**Protection analysis of *Cryptococcus neoformans* vaccine strain HK-fbp1 against fungal infections**

**Experimental design**

**Vaccine strain preparation**

*C. neoformans* wild type H99 and its mutant *fbp1*Δ were heat-killed following a procedure below. Fungal cells from YPD overnight cultures were precipitated and washed twice with sterile PBS. The cell suspension with the correct concentration was then aliquoted into Eppendorf tubes and heated on a hot plate at 75°C for 60 min. The viability of the cells following heat treatment was examined by plating the processed cell suspension on YPD agar plates; no colonies were recovered after incubation at 30°C for 3 days.

**Mouse vaccination**

Mice were vaccinated intranasally with 5 x $10^7$ heat-killed fungal cells in 50 µl volume per animal at day -42 unless otherwise specified. Each group of 10 mice were vaccinated again with the same dose of heat-killed fungal strains at day -12. A group of unvaccinated mice were served as a control.

**Challenge with *Cryptococcus* strains**

The vaccinated groups and unvaccinated control group were challenged with either 1 x $10^4$ live *C. neoformans* or *C. gattii* cells via intranasal inoculation. Infected animals were weighed and monitored daily for disease progression, and moribund mice were euthanized. All survivors were euthanized on day 65 after challenge with live H99 cells unless otherwise specified.

To test the vaccine protection against *Cryptococcus* strains in CD4 depleted mice, mice were treated via intraperitoneal administration of anti-CD4 (GK1.5, rat IgG2b) antibody (BioxCell, CAT#BE0003-1). Each mouse received 200 µg of GK1.5 or isotype (LTF-2, rat IgG2b) control antibody (BioxCell, CAT# BE0090-A050MG) antibodies in a volume of 200 µl PBS 9 days prior to the first vaccination, and weekly thereafter during the observation period. Efficient depletion was confirmed by measuring the prevalence of $CD4^+$ T cells in blood samples by flow cytometry on the day before the first vaccination (day -43) and the day before challenge (day -1). The depletion was also confirmed by measuring the prevalence of $CD4^+$ T cells in BALF and lung tissues by flow cytometry at the endpoint of the experiment.

**Challenge with *Aspergillus fumigatus***

We will induce and maintain neutropenia in mice before infection.

To induce and maintain neutropenia in mice, six-eight weeks old mice will be given an initial 150 mg/kg dose of cyclophosphamide (Cytoxan) via IP injection four days prior to challenge with *Aspergillus*. At day -1 prior to infection and day +2 post infection, mice will be given 100 mg/kg of cyclophosphamide via IP to maintain neutropenia. At day -1 prior to infection, mice will receive a single dose of Hydrocortisone Acetate (300 mg/kg) subcutaneously to impair macrophage clearance of *A. fumigatus*. The mice will be given sterile water and rodent chow containing Doxycycline (DoxDiet) to prevent opportunistic bacterial infections and will be housed in sterile caging at the ICPH ABSL-2 RAF throughout the study including the acclimation period.

For *Aspergillus* infection, the immunocompromised mice will be immobilized with a single IP injection of Ketamine/Xylazine (65/5mg/kg) then inoculated intranasally with a non-lethal dose of ~1.0 x10^6 spores of *A. fumigatus*. Mice developed lethal infection will be

euthanized by $CO_2$ inhalation. Lungs will be harvested and assessed for microbial burden by genotype specific qPCR and quantitative CFU assay. The mice will be monitored twice daily by the animal care staff or the Principal Investigator's research staff listed on this protocol. Mice will be observed for any signs of illness such as ruffled fur, recumbency, or inability to eat or drink. Those animals will be immediately euthanized by the veterinary, animal care or PI staff.

**References:**

Masso-Silval J, Espinosal V, Liu T, Wang Y, Xue C*, and Rivera A*. (2018). The development of protective immunity against *Cryptococcus neoformans* is controlled by the novel virulence factor Fbp1**.** mBio 9:e01828-17

Wang Y, Wang K, Rivera A*, and Xue C*. (2019) A heat-killed *Cryptococcus* mutant strain induces host protection against multiple invasive mycoses in a murine vaccine model. mBio 10:e02145-19