

Supplementary Information

Intramuscular immunization with a CpG-adjuvanted *Cryptococcus neoformans* vaccine induces sterilizing immune protection

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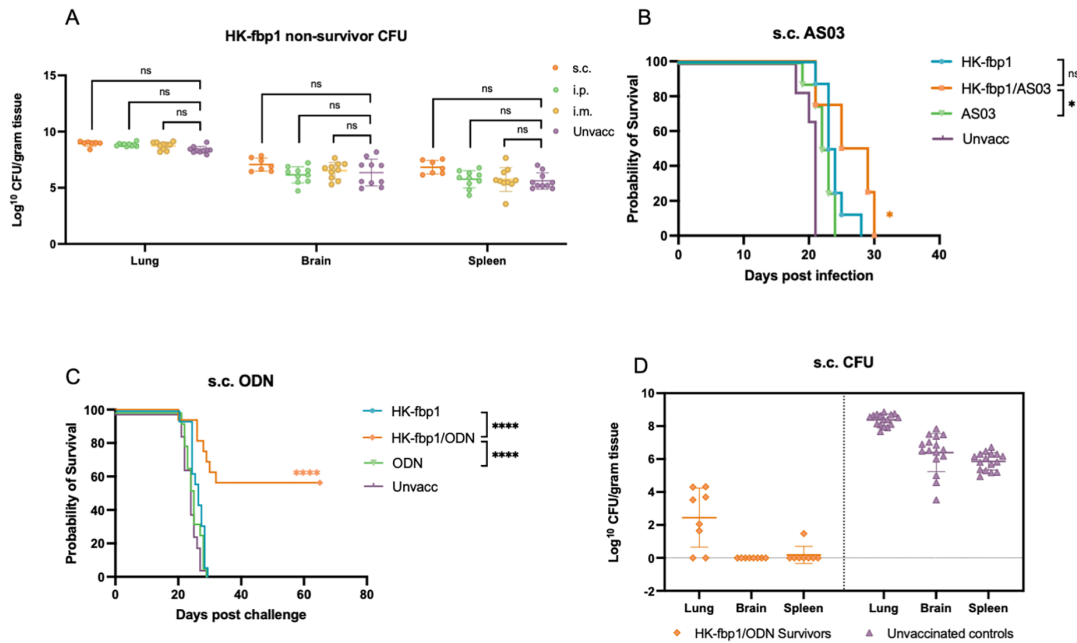


Figure S1. The AS03 adjuvanted HK-fbp1 vaccine (HK-fbp1/AS03) provides no protection against *C. neoformans* challenge in parental vaccination model. **A.** Fungal burdens in the lungs, brains, and spleens of non-surviving vaccinated mice and unvaccinated controls shown in (1B). $N = 7-10$ per group. **B.** Survival curves of wild-type BALB/c mice vaccinated with 5×10^7 HK-fbp1, 5×10^7 HK-fbp1 formulated with AddaS03, or AddaS03 alone via subcutaneous injection, followed by intranasal challenge with H99. $N = 8$ per group. **C.** Survival curves of wild-type BALB/c mice vaccinated with 5×10^7 HK-fbp1, 5×10^7 HK-fbp1 formulated with ODN2395, or ODN2395 alone via subcutaneous injection, followed by intranasal challenge with H99. $N = 16$ per group. **D.** Fungal burdens in the lungs, brains, and spleens of vaccinated surviving BALB/c mice at the study endpoint and of non-survivor unvaccinated controls shown in (S1C). $N = 8-16$ per group.

Each symbol represents an individual mouse. Survival data were analyzed by the Kaplan–Meier method., and statistical significance was determined using the log-rank (Mantel–Cox) test with Holm–Šidák correction for multiple comparisons (B, C). ****, $P < 0.0001$; *, $P < 0.05$. CFU data are presented as mean $\pm$ SD, and statistical significance was determined using one-way ANOVA followed by Tukey’s multiple-comparison test within each tissue (A).

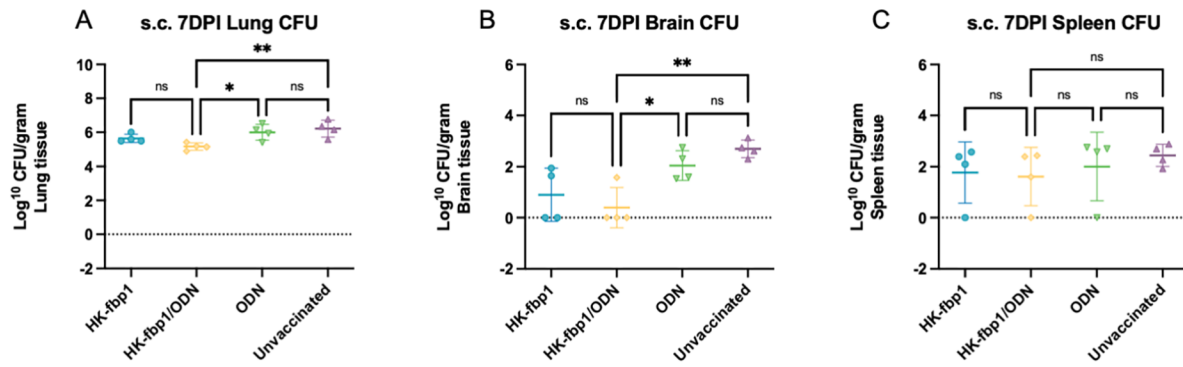


Figure S2. Mice s.c. vaccinated with HK-fbp1/ODN and challenged with wild type showed similar dissemination as unvaccinated mice at the early infection stage. A–C. Fungal burdens in the lungs (A), brains (B), and spleens (C) at 7 days post-infection in subcutaneously vaccinated and unvaccinated mice. Each symbol represents one mouse. CFU data are presented as mean $\pm$ SD. Statistical analysis of CFU data was performed using one-way ANOVA with Tukey's multiple-comparison test. **, $P < 0.01$; *, $P < 0.05$. $N = 4$ per group.

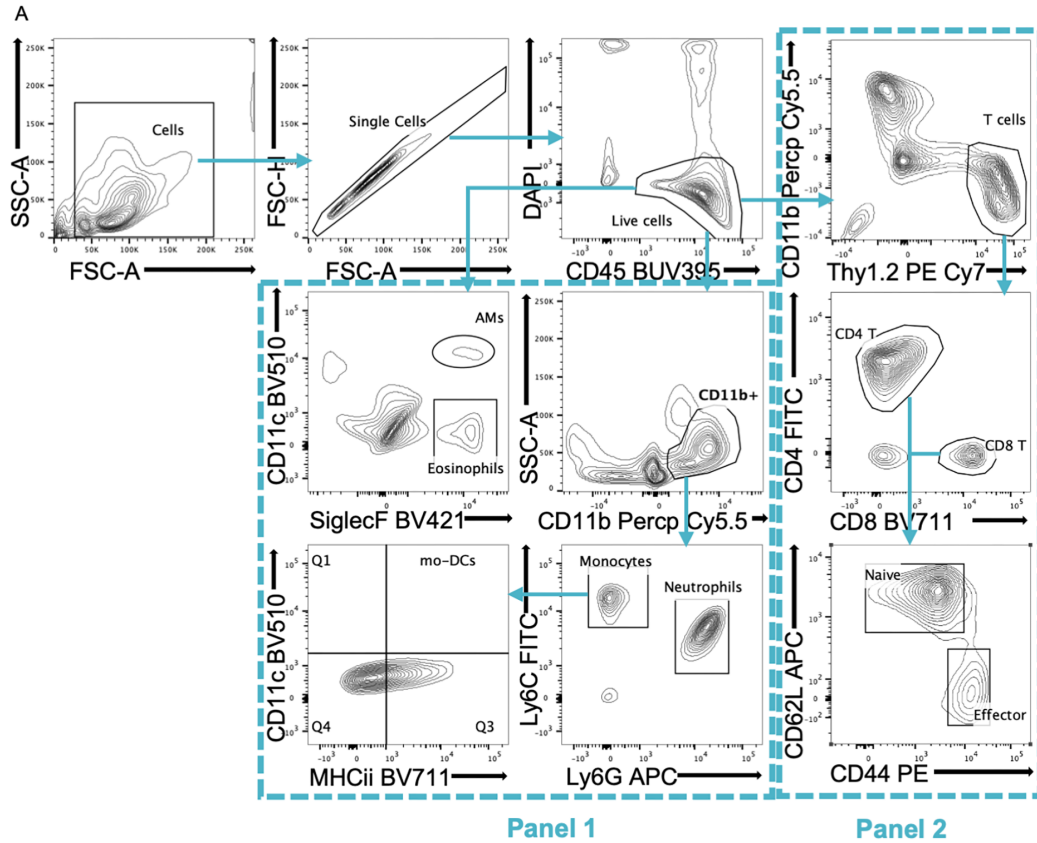
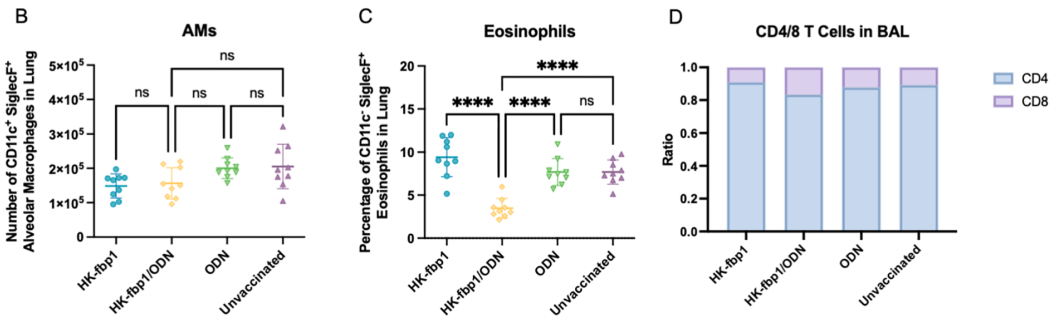


Figure S3. 7 days post H99 challenge, i.m. HK-fbp1/ODN vaccinated mice displayed limited eosinophil recruitment in lung and higher CD8 expansion in BALF. A. Representative flow cytometry plots illustrating the sequential gating strategy used to identify lung myeloid and T-cell subsets. Live leukocytes were first gated as DAPI⁻ CD45⁺ single cells. Alveolar macrophages (AMs) and eosinophils were further identified as CD11c⁺ Siglec-F⁺ and CD11c⁻ Siglec-F⁺ cells, respectively. Within the CD11b⁺ compartment, monocytes were gated as CD11b⁺ Ly6C⁺ Ly6G⁻, neutrophils as CD11b⁺ Ly6C^{int} Ly6G⁺, and monocyte-derived dendritic cells (mo-DCs) as CD11b⁺ Ly6C⁺ CD11c⁺ MHC-II⁺. T cells were identified as CD11b⁻ Thy1.2⁺ cells expressing either

CD4 or CD8. Activated subtype T cells were further defined as CD62L⁻ CD44^{hi}, whereas naïve T cells displayed the opposite phenotype. **B–C.** Numbers of alveolar macrophages (B) and percentages of eosinophils (C) in the lungs of vaccinated and unvaccinated BALB/c mice at 7 days post-infection (7DPI). Each symbol represents one mouse. $N = 9$ per group. Cell populations were identified as shown in Figure S3A. ****, $P < 0.0001$. Statistical significance was determined using one-way ANOVA. **D.** Proportions of CD4⁺ and CD8⁺ T lymphocytes among total T lymphocytes in BALF across experimental groups at 7DPI.

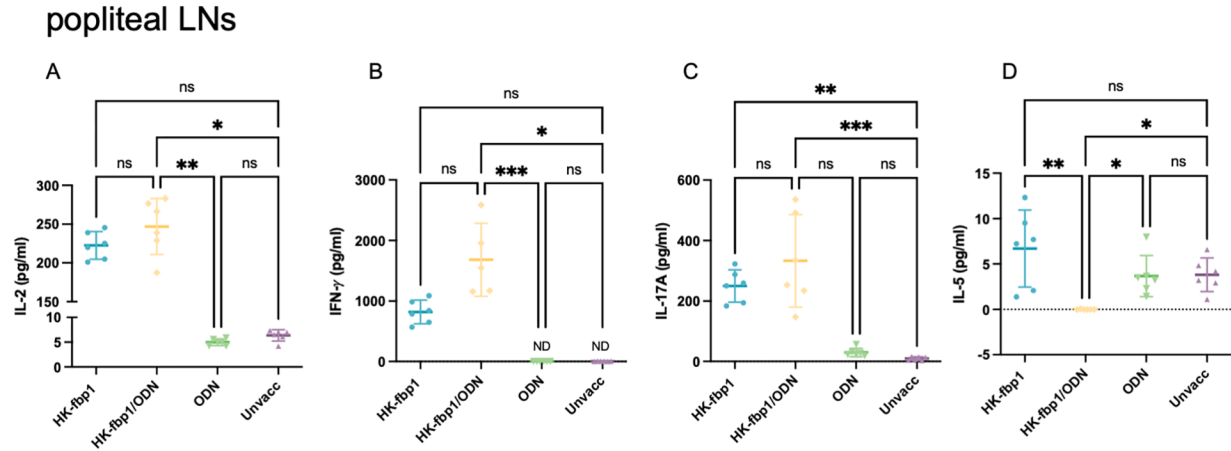


Figure S4. Muscle-draining popliteal LN at vaccination site exhibited similar CD4⁺ T cell cytokine profile as inguinal LN. A-D. Cytokine levels produced by CD4⁺ T cells in the popliteal lymph nodes. Each group contained five to six technical replicates. Data are presented as mean $\pm$ SD. Statistical significance was determined using one-way ANOVA. ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

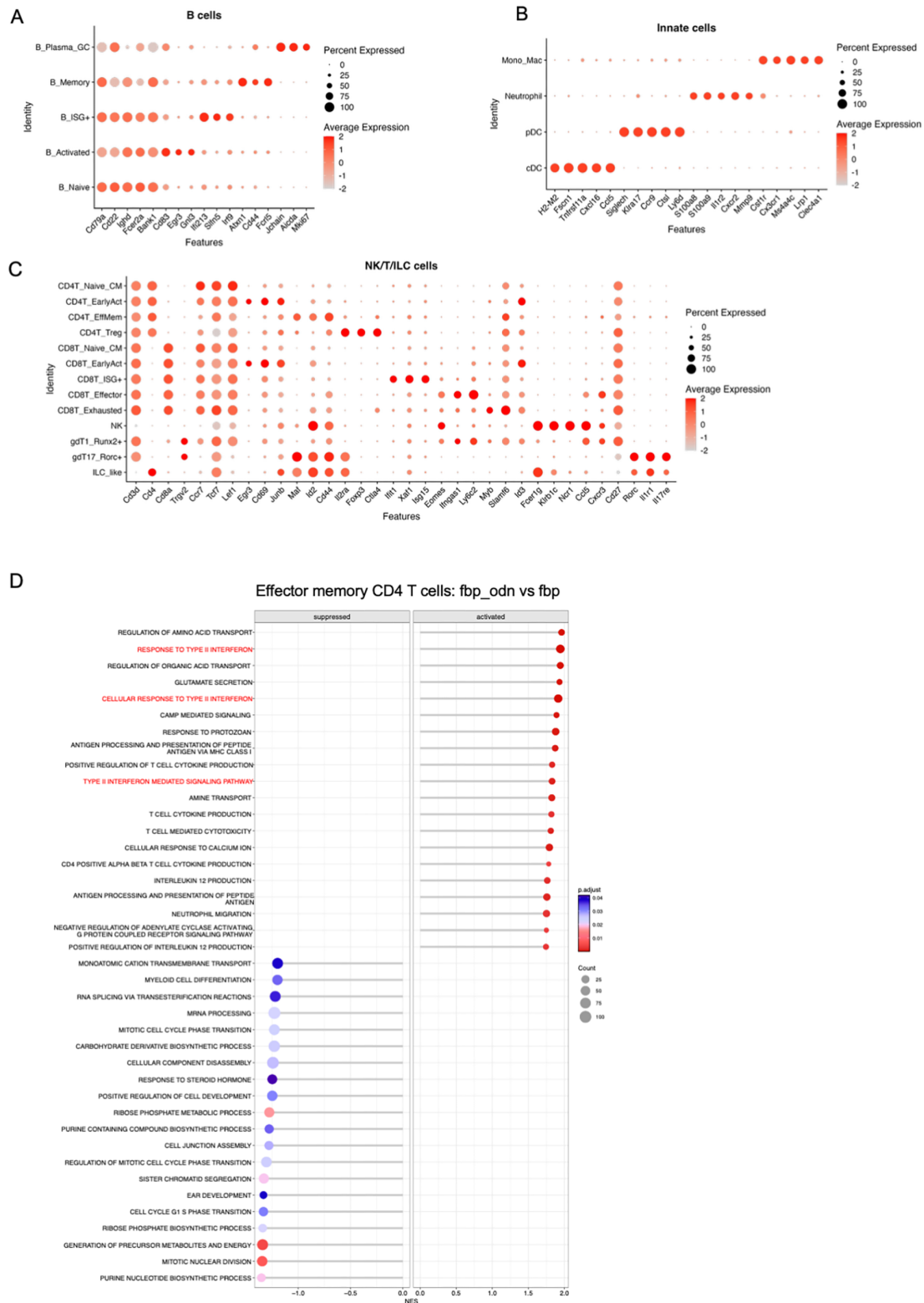


Figure S5. Effector memory CD4⁺ T cells in HK-fbp1/ODN group exhibited strong enrichment of IFN-II-related pathways. A-C. Bubble plots showing the expression levels of

signature genes in each cell cluster of each cell population, including B cells (A), myeloid cells (B), and ILC/NK/T cells (C). **D.** Gene set enrichment analysis of GO biological processes in CD4 effector-memory T cells comparing HK-fbp1/ODN versus HK-fbp1 vaccination. Dots represent the top 20 pathways ranked by normalized enrichment score, with color indicating enrichment direction and size reflecting nominal p value.

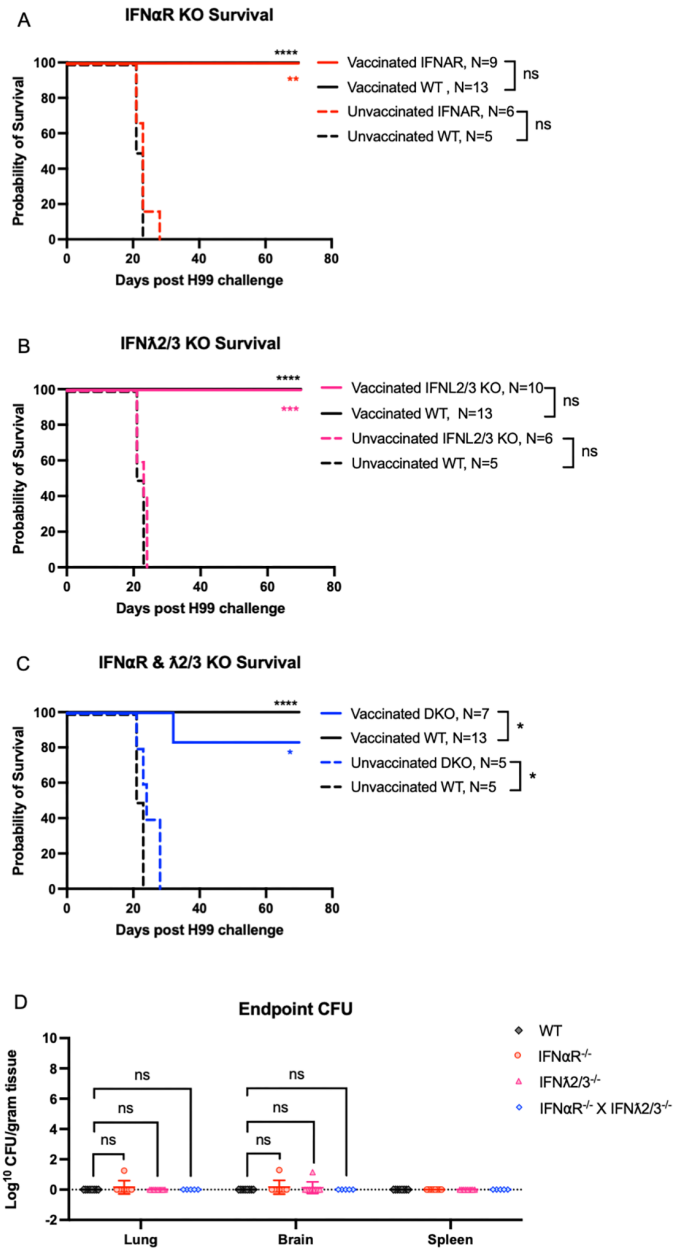


Figure S6. Type I and type III interferones are not required for HK-fbp1/ODN mediated vaccine protection in our i.m. vaccination model. A–C. Survival curves of WT C57BL/6, IFN α R^{-/-} (A), IFN λ 2/3^{-/-} (B), and double knockout IFN α R^{-/-} × IFN λ 2/3^{-/-} (C) mice that were vaccinated or left unvaccinated with the intramuscular HK-fbp1/ODN vaccine and subsequently challenged intranasally with H99. The number of mice in each group is indicated in the legend. Survival data were analyzed using the Kaplan–Meier method, and statistical significance was determined by the log-rank (Mantel–Cox) test with Holm–Šidák correction for multiple

comparisons. ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. **D.** Fungal burdens in the lungs, brains, and spleens of surviving vaccinated mice at the endpoint of the study shown in (A–C). $N = 5$ –9 per group. Each symbol represents an individual mouse. Data are presented as mean $\pm$ SD, and statistical significance was determined using one-way ANOVA followed by Tukey’s multiple-comparison test within each tissue.

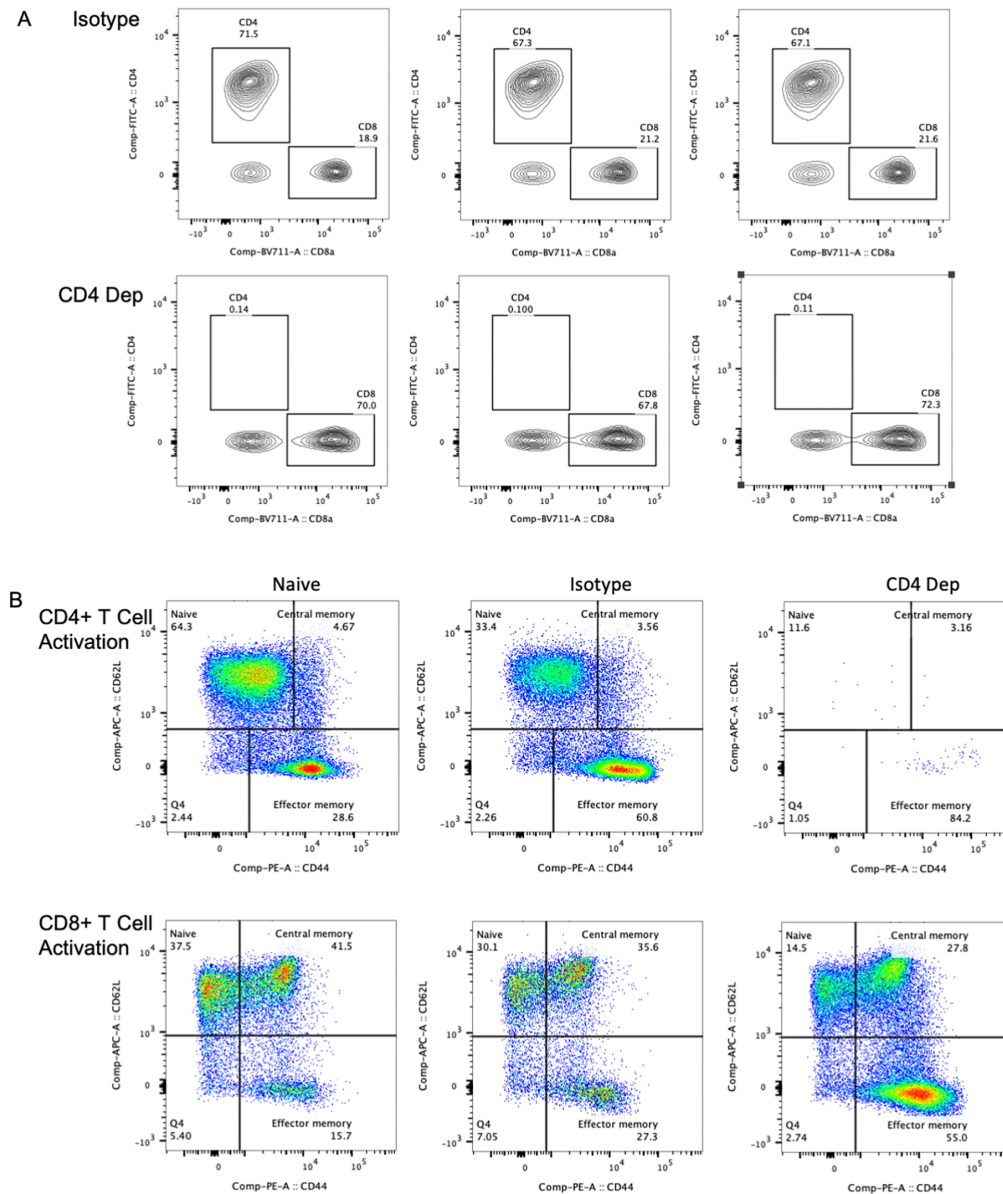


Figure S7. HK-fbp1/ODN vaccinated CD4⁺ T cell-deficient mice displayed vigorous CD8⁺ T cells activation in the lung. A. Confirmation of anti-CD4 (GK1.5) antibody–induced CD4⁺ T cell depletion in peripheral blood before H99 challenge, assessed by flow cytometry. **B.** Representative flow cytometry plots showing activation of lung CD4⁺ (top) and CD8⁺ (bottom) T cells in naïve, and intramuscularly vaccinated isotype control– and anti-CD4 antibody–treated surviving mice at the study endpoint.

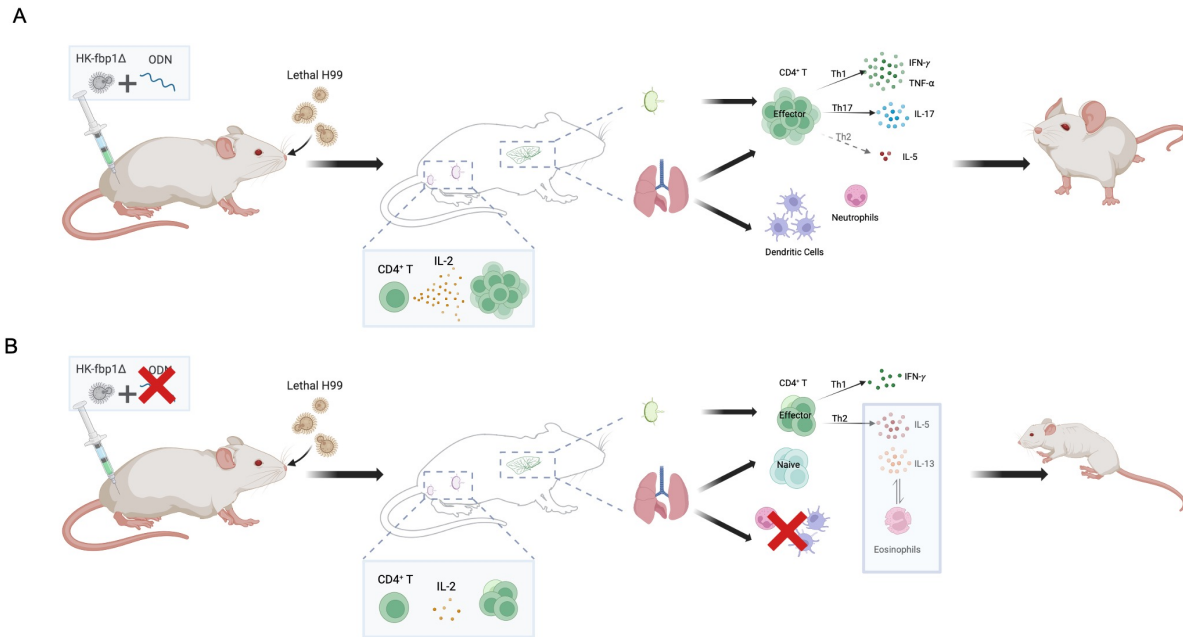


Figure S8. Proposed working model of intramuscular HK-fbp1/ODN vaccine-mediated immune protection against *C. neoformans*. **A.** Following intramuscular immunization with HK-fbp1/ODN and subsequent H99 challenge, antigen-specific CD4⁺ T cells in muscle-draining lymph nodes undergo robust proliferative priming when re-encounter antigens, as reflected by elevated IL-2 production. Upon infection, monocytes and mo-DCs, together with neutrophils, are efficiently recruited to the lung, supporting antigen presentation and a protective effector milieu. In parallel, antigen-specific CD4⁺ T cells in the lung draining lymph nodes exhibit a Th1/Th17-skewed program characterized by high IFN- γ and IL-17A with minimal Th2 cytokines, resulting in effective fungal control and survival. **B.** In contrast, intramuscular immunization with HK-fbp1 alone elicits weaker priming in muscle-draining lymph nodes, with reduced IL-2 output and limited expansion potential. Following H99 challenge, recruitment of mo-DCs and neutrophils to the lung is diminished, and CD4⁺ T-cell responses are comparatively blunted and/or skewed toward non-protective Th2-associated cytokines, accompanied by increased eosinophilia. This insufficiently coordinated priming-effector response fails to achieve protective immunity, leading to progressive infection and mortality.