

Supplementary Information

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

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Figure S1

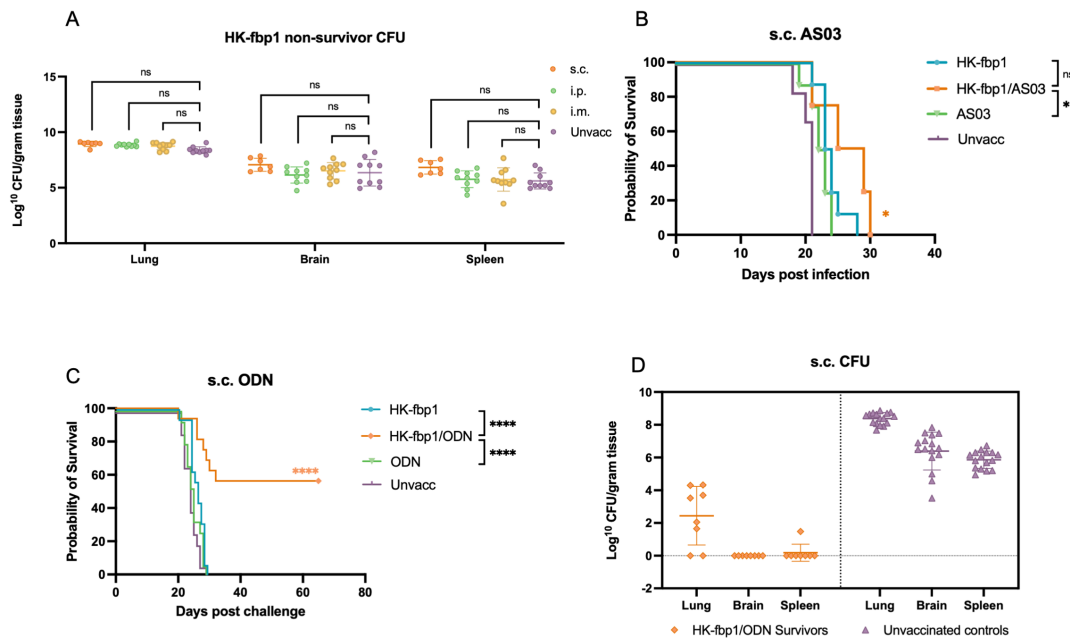


Figure S1. The AS03 adjuvanted HK-fbp1 vaccine (HK-fbp1/AS03) provides no protection against *C. neoformans* challenge in parental vaccination model. **A.** Fungal burdens in the lungs, brains, and spleens of non-surviving vaccinated mice and unvaccinated controls shown in (1B). $N = 7\text{--}10$ per 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). $N = 8\text{--}16$ 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). Each symbol represents an individual mouse.

Figure S2

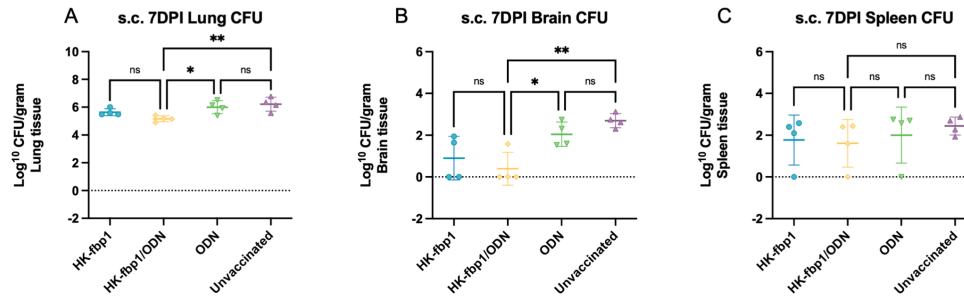


Figure S2. Mice s.c. vaccinated with HK-fbp1/ODN and challenged with wild type showed similar dissemination as unvaccinated mice at the early infection stage. A–C. Fungal burdens in the lungs (A), brains (B), and spleens (C) at 7 days post-infection in subcutaneously vaccinated and unvaccinated mice. Each symbol represents one mouse. CFU data are presented as mean ± 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

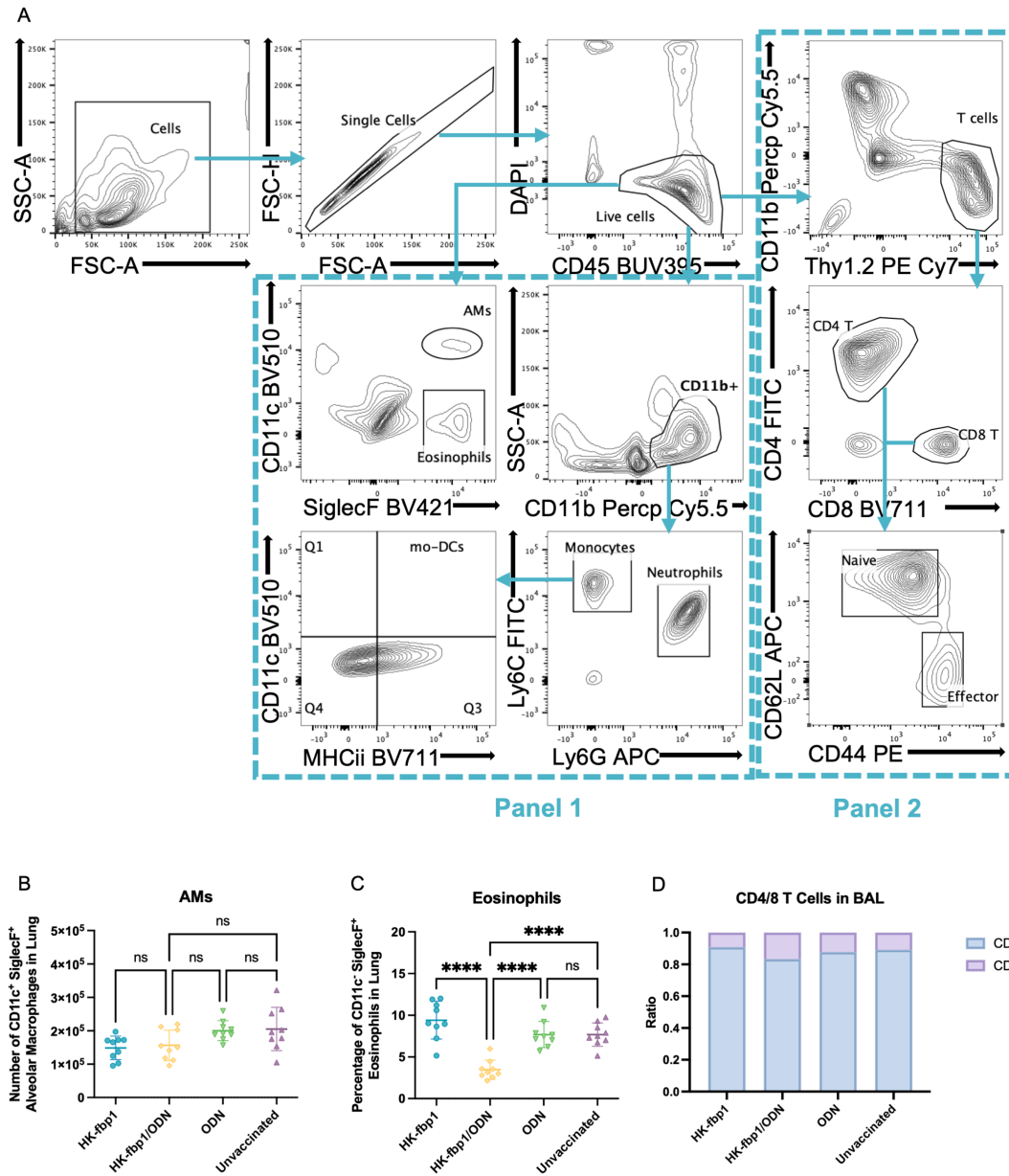


Figure S3. 7 days post H99 challenge, i.m. HK-fbp1/ODN vaccinated mice displayed limited eosinophil recruitment in lung and higher CD8⁺ T cells expansion in BALF. A. Representative flow cytometry plots illustrating the sequential gating strategy used to identify lung myeloid and T-cell subsets. Live 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. **D.** Proportions of CD4⁺ and CD8⁺ T lymphocytes among total T lymphocytes in BALF across experimental groups at 7DPI.

Statistical significance was determined using one-way ANOVA with Tukey's multiple-comparison test. (B–C). ****, *P* < 0.0001.

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Figure S4

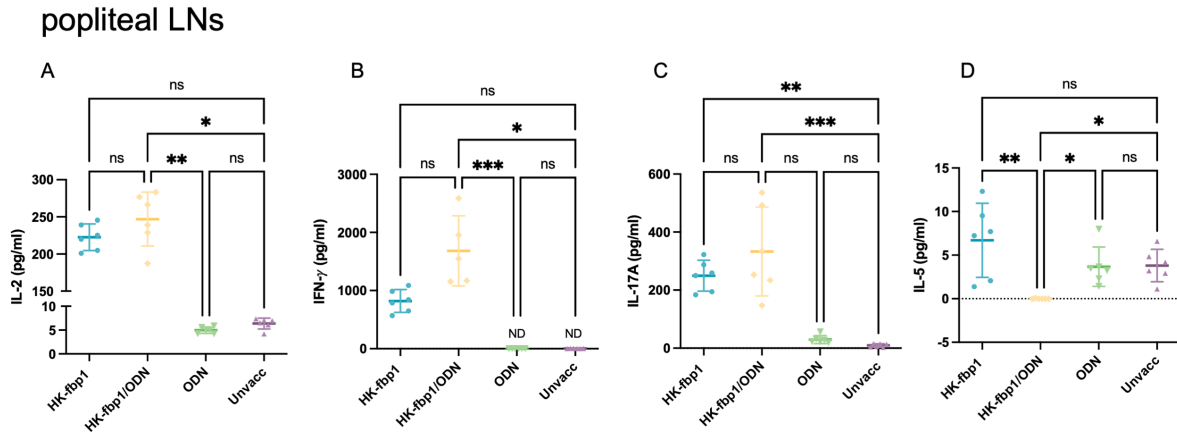


Figure S4. Muscle-draining popliteal LN at vaccination site exhibited similar CD4⁺ T cell cytokine profile as inguinal LN. A-D. Cytokine levels produced by CD4⁺ T cells in the popliteal lymph nodes. Each group contained five to six technical replicates. Data are presented as mean $\pm$ SD. Statistical significance was determined using one-way ANOVA with Tukey's multiple-comparison test. ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

Figure S5.

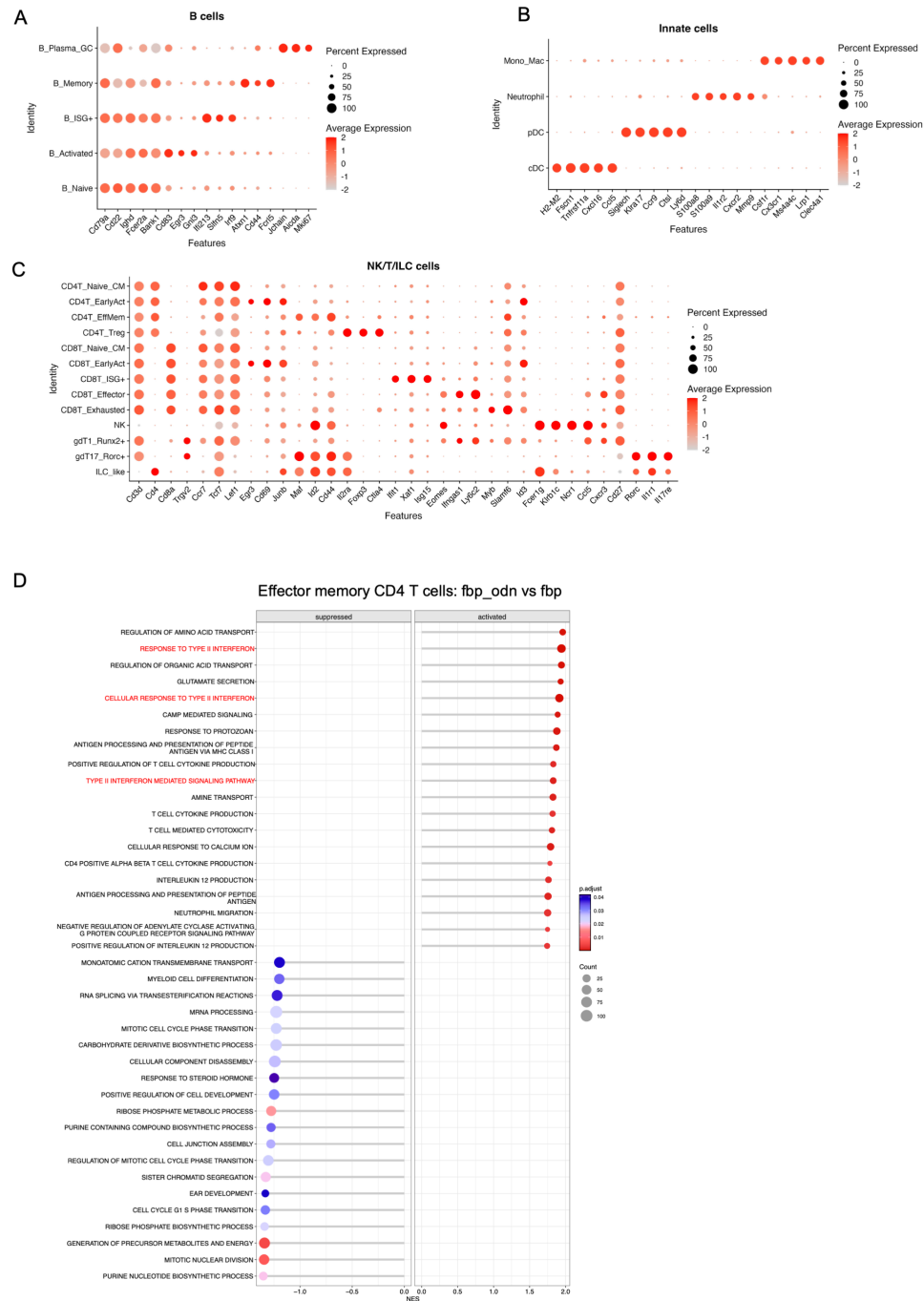


Figure S5. Effector memory CD4⁺ T cells in HK-flp1/ODN group exhibited strong enrichment of IFN-II-related pathways. A-C. Bubble plots showing the expression levels of signature genes in each cell cluster of each cell population, including B cells (A), myeloid cells

(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

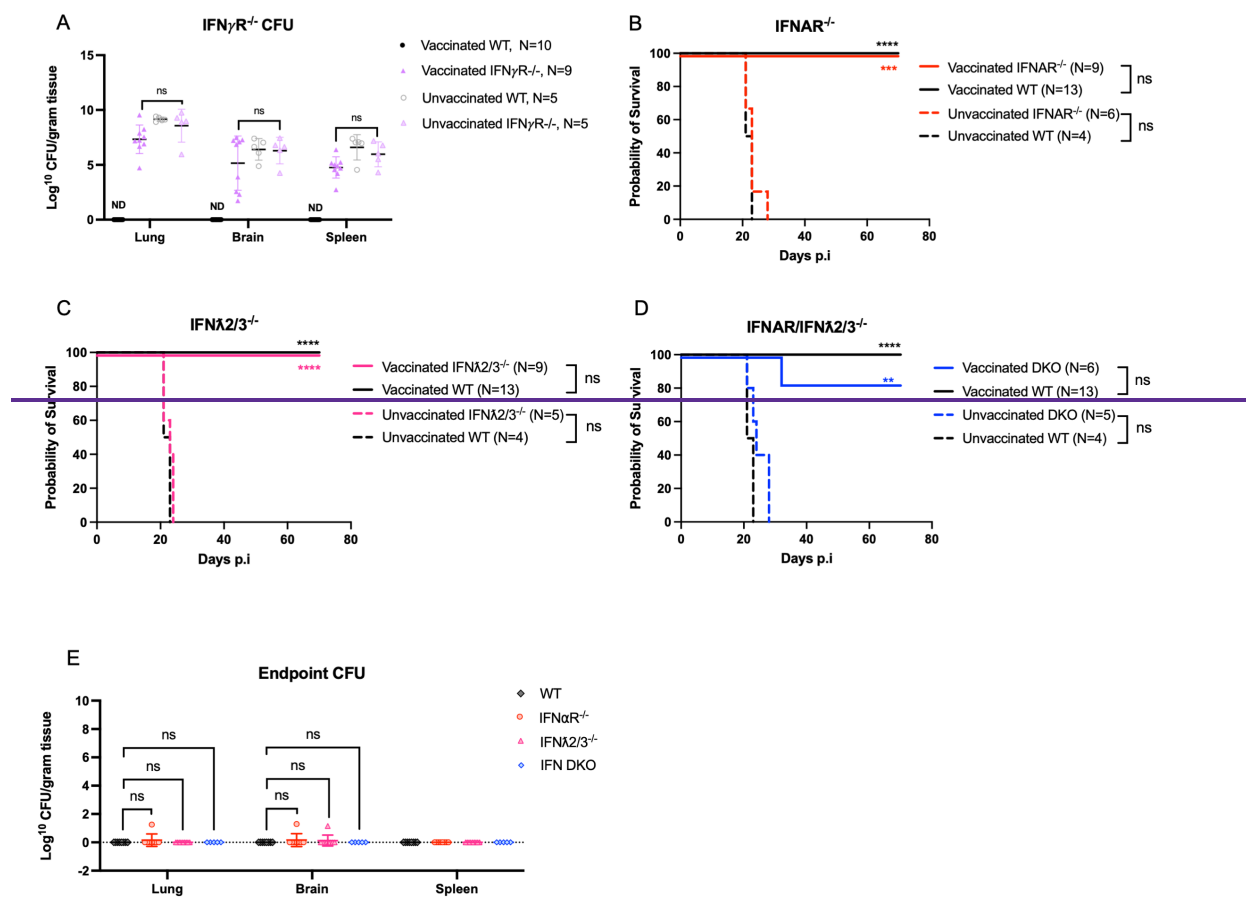


Figure S6.

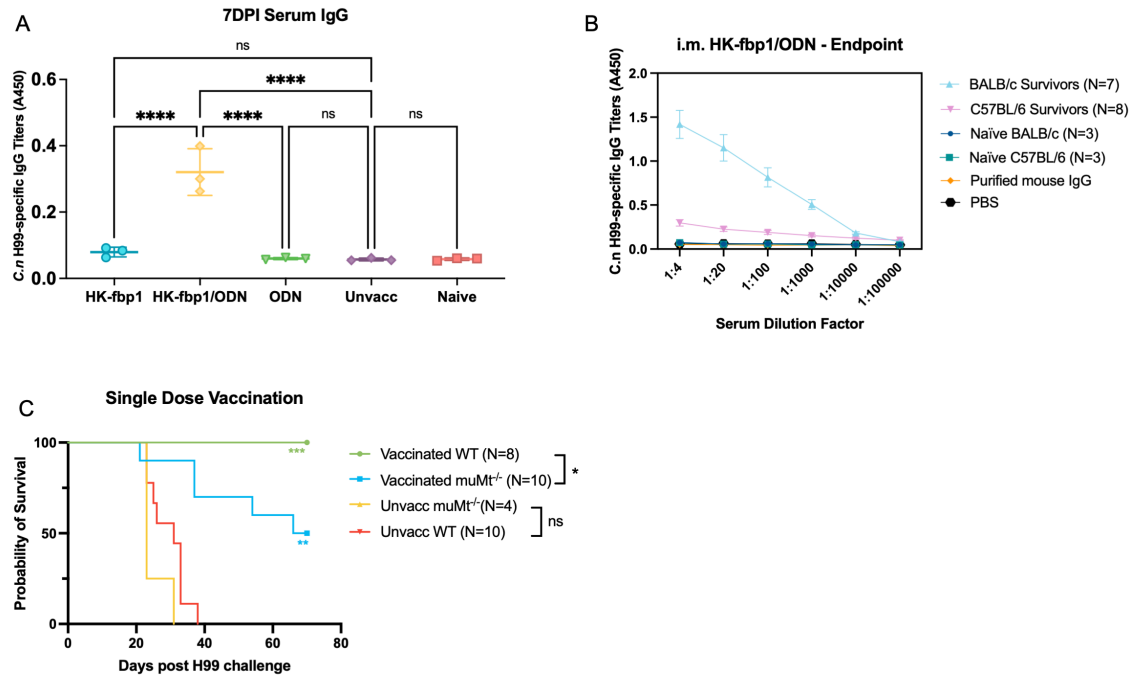


Figure S6. Antibody response and B cell contribution to vaccine-induced protection. A. H99 protein-specific serum IgG levels of vaccinated mice at 7 DPI, $N = 3$ per group. **B.** H99 protein-specific serum IgG in C57BL/6 and BALB/c mice at the experimental endpoint after i.m. vaccination with two-dose HK-fbp1/ODN. $N = 7-8$ per vaccinated group, $N = 3$ per control group. Purified mouse IgG and PBS were included as negative controls. **C.** Survival curves of WT C57BL/6 and muMt^{-/-} mice that were vaccinated with i.m. HK-fbp1/ODN once or left unvaccinated and subsequently challenged intranasally with H99. The number of mice in each group is indicated in the legend.

Antibody response data are presented as mean $\pm$ SD (A) or mean $\pm$ SEM (B). Statistical significance of IgG data was determined using one-way ANOVA followed by Tukey's multiple-comparison test (A). ****, $P < 0.0001$. Survival data were analyzed using the Kaplan–Meier method, and statistical significance was determined by the log-rank (Mantel–Cox) test with Holm–Šídák correction for multiple comparisons. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

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.** Fungal burdens in the lungs, brains, and spleens of surviving vaccinated mice at the endpoint of the study shown in (Main Figure. 7A). $N = 5-9$ per group. **B-D.** Survival curves of WT C57BL/6^{IFN α R^{-/-}} (B), IFN λ 2/3^{-/-} (C), and double knockout IFN α R^{-/-} $\times$ IFN λ 2/3^{-/-} (D) 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. ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

Figure S7

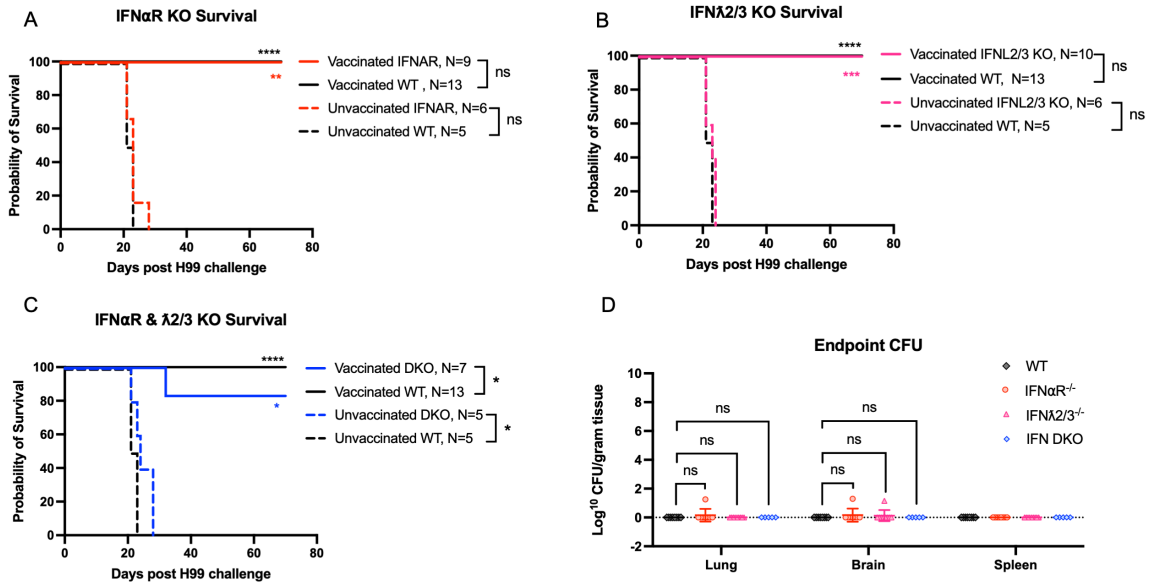


Figure S7. Type I/III interferons are not required for two-dose HK-fbp1/ODN mediated vaccine protection, while ODN adjuvantation shifts lung cytokine transcripts toward a Th1/Th17-associated profile. A–C. Survival curves of WT C57BL/6, IFN α R^{-/-} (A), IFN λ 2/3^{-/-} (B), and double knockout IFN α R^{-/-} × IFN λ 2/3^{-/-} (C) mice that were vaccinated twice with the intramuscular HK-fbp1/ODN vaccine or left unvaccinated and subsequently challenged intranasally with H99. **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.

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. The number of mice in each group is indicated in the legend. CFU data is presented as mean ± SD, and statistical significance was determined using one-way ANOVA followed by Tukey’s multiple-comparison test within each tissue. Each symbol represents an individual mouse. ****, *P* < 0.0001; ***, *P* < 0.001; **, *P* < 0.01; *, *P* < 0.05.

Figure S8

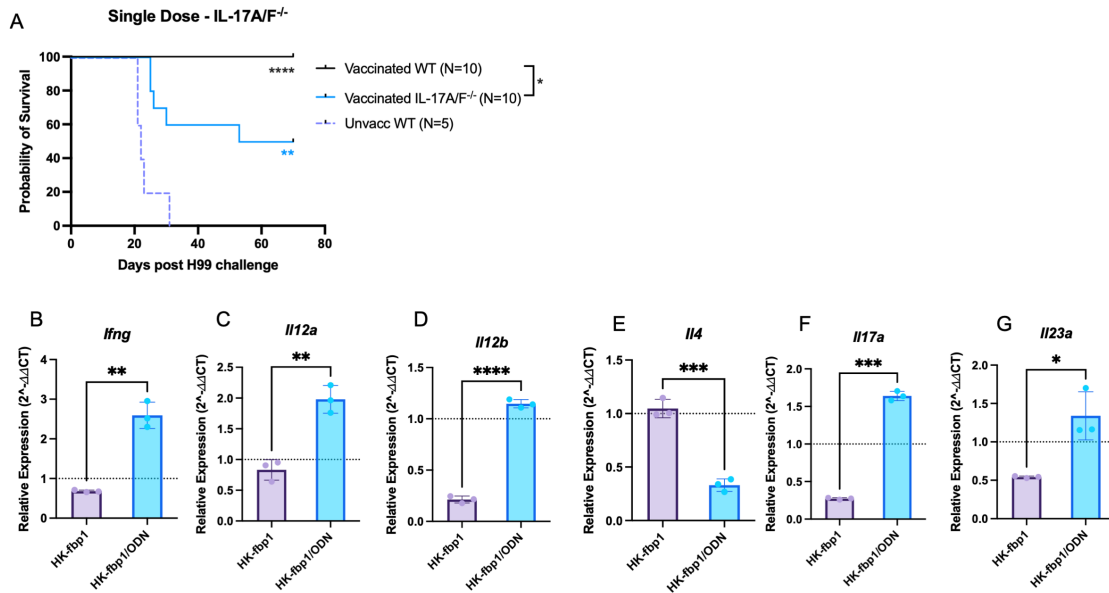


Figure S8. IL-17A/F contributes to protection and is transcriptionally induced in the lungs after single-dose i.m. HK-fbp1/ODN vaccination. **A.** Survival curves of WT C57BL/6 and , IL-17A/F^{-/-} mice that were vaccinated once with the intramuscular HK-fbp1/ODN vaccine or left unvaccinated and subsequently challenged intranasally with H99. The number of mice in each group is indicated in the legend. **B-G.** Relative expression of *Ifng* (B), *Il12a* (C), *Il12b* (D), *Il17a* (E), *Il23a* (F) and *Il4* (G), in lung tissue at 14 days post infection, measured by real-time PCR and normalized to *Gapdh*. Mice were left unvaccinated or vaccinated once with i.m. HK-fbp1 or HK-fbp1/ODN and challenged with H99.

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. qPCR data is presented as mean ± SD, and statistical significance was determined using Mann–Whitney U test (non-parametric) (B–G). Each symbol represents an individual mouse. ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

E. Fungal burdens in the lungs, brains, and spleens of surviving vaccinated mice at the endpoint of the study shown in (B–D). $N = 5–9$ 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

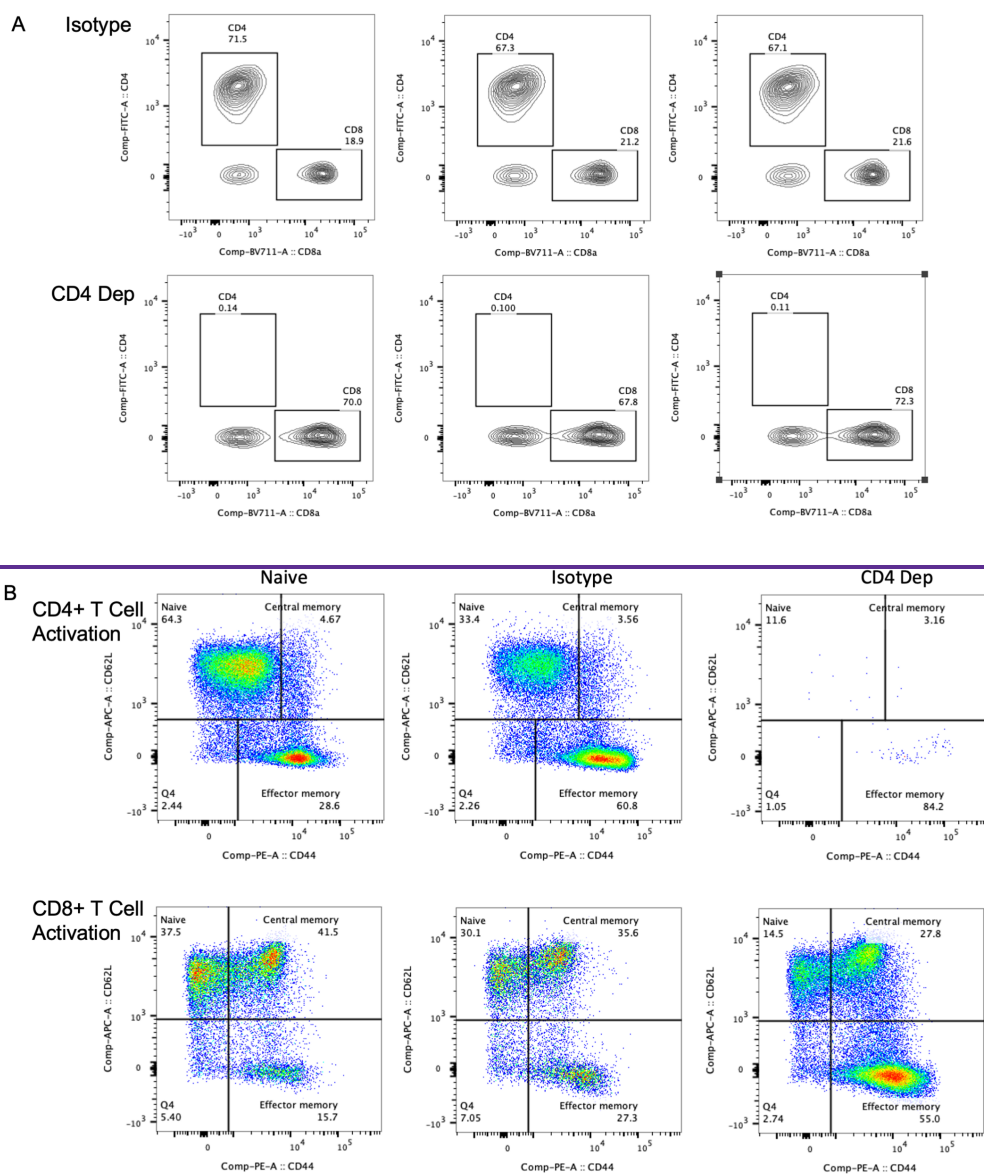


Figure S9

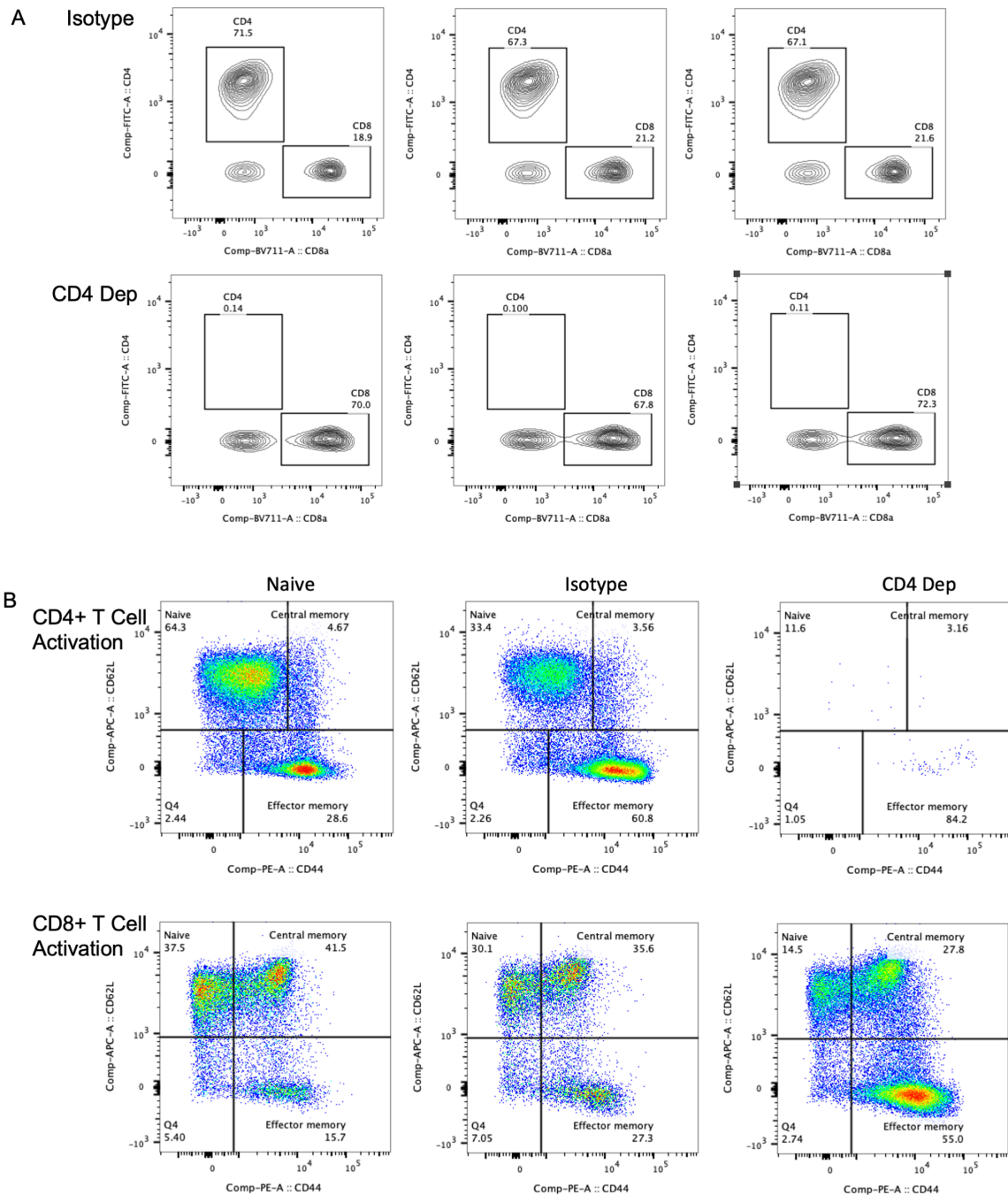


Figure S97. HK-fbp1/ODN vaccinated CD4⁺ T cell-deficient mice displayed vigorous CD8⁺ T cells activation in the lung. **A.** Confirmation of anti-CD4 (GK1.5) antibody–induced CD4⁺ T cell depletion in peripheral blood before H99 challenge, assessed by flow cytometry.

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.

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Figure S10

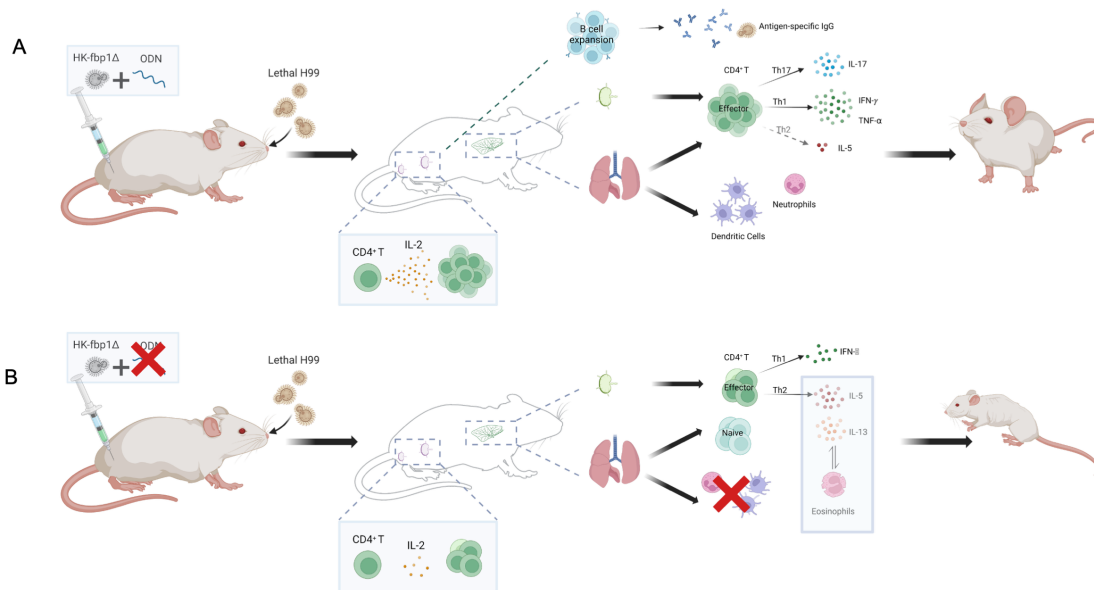


Figure S8

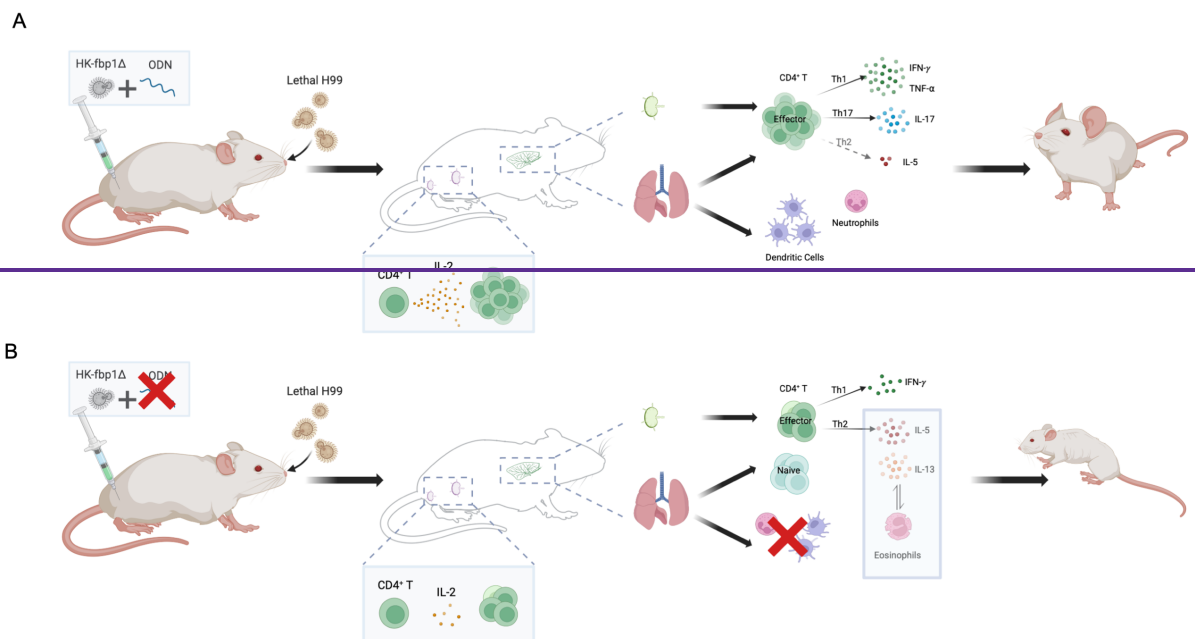


Figure S108. Proposed working model of intramuscular HK-fbp1/ODN vaccine-mediated

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, B cells produce antigen-specific IgG response systemically, and 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.