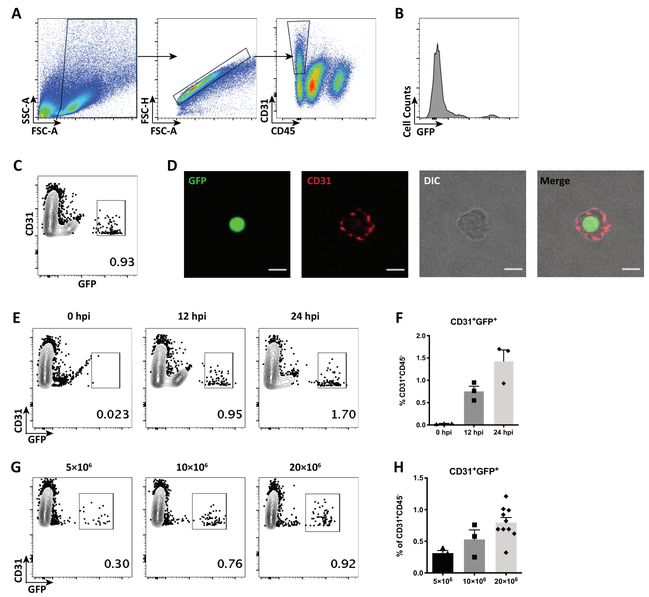

FIG. 3 Quantitative analysis of internalization of GFP-labeled C. neoformans by brain endothelization in vivo.
(A and B) Gating strategy of CD31+GFP+ cells. Endothelial cells were isolated from the brain of mice 24 h after i.v. infection with 20×10^6 GFP-labeled C. neoformans. Endothelial cells were defined as CD31+CD45-. CD45+ leukocytes and CD45 intermediate microglia were gated out after selecting CD45- population (A). A representative flow cytometry histogram showing the expression of GFP by CD31+CD45- cells (B). (C and D) Mice were infected with 20×106 GFP-labeled C. neoformans H99. Brain endothelial cells were purified 24 h after infection. (C). CD31+GFP+ cells were sorted by fluorescence-activated cell sorting (FACS). The sorted cells were subjected to analysis by confocal microscopy (D). (E and F) Mice were infected with 20×10^6 GFP-labeled C. neoformans H99. The frequency of CD31+GFP+ cells was determined 0, 12, and 24 h post infection by flow cytometry. Representative dot plots (E) and quantification (F). (G and H) Mice were infected with 5×10^6, 10×10^6, or 20×10^6 GFP-labeled C. neoformans H99. The frequency of CD31+GFP+ cells was determined 24 h post infection by flow cytometry. Representative dot plots (G) and quantification (H). Data shown are the mean ± SEM. Dots represent individual mice. Data are a representative of two independent experiments. Scale bars: 5 μm.

Using CFSE-labeled C. neoformans to quantitatively analyze the interaction between C. neoformans and brain endothelial cells in vivo

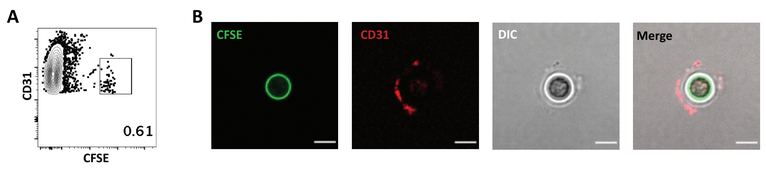

FIG. 4 Quantitative analysis of internalization of CFSE-labeled *C. neoformans* by brain endothelization in vivo.
(A and B) Mice were infected with 20×106 CFSE-labeled C. neoformans H99. Brain endothelial cells were purified 24 h after infection. The frequency of CD31+CFSE+ cells (gated on CD31+ CD45- cells) was determined by flow cytometry (A). CD31+CFSE+ cells were sorted by fluorescence-activated cell sorting (FACS). The sorted cells were subjected to analysis by confocal microscopy to determine the colocalization of brain endothelial cells (CD31+) and the yeast cells (CFSE+) (B). Scale bars: 5 μm.

Mice infected with acapsular C. neoformans exhibit a lower frequency of endothelial cells containing the yeast cell

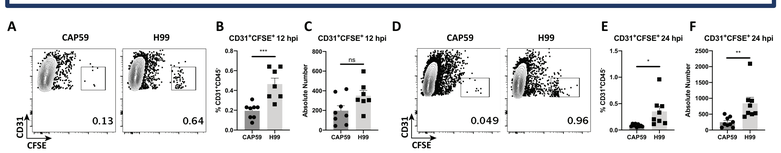

FIG. 5 Mice infected with acapsular *C. neoformans* display a lower frequency of infected brain endothelial cells compared to wild-type *C. neoformans*.
Mice were infected with 20×106 CFSE-labeled acapsular strain CAP59 or wild-type C. neoformans H99. Brain endothelial cells were purified 12 h (upper panel) or 24 h (lower panel) after infection. The frequency of CD31+CFSE+ cells (gated on CD31+CD45- cells) was determined by flow cytometry. (A and D) representative dot plots, (B and E) frequency, and (C and F) absolute number. Data from two independent experiments were pooled and are presented as mean ± SEM. Dots represent individual mice. ns, not significant; * p<0.05, ** p<0.01, *** p<0.001.

Mice infected with heat-killed C. neoformans display a lower frequency of endothelial cells containing the yeast cell

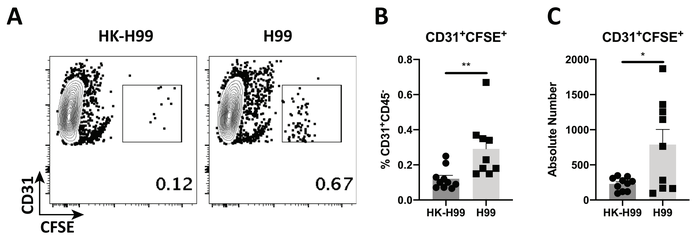

FIG. 6 Mice infected with heat-killed *C. neoformans* display a lower frequency of infected brain endothelial cells compared to viable *C. neoformans*.
Mice were infected with 20×106 CFSE-labeled heat-killed (HK-99) or viable C. neoformans H99. Brain endothelial cells were purified 24 h after infection. The frequency of CD31+CFSE+ cells (gated on CD31+CD45- cells) was determined by flow cytometry. (A) representative dot plots, (B) frequency, and (C) absolute number. Data from two independent experiments were pooled and are presented as mean ± SEM. Dots represent individual mice. * p<0.05, ** p<0.01.

Brain endothelial cells are invaded by serotype A of C. neoformans at a higher rate than serotype D strain

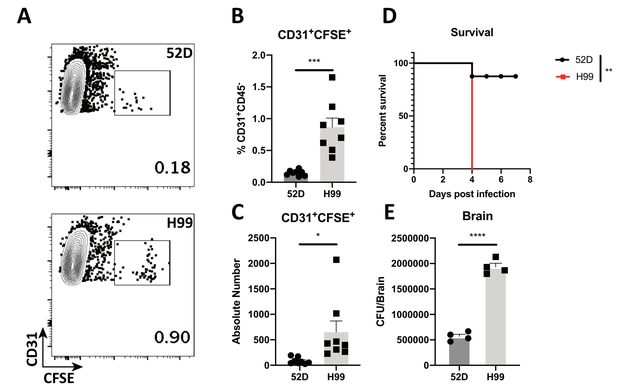

FIG. 7 The frequency of brain endothelial cells invaded by serotype A of *C. neoformans* is higher compared to serotype D.
(A-C) Mice were infected with 20×106 CFSE-labeled C. neoformans H99 (serotype A) or 52D (serotype D). Brain endothelial cells were purified 24 h after infection. The frequency of CD31+CFSE+ cells (gated on CD31+CD45- cells) was determined by flow cytometry. (A) representative dot plots, (B) frequency, and (C) absolute number. (D and E) Mice were infected with 20×106 C. neoformans H99 or 52D. (D) The survival was determined (n=5 mice/group). (E) The fungal burdens in the brain were determined on day 3 after infection (n=4 mice/group). Data from two independent experiments were pooled for (B) and (C), and data are presented as mean ± SEM. Dots represent individual mice. * p<0.05, ** p<0.01, ***p<0.001, **** p<0.0001.

Mice deficient in CD44 show a slightly lower frequency of brain endothelial cells containing C. neoformans.

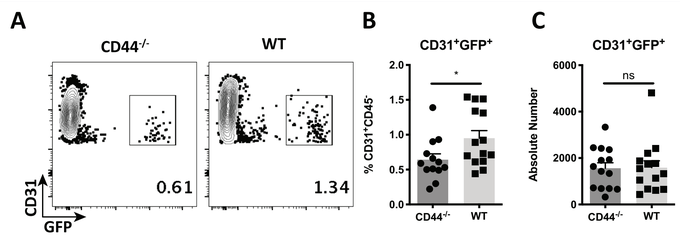

FIG. 8 CD44$^{-/-}$ mice exhibit a slightly lower frequency of brain endothelial cells invaded by *C. neoformans* compared to wild-type mice.
CD44-/- and wild-type (WT) mice were infected with 20×106 GFP-labeled C. neoformans H99. Brain endothelial cells were purified 24 h after infection. The frequency of CD31+GFP+ cells (gated on CD31+CD45- cells) was determined by flow cytometry. (A) representative dot plots, (B) frequency, and (C) absolute number. Data from three independent experiments were pooled and are presented as mean ± SEM. Dots represent individual mice. ns, not significant; * p<0.05.

Conclusion

We have established an approach to quantitatively analyze the interaction between C. neoformans and brain endothelial cells in vivo during infection by using flow cytometry.

Acknowledgement

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