

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

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Article

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Posted Date: October 23rd, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-8603960/v1>

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Additional Declarations: There is **NO** Competing Interest.

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

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Abstract

Despite the rising burden of invasive fungal disease, no fungal vaccine is available to use. *Cryptococcus neoformans* is a fungal pathogen that initiates pulmonary infection and can disseminate to cause fatal meningoencephalitis. We previously identified HK-fbp1, a heat-inactivated *C. neoformans* mutant strain lacking the F-box protein Fbp1, that confers durable mucosal protection. Here, we combine HK-fbp1 with the type-C CpG adjuvant ODN2395, enabling effective intramuscular immunization to provide robust immune protection against pulmonary challenge, with mostly complete clearance of fungal burden and blockade of extrapulmonary dissemination. Protection correlated with strong recruitment of innate immune cells to the lung and a coordinated CD4⁺ T-cell response. *Cryptococcus*-specific CD4⁺ T cells produced IFN- γ and IL-17A in lung-draining lymph nodes, whereas muscle-draining nodes exhibited strong IL-2, consistent with coordinated priming and Th1/Th17 effector programming. Protection required IFN- γ , but not other interferons, and persisted in CD4⁺ T-cell- or B-cell-deficient mice, supporting development for immunocompromised hosts. Together, these findings establish CpG-adjuvanted intramuscular HK-fbp1 vaccination as a clinically tractable strategy that can elicit near-sterilizing antifungal immunity and provide a foundation for advancing HK-fbp1-based antifungal vaccines.

Keywords: *Cryptococcus neoformans*, intramuscular vaccination, Fbp1, CpG adjuvant, innate immunity, adaptive immunity, mice.

Introduction

Invasive fungal infections (IFIs) are a significant global health concern, with an estimated annual incidence of 6.5 million cases and 3.8 million associated deaths ¹. Cryptococcosis, caused by *Cryptococcus neoformans* and *C. gattii* species complex, is a life-threatening disease when disseminates through the bloodstream to the brain, disproportionately affecting immunocompromised individuals, such as HIV/AIDS patients, organ transplant recipients and patients undergo chemotherapy, etc. Cryptococcal meningitis is account for ~ 20% AIDS related

deaths worldwide ². Hence *C. neoformans* has been recognized as a critical priority fungal pathogen by World Health Organization due to its medical significance.

Significant efforts have been dedicated to combating this human pathogen. However, owing to the limitations of current antifungals and the lack of effective preventive strategies, the mortality rate of cryptococcosis remains alarmingly high. Current antifungals on the market include three major classes: polyenes, azoles, and echinocandins. Polyenes are fungicidal but exhibit substantial toxicity against hosts. Azoles are fungistatic and frequently induce drug resistance. Echinocandins are ineffective against cryptococcal infections ³. Whereas antiretroviral therapy (ART) and cryptococcal antigen screening can reduce mortality in high-risk and asymptomatic populations, the adoption of these strategies remains limited in low- and middle-income settings due to resource constraints ^{4, 5, 6}. In addition, vaccination has been a highly effective disease prevention and control strategy in dealing with viral infections and bacterial infections. However, there is no fungal vaccine currently available for clinical use. Therefore, there is an urgent need to develop new strategies against cryptococcal infections to protect not only high-risk populations but also healthy individuals who may later experience immune system weakening, leading to the reactivation of dormant fungal cells.

Although no vaccine studies against cryptococcal infection have reached clinical phase, preclinical research has developed a number of vaccine candidates that showed promising outcome in animal models, including glycoconjugate vaccine, targeting capsular polysaccharide ⁷; a subunit vaccine formulated as glucan particles, delivered a peptide sequence from Cda2, a stimulatory antigen of *C. neoformans* ⁸; and whole cell-based vaccine candidates. Studies from different groups demonstrated that intranasal whole-cell-based immunization can elicit robust immune responses, significantly reduce fungal burden, and improve survival rates in animal models ^{9, 10, 11, 12, 13, 14, 15, 16, 17}. The immune response to whole-cell-based vaccines involves a complex interplay between innate and adaptive immunity. Innate immune cells, such as monocytes/macrophages, monocyte-derived dendritic cells, and neutrophils are pivotal in the early stages of infection, controlling fungal dissemination and activating adaptive immune responses ^{10, 17}. Adaptive immunity, particularly CD4⁺ T-cells, is crucial for long-term fungal control, as evidenced by the increased susceptibility of individuals with low CD4⁺ T-cell numbers, such as HIV/AIDS patients ^{2, 18}. However, recent studies indicate that existence of one arm of T cell response, either CD4⁺ T-cells

or CD8⁺ T-cells, are sufficient for pre-clinical vaccine candidates^{9, 11, 12, 13, 15, 16}. In addition, both immunocompetent and immunodeficient mice intranasally immunized with a modified *C. neoformans* strain expressing IFN- γ (H99 γ) successfully resolved subsequent wild-type infection, highlighting the importance of IFN- γ in protective immunity^{19, 20}. This is further evident by the loss of protection in IFN- γ Receptor IFN γ R^{-/-} mice, in contrast to the full protection achieved in wild-type mice following vaccination¹⁵.

Our previous studies identified a promising whole-cell vaccine based on a *C. neoformans* mutant strain lacking Fbp1, a key component of the SCF E3 ubiquitin ligase system. The absence of Fbp1 prevents degradation of its downstream substrate proteins, leading to induction of protective immunity and loss of fungal virulence^{15, 16, 17, 21, 22, 23}. The intranasal immunization of heat-killed *fbp1* Δ cells (HK-fbp1) showed strong protection in both naïve and CD4⁺ T-cell-depleted mice from subsequent challenge with the virulent parental strain by eliciting robust pulmonary Th1/Th17 immunity, primarily driven by CD4⁺ T cell or compensated by CD8⁺ T cell in the absence of CD4⁺ T cells^{15, 16}. The HK-fbp1 vaccine also showed heterologous protection against other invasive fungal infections, e.g., *Aspergillosis*, indicating a potential fungal vaccine with broad protection spectrum.

To further expand the utility of our HK-fbp1 vaccine beyond intranasal route, here we incorporated a CpG based adjuvant (ODN2395) into the HK-fbp1 vaccine. We found that this combinational vaccine not only improved the efficacy in the intranasal immunization model but also provided full protection in an intramuscular vaccination model for the first time. We demonstrated that intramuscular administration of ODN adjuvanted HK-fbp1 (HK-fbp1/ODN) effectively resolved subsequent *C. neoformans* lung infection and prevented its extrapulmonary dissemination. Importantly, the adjuvanted immunization enhanced T-cell proliferative responses in the muscle draining lymph node and promoted robust Th1/Th17 effector responses at the site of infection, indicating establishment of a coordinated priming-effector T cell axis that underlies the vaccine's protection. Single-cell transcriptomics showed that the effective HK-fbp1/ODN vaccine markedly enhances B cell activation programs, indicating substantial remodeling of humoral immunity. Yet HK-fbp1/ODN also protects B cell-deficient muMt mice, where vaccination drives heightened T-cell cytokine responses, suggesting a shift toward T cell-mediated protection in the absence of B cells. In aggregate, these findings established a novel anti-cryptococcosis vaccine

capable of mobilizing both innate and adaptive immunity mediated protection via intramuscular vaccination, a clinically preferred vaccination route. Moreover, our study elucidates the immune mechanism underlining this enhanced immune protection in this intramuscular vaccination model.

Results

Immunization with adjuvanted HK-fbp1 vaccine via intramuscular, subcutaneous, and intranasal routes demonstrated strong protection against cryptococcosis.

In previous studies, we developed a potent vaccine based on heat-killed fbp1 mutant cells (HK-fbp1) that provided complete protection against *C. neoformans* in both wild-type and CD4⁺ T cell-depleted mice via intranasal vaccination (i.n.) method (Figure 1A)^{15, 16}. To broaden the potential applicability of this vaccine for future development, we next evaluated the protective efficacy of HK-fbp1 vaccine delivered via other common immunization routes, including intraperitoneal (i.p.), subcutaneous (s.c.), and intramuscular (i.m.), using the same two-dose vaccination strategy. Upon challenge with wild-type strain H99, mice vaccinated through these routes developed lethal infection at a similar rate as that of unvaccinated controls, indicating that HK-fbp1 vaccine provided no protection when administered through these parenteral routes (i.p., s.c., or i.m.), which predominately induce systemic rather than mucosal immunity (Figure 1B and S1A).

Considering that many licensed vaccines employ adjuvants to augment both the magnitude and durability of protective immunity^{24, 25}, we posited that incorporating an adjuvant into our HK-fbp1 vaccine would enhance its efficacy and expand its utility. In this study, we tested two commercial adjuvants, AddaS03 and ODN2395. AddaS03 (AS03) is an oil-in-water nano-emulsion adjuvant containing squalene and DL- α -tocopherol, and has been utilized in influenza vaccines such as Pandemrix²⁶. AS03 augments cytokine and chemokine production (e.g., CCL2, IL-6) at the draining lymph nodes, thereby improving antigen uptake and activating cell-mediated immunity^{26, 27, 28}. Meanwhile, ODN2395 (ODN) is a synthetic CpG oligonucleotide that is recognized by TLR9 to induce Th1-dominated immune responses^{29, 30, 31}. We tested the AS03 adjuvanted HK-fbp1 vaccine (HK-fbp1/AS03) in i.m. and s.c injection vaccination models in mice following our two-dose vaccination strategy. Our data showed that mice i.m. or s.c. vaccinated

with HK-fbp1/AS03 vaccine succumbed to H99 challenge at approximately the similar time as the unvaccinated controls, suggesting that AS03 adjuvant did not provide beneficial effect on vaccine protection (Figure 1C and S1B). Excitingly, mice vaccinated with the ODN adjuvanted HK-fbp1 vaccine (HK-fbp1/ODN) achieved strong protection against virulent H99 challenge when using i.m. injection method (Figure 1D and 1E). Over 75% vaccinated BALB/c mice and 100% C57BL/6 mice survived in the i.m. vaccination model. Furthermore, mice s.c. vaccinated with the HK-fbp1/ODN vaccine also showed over 60% survival rate after challenged with H99 (Figure S1C). In contrast, mice vaccinated with either HK-fbp1 or ODN adjuvant alone did not provide any protection in these injection models, and mice were succumbed to challenge at the same time as the unvaccinated control (Figure 1D and 1E). The endpoint CFU data indicated that most of the survivors received combined i.m. vaccine have completely cleared infection, except only a few mice showed low-level fungal burden remaining in the lung and brain (Figure 1F and 1G). Notably, no CFU was recovered from spleens of surviving animals (Figure 1F and 1G). Analysis of mouse lung tissues stained with hematoxylin and eosin (H&E) further supports the significant inhibition of *Cryptococcus* challenge (Figure 1H). At the end point of the experiment, complete clearance of *Cryptococcus* cells was observed exclusively in the lung tissues of HK-fbp1/ODN-vaccinated mice. In contrast, *Cryptococcus* infection in HK-fbp1 or ODN immunized (data not shown) and unvaccinated control groups resulted in extensive alveolar space enlargement and necrotic lesions (Figure 1H). A similar pattern was also observed in the s.c. model, indicating effective blockade of extrapulmonary dissemination (Figure S1D). These results demonstrated that the ODN and HK-fbp1 combination vaccine (HK-fbp1/ODN) provided unique immune protection in vaccinated animals and achieved near-complete fungal clearance against infection.

These findings demonstrate that only the CpG-adjuvanted formulation (HK-fbp1/ODN), but not HK-fbp1/AS03, HK-fbp1 alone, or ODN alone, confers robust protection and near-sterilizing immunity following parenteral immunization. Given that the intramuscular route yielded the most consistent and potent protection, and is broadly used in licensed vaccines, we focused on this model to dissect the underlying immune mechanisms in subsequent experiments.

HK-fbp1/ODN vaccine via intramuscular administration restricted brain dissemination and maintained normal lung structures.

To further understand the dynamic of the vaccination-mediated inhibition of fungal proliferation in vivo, we measured the fungal burden in the lungs, brains and spleens at day 7 post H99 challenge (7 DPI, Figure 2A). Most HK-fbp1/ODN vaccinated mice had no spleen or brain dissemination, and significant lower lung fungal burden compared to unvaccinated and/or single-component vaccinated littermates, which contributes to the profound survival profile induced by combined vaccine at the end of this study (Figure 2B to 2D). Moreover, H&E staining of lung tissues further supported the marked inhibition of *Cryptococcus* infection achieved by the HK-fbp1/ODN vaccine (Figure 2E). At 7 DPI, *Cryptococcus* cells in intramuscularly vaccinated HK-fbp1/ODN mice were tightly encircled by dense infiltrates of immune cells, forming a compact inflammatory cuff that was not observed in any single-component vaccine group or in unvaccinated controls. In contrast, HK-fbp1 vaccinated mice exhibited loose and incomplete immune cell encapsulation of fungal cells, ODN alone and unvaccinated animals displayed extensive alveolar space enlargement and necrotic lesions, pathological features that were absent in both HK-fbp1 and HK-fbp1/ODN groups (Figure 2E). In s.c. model, HK-fbp1/ODN vaccinated mice displayed significantly lower fungal burdens than ODN alone or unvaccinated controls, and showed a reduction relative to HK-fbp1, although this difference did not reach statistical significance (Figure S2). These results indicate a positive correlation between vaccine-induced lymphocytic infiltration into the lungs, the prevention of brain dissemination during the early phase of infection, and the overall protection observed in the later phase.

HK-fbp1/ODN vaccinated mice elicit strong innate immunity and T cell-mediated adaptive immunity in the lung.

To elucidate the immune mechanisms underlying the protective effects observed in i.m. HK-fbp1/ODN vaccinated mice, we analyzed pulmonary immune responses at 7 DPI as outlined in Figure 2A, and the gating strategy used to define lung immune cell populations is shown in Figure S3A. We detected a significantly increased monocyte population in the lungs of mice vaccinated with HK-fbp1/ODN, which is consistent with their role as precursors of monocyte-derived dendritic cells (mo-DCs) and macrophages (Figure 3A). In parallel, mo-DC frequencies were also elevated (Figure 3B), delineating a lineage continuum that supports antigen acquisition and efficient priming of CD4⁺ T cells. By contrast, alveolar macrophage abundance, a key determinant of antifungal effector activity against *Cryptococcus*, was similar across groups at this time point

(Figure S3B), indicating that early differences are unlikely to be explained by alveolar macrophage numbers. Among all groups, the HK-fbp1/ODN treated cohort exhibited the highest neutrophil levels (Figure 3C) and the lowest eosinophil levels (Figure S3C), a pattern consistent with an inflammatory milieu biased toward Th1 and Th17 responses rather than Th2 immunity. These findings suggest that ODN supports the injected HK-fbp1 antigen in initiating early proinflammatory innate immune responses, particularly by activating cellular effectors involved in promoting antigen presentation, enhancing phagocytic capacity and the subsequent orchestration of adaptive immunity. In terms of cellular-mediated adaptive immunity, we found that mice immunized with HK-fbp1/ODN exhibited the highest frequencies of CD4⁺ and CD8⁺ T cell in the lungs, followed by those receiving HK-fbp1, while comparable low levels were observed in ODN only and unvaccinated controls, indicating that HK-fbp1 antigen is required in the i.m. vaccination strategy to promote T-cell recruitment to the site of infection (Figure 3D).

In addition, CD4⁺/CD8⁺ T cell activation followed a trend similar to that of T cell recruitment (Figure 3E to 3G). These results indicated that the HK-fbp1/ODN vaccination significantly enhanced the differentiation of naïve CD4⁺/CD8⁺ T cells into effector memory cells, which serve as the precursors for polarized Th1/Th2/Th17 cells in upcoming stages of vaccine-induced immune response. We observed that CD4⁺ T cells constituted the majority of the T cell population not only in the lung but also in the bronchoalveolar lavage fluid (BALF) (Figure 3D and S3D). To further elucidate CD4⁺ T cell differentiation patterns induced by vaccination, we assessed cytokine production in BALF at 7 DPI (Figure 3H and 3I). In BALF, T cell cytokines are predominantly produced by CD4⁺ T cells over CD8⁺, indicating a CD4⁺ T cell-dominant immunity (Figure S3D). Th1 cytokines, including IFN- γ and TNF- α , showed highest levels in HK-fbp1/ODN groups followed by HK-fbp1, exhibited markedly higher induction compared to the ODN alone and control groups (Figure 3H and 3I). This suggests that the intramuscular administration of HK-fbp1 is the key driver of the Th1 response. In addition, HK-fbp1/ODN vaccinated mice exhibited a significant increase in IL-17A levels and a minimal level of IL-13 from CD4⁺ T cells compared to all other littermates (Figure 3H and 3I). Because IL-17A and TNF- α provide powerful signals to recruit neutrophils and promote sustained neutrophils presence, the high IL-17A and TNF- α production in the lung is consistent with our observation of increased neutrophils recruitment (Figure 3C). However, the roles of neutrophils in our combination i.m. vaccine model remain under

investigation. Together, these findings indicate that i.m. HK-fbp1/ODN vaccination establishes a lung environment that favors Th1/Th17-skewed immunity while suppressing Th2 responses, consistent with the observed patterns of monocyte–moDC activation, T-cell recruitment/activation, and neutrophil/eosinophil balance.

CD4⁺ T cells are highly proliferative in the muscle draining lymph nodes (LNs), but only produce high effector Th1/Th17 cytokines in the lung draining LN.

Our previous studies have demonstrated that CD4⁺ T cells play a crucial role as effector cells in vaccine-induced immunity¹⁵. Although the HK-fbp1/ODN combination vaccine elicited the strongest innate and adaptive responses, i.m. HK-fbp1 alone also triggered moderate CD4⁺ T-cell activation, neutrophil recruitment, and Th1/Th17 cytokine production (Figure 3). This raises the question of why HK-fbp1, despite inducing measurable immunity, nevertheless failed to protect against H99. Examination of the 7DPI lung immune landscape suggests that HK-fbp1 induces a response broadly comparable to HK-fbp1/ODN, albeit with markedly attenuated magnitude. Despite inflammatory cell encapsulation of fungal cells, colony counts remain higher (Figure 2E). These findings suggest that i.m. HK-fbp1 generates a qualitatively appropriate but quantitatively insufficient response, unable to surpass the threshold required for fungal control.

This discrepancy prompted us to investigate how the CpG adjuvant enhances HK-fbp1 to achieve protective immunity, leading us to dissect the priming-to-effector axis of CD4⁺ T-cell responses (Figure 4). To test this hypothesis and investigate the underlying mechanisms, we conducted a CD4⁺ T cell recall experiment using both distal and proximal secondary lymph nodes (LNs) to evaluate the importance of vaccination site T-cell activation and effector cytokine production at the site of infection, a response uniquely induced by i.m. HK-fbp1/ODN immunization (Figure 4A). CD4⁺ T cells isolated from proximal lung-draining (mediastinal) lymph node (mLN) or distal muscle-draining lymph nodes (inguinal LN, iLN or popliteal LN, pLN) were co-cultured with naïve antigen-presenting cells (APCs) and *C. neoformans* antigens from H99, followed by the measurement of cytokine levels in the supernatant. To monitor T-cell proliferation capacity, we measured IL-2 levels and observed significant augmentation of IL-2 production in distal pLN and iLN of HK-fbp1/ODN vaccinated mice than HK-fbp1 or ODN alone following H99 challenge (Figure 4B and S4A). Meanwhile, distal IL-2 levels in ODN vaccinated mice were

comparable to those in unvaccinated controls. In addition, IL-2 production was over 100-fold lower in lung mLNs than muscle draining iLN of HK-fbp1/ODN group (Figure 4B). We also measured effector cytokines such as IL-17A and IFN- γ . In contrast to IL-2, the overall levels of IL-17A and IFN- γ were significantly higher, being elevated exclusively in proximal mLN, but not in distal iLN and pLN (Figure 4C-D and S4B-C). In mLNs, H99-specific IFN- γ production by CD4⁺ T cells was the highest in HK-fbp1/ODN vaccinated mice, approximately 3-fold higher than in the HK-fbp1 group (Figure 4C). Similar patterns were observed in distal lymph nodes (iLNs and pLNs), but absolute IFN- γ responses were uniformly lower than in mLNs (Figure 4C and S4B). IL-17A production mirrored IFN- γ across sites; however, the difference between HK-fbp1/ODN and HK-fbp1 was far greater (~20-fold), consistent with high IL-17A being detected only in BALF CD4⁺ T cells from the adjuvanted group (Figure 4D and S4C). By contrast, IL-5 exhibited the opposite pattern: its levels were elevated in mLNs but remained low in iLNs and pLNs, and among all groups the adjuvanted regimen showed the lowest IL-5 (Figure 4E and S4B).

Together, these data reveal a compartmentalized CD4⁺ T cell response induced by intramuscular HK-fbp1/ODN immunization, marked by robust proliferative priming in distal muscle-draining lymph nodes and potent Th1/Th17 effector cytokine production in proximal lung-draining lymph nodes. This priming-effector axis was specific to the adjuvanted vaccine. In contrast, unprotected groups displayed weak antigen specific IFN- γ and IL-17A responses alongside disproportionately elevated IL-5, indicating a detrimental Th2-skewed environment. The notably higher IL-2 levels in distal lymph nodes, combining with the strong IFN- γ and IL-17A responses restricted to the lung-draining nodes, support a model that HK-fbp1/ODN promotes effective CD4⁺ T cell expansion at the vaccination site, followed by their deployment as Th1/Th17 effectors at the site of infection. The IFN- γ and IL-5 production observed in unvaccinated mice likely reflects an inflammatory burst derived from a limited pool of *C. n.*-specific CD4⁺ T cells, insufficient to confer protection in the lung.

Single-cell RNA sequencing identifies enhanced immune activation and effector functions induced by HK-fbp1/ODN vaccination.

Our previous work using the HK-fbp1 i.n. vaccination model revealed the critical role of T cell-mediated responses in vaccine-induced protection. The HK-fbp1/ODN combination, but not HK-

fbp1 alone, provided strong protection in our i.m. vaccination model suggests that additional immune mechanisms likely contribute to the efficacy of adjuvanted vaccine. To better understand the immune factors contributing to the protection in the i.m. HK-fbp1/ODN vaccination mode, we surveyed the immune landscape induced by i.m. HK-fbp1 alone or HK-fbp1/ODN vaccination through single-cell RNA sequencing (scRNA-seq) of vaccine-site draining inguinal lymph nodes (iLNs) collected at 12 days following booster administration (Figure 5A). UMAP clustering revealed consistent identification of major immune cell types across all experimental groups-naïve control (ctrl), HK-fbp1 alone (fbp), and HK-fbp1 with adjuvant ODN (fbp_odn) combination (Figure 5B). The identification of each immune cell subset was based on the expression of top differentially expressed markers and established canonical markers (Figure S5A–C).

Our scRNA-seq analysis revealed a higher abundance of B cells in immunized mice, with the most pronounced expansion in the adjuvanted (HK-fbp1/ODN) group, indicating a strong B-cell response elicited by the two-dose adjuvanted regimen (Figure 5B and 5C). To determine how non-protective and protective vaccination reshape the immune landscape, we quantified changes in subtype cell-type composition across treatment groups (Figure 5C). Compared with controls, HK-fbp1 vaccination induced a skewed and only partial immune expansion, enriching ISG⁺, memory-like B and germinal center/plasma lineage B cell subsets, naïve, effector-memory and Treg CD4 populations, ISG⁺ CD8 populations, as well as pDC/monocyte-macrophages/Runx2⁺ gdT subtype 1 cells, while contracting naïve/activated B cells, most CD8 subsets, and multiple innate effectors. The protective HK-fbp1/ODN vaccine drove a more coherent effector-oriented profile, with enrichment of activated, ISG⁺ and GC/memory B cells, CD4 effector-memory and regulatory subsets, CD8 ISG⁺/exhausted populations, and inflammatory myeloid and gdT1_Runx2⁺ cells with strong enrichment of neutrophils (Figure 5C).

To dissect how these vaccines differentially imprint B-cell states, we analyzed differential gene expression focusing on the most abundant antigen-experienced B cell subset-memory B cells (Figure 5D) and performed Gene Ontology (GO) biological process Gene Set Enrichment Analysis (GSEA) (Figure 5E). In the HK-fbp1 vs. control comparison, memory B cells failed to engage core B-cell functional programs-including BCR signaling, activation, proliferation and differentiation, and showed reduced enrichment of T-cell activation and T cell-mediated immunity pathways.

311 Instead, HK-fbp1 induced a dominant interferon signature, with marked upregulation of type I and
312 type II interferon responses, suggesting a predominantly inflammatory state.

313 Incorporating ODN into HK-fbp1 resulted a qualitative shift from this IFN-dominant state
314 to a more coordinated adaptive immune program. ODN adjuvantation was associated with
315 upregulation of pathways involved in B-cell activation, proliferation and differentiation, together
316 with enhanced T-cell activation, CD8⁺ T-cell differentiation, and positive regulation of T-cell
317 cytotoxicity. We also observed strong enrichment of GO terms associated with antigen processing
318 and presentation, including MHC class I- and class II-related pathways within memory B cells.
319 While these transcriptional signatures do not establish antigen-presenting activity directly in our
320 system, they are consistent with published reports describing the potential of activated B cells to
321 contribute to antigen presentation under certain immunological contexts ^{32,33,34}. Furthermore, HK-
322 fbp1/ODN also amplified type II interferon signaling without broadly increasing type I interferon
323 pathways, indicating an IFN- γ -biased environment that supports enhanced effector differentiation.
324 HK-fbp1/ODN vaccination also upregulated the proteostasis and stress-resilience pathways,
325 including protein folding, refolding, chaperone-mediated complex assembly, PERK- and ATF6-
326 mediated unfolded protein responses, translational initiation, and autophagy-signatures
327 characteristic of metabolically fit, long-lived memory lymphocytes. In addition to transcriptional
328 remodeling within memory B cells, we also observed differential programming among T-cell
329 subsets. Notably, in the CD4 effector-memory population, HK-fbp1/ODN vaccination enriched
330 multiple IFN-II-related pathways, including “response to type II interferon,” “cellular response to
331 type II interferon,” and “type II interferon-mediated signaling” compared with HK-fbp1 (Figure
332 S5D). These IFN- γ -responsive signatures suggest that CD4 effector-memory T cells in the
333 adjuvanted group acquire a more inflammatory and helper-competent state that is well positioned
334 to support downstream immune functions, including enhanced cytotoxic T-cell activation and
335 improved orchestration of B-cell responses. Such coordinated reinforcement of IFN- γ -driven
336 programming across both B-cell and CD4 T-cell compartments likely contributes to the superior
337 protective efficacy of HK-fbp1/ODN.

338 Together, these data indicate that the unprotective HK-fbp1 vaccine is associated with an
339 interferon-driven inflammatory state without engaging antigen-processing pathways in functional
340 B cells. In contrast, ODN adjuvantation promotes coordinated activation of adaptive immunity by

enhancing B-cell activation associated programs, enriching GO terms linked to antigen processing and presentation, cytotoxicity and CD4⁺ T effector cell pathways carrying IFN-II-responsive signatures. This broader remodeling of humoral and cellular compartments may help explain the superior protective efficacy of HK-fbp1/ODN.

B cells are dispensable for two-dose HK-fbp1/ODN vaccination mediated protection against *C. neoformans* challenge but contribute to suboptimal single-dose vaccination.

To determine the potential role of B cells in combination vaccine mediated protection, we tested the B cell deficient mice muMt^{-/-} (*Ighm*^{-/-}, Jackson labs) in our two-dose i.m. vaccination model (Figure 1A). While the unvaccinated wild type (WT) mice and muMt^{-/-} mice succumbed to H99 infection within 45 days, both WT and muMt^{-/-} mice vaccinated with HK-fbp1/ODN survived H99 challenge (Figure 6A), indicating that both genotypes remain fully protected by i.m. vaccination. We measured the endpoint fungal burden in the lung, brain and spleen of survival mice at 60 days post-challenge and detected no CFU in all these mouse organs except one mouse brain (Figure 6B). This result, however, does not exclude the possibility that B cells may help regulate T cell function in WT mice. To test this hypothesis, we measured the frequency of the IFN- γ , TNF- α and IL-17A producing CD4⁺ T cell populations in BALF of surviving mice at the endpoint. Indeed, we detected an increased CD4⁺ T cell population producing these Th1/Th17 response cytokines in the muMt^{-/-} mouse BALF compared to WT mice (Figure 6C-6E). Together, these findings indicate that B cells are dispensable for i.m. HK-fbp1/ODN vaccine-mediated protection under the two-dose regimen, despite the substantial B-cell expansion and activation observed after vaccination. Given this pronounced B-cell response, we next asked whether it translated into a functional humoral response. In BALB/c mice, HK-fbp1/ODN induced significantly higher H99 protein-specific serum immunoglobulin G (IgG) at 7 DPI than either HK-fbp1 or ODN alone (Figure S6A). Likewise, vaccinated C57BL/6 surviving mice also produced detectable antigen-specific IgG (Figure S6A). However, the magnitude of humoral response did not track with protection, as at the study endpoint, surviving BALB/c mice exhibited higher IgG titers than C57BL/6 mice despite lower protection (Figure S6B). Therefore, B cells are engaged by vaccination and mount a humoral response, but neither the presence of B cells nor the magnitude of the antigen-specific antibody response determines protective outcome under this regimen. Because two-dose vaccination confers near-complete protection, the contribution of any

individual effector arm may be obscured. We then reduced immunization to a single dose strategy as a less stimulating condition. Following this suboptimal regimen, HK-fbp1/ODN vaccinated $\mu\text{Mt}^{-/-}$ mice showed significantly reduced survival relative to fully protected WT mice (Figure S6C), which indicates the immunization dose determines the requirement for B cells. These data demonstrate a context-dependent role of B cells in HK-fbp1/ODN-mediated protection. B cells are functionally engaged by vaccination and contribute to protection when immunization is limited but are dispensable under the optimal two-dose regimen, where the effector function of CD4^{+} T cells is maintained in the absence of B cells. Thus, we accordingly focused on defining the cellular immunity that mediate vaccine-induced protection in this study.

IFN- γ is required for i.m. HK-fbp1/ODN vaccination induced protection, whereas type I/III IFNs play a dispensable role.

Given the robust IFN- γ production observed in HK-fbp1/ODN i.m. vaccinated mice throughout the vaccination study, we evaluated the necessity of IFN- γ in this vaccine model using the IFN- γ receptor knockout mice (IFN- $\gamma\text{R}^{-/-}$). Compared to vaccinated wild type C57BL/6 mice, IFN- $\gamma\text{R}^{-/-}$ mice all succumbed to lethal infection within 60 days following H99 challenge although displayed a prolonged survival profile, suggesting that IFN- γ is a critical contributor in vaccination protection but may not be the sole factor (Figure 7A). Measurement of fungal burden in the infected lungs, brains and spleens at the endpoint of the experiment detected high CFU in all animals, indicating vaccinated IFN- $\gamma\text{R}^{-/-}$ mice were not protected from pulmonary H99 colonization and extrapulmonary dissemination following challenge (Figure 7B). We then undertook further investigation of the contribution of other two IFN subtypes I and III by testing IFN- α receptor knock-out (IFN- $\alpha\text{R}^{-/-}$), IFN- λ subtype 2/3 knock-out (IFN- λ 2/3 $^{-/-}$), and double knock-out (IFN- $\alpha\text{R}^{-/-}$ IFN- λ 2/3 $^{-/-}$) mice (Figure S7A to S7C). We vaccinated mice lacking either IFN- αR or IFN- λ 2/3 or both (DKO) and challenged with H99 using our standard vaccination strategy. Our data showed that IFN- $\alpha\text{R}^{-/-}$ or IFN- λ 2/3 $^{-/-}$ group survived the challenge of H99, indicating a full protection similar to WT mouse group (Figure S7A and S7B). The double KO mice showed 80% survival following H99 challenge (Figure S7C). These results demonstrated that IFN- α and IFN- λ did not play a major role in the HK-fbp1/ODN vaccine mediated protection in this vaccine model. The IFN- $\alpha\text{R}^{-/-}$, IFN- λ 2/3 $^{-/-}$, and double-knockout mice exhibited near-complete fungal clearance of *C. neoformans* infection comparable to that of wild-type mice at the

experimental endpoint (Figure S7D). Together, these results indicate that protection conferred by i.m. HK-fbp1/ODN vaccination depends on IFN- γ receptor signaling, but is independent of type I and type III IFN signaling.

IL-17A/F is dispensable in the standard two-dose vaccination model but contributes to protection under suboptimal single-dose regimen.

Since strong IL-17A production was also observed from adjuvanted HK-fbp1 vaccinated mice, we tested the vaccine-induced protection in IL-17A/F double-knockout mice (IL-17A/F^{-/-}, Jackson laboratories). With the two-dose vaccination, both WT mice and double-knockout mice survived to the study endpoint, suggesting IL-17A/F is dispensable for vaccine mediated protection under this regimen (Figure 7B). We hypothesized that the two-dose regimen may induce a level of protective immunity at which individual effector pathways become functionally redundant, such that an intact IFN- γ -dependent response is sufficient to maintain protection in the absence of IL-17A/F. To test this, we repeated vaccination using the less stringent single-dose regimen. Under this condition, WT mice remained fully protected against challenge, whereas IL-17A/F^{-/-} mice were only partially protected (Figure S8A), indicating that IL-17A/F does contribute to vaccine-induced protection but that its contribution is redundant under the highly protective two-dose regimen. To define the signals associated with IFN- γ and IL-17 induction under single-dose vaccination, we measured cytokine transcripts in lung tissue by real-time PCR at 14 DPI, using unvaccinated but infected mice as a control and *Gapdh* as the housekeeping gene. Relative to mice vaccinated with HK-fbp1 alone, HK-fbp1/ODN vaccinated mice showed significantly higher expression of *Ifng*, *Il12a*, *Il12b*, *Il23a* and *Il17a* (Figure S8B to S8F). In contrast, *Il4* expression was markedly downregulated, at a level comparable to that of unvaccinated infected mice (Figure S8G). The concomitant upregulation of *Il12a*, *Il12b* and *Ifng* is consistent with activation of an IL-12/IFN- γ axis following HK-fbp1/ODN vaccination. Although these transcriptional data alone do not establish a requirement for IL-12, our data suggests IL-12 as a potential upstream contributor to the enhanced IFN- γ response. Because *Il12b* encodes the p40 subunit shared by IL-12 and IL-23, the coordinated increase in *Il12b*, *Il23a* and *Il17a* is also consistent with engagement of the IL-23/IL-17 axis under this condition. Together with the limited *Il4* expression, these data indicate that ODN adjuvantation shifts the pulmonary transcriptional response toward a Th1/Th17-associated rather than a type 2-associated profile in single-dose vaccinated mice. Taken together,

our data demonstrate that IFN- γ is an essential protective cytokine in this vaccine model, whereas IL-17A/F are dispensable under the standard two-dose regimen. In the case of IL-17A/F, a contribution becomes significant when received suboptimal vaccination.

CD8⁺ T cells compensate for CD4⁺ T cell deficiency as a source of effector cytokines, including IFN- γ .

As previous data has established that IFN- γ is required for vaccine-induced protection, we next asked the provider cells are. IFN- γ is predominantly produced by CD4⁺ Th1 cells, CD8⁺ cytotoxic T cells, NK/T cells, and type I innate lymphoid cells (ILC1). To identify the cellular sources responsible for the IFN- γ -dependent protection in this vaccination model, we performed survival studies in CD4⁺ T cell-depleted CBA/J mice that were i.m. vaccinated with HK-fbp1/ODN twice and subsequently challenged with H99 as previously described ¹⁵. CD4⁺ T cell depletion was achieved by weekly intraperitoneal injection of CD4-depleting monoclonal antibody clone GK1.5, and CD4⁺ T cell levels were assessed at three time points: prior to vaccination, prior to challenge, and at the experimental endpoint. At all-time points measured, CD4⁺ T cells were completely cleared in anti-CD4 mAb treated mice, while isotype mAb treated mice showed no change in T cell phenotype (Figure S9A). Interestingly, depletion of CD4⁺ T cells did not compromise the protective efficacy of HK-fbp1/ODN vaccination in CBA/J mouse model (Figure 7D). Endpoint fungal burden analysis from both isotype and CD4-depleted mice showed extended H99 proliferation in the lung but no difference of H99 dissemination to the other tested organs (Figure 7E). These data provide support to the potential application of HK-fbp1/ODN vaccine to certain immunocompromised populations, such as patients with HIV/AIDS, who also possess a weakened immune system due to diminished CD4⁺ T cell counts.

To further investigate the mechanisms by which mice survived H99 infection despite CD4⁺ T cell deficiency, we analyzed the airway T cell composition and T cell-associated cytokine profiles including IFN- γ at the experimental endpoint. In the lung of naïve mice, most T cells express high CD62L, while in vaccinated isotype treated mice, a larger proportion of both CD4⁺ and CD8⁺ T cells differentiated into effector phase with increased CD44 expression. In CD4 depleted mice, the extent of CD62L⁻ CD44^{high} CD8⁺ T cells differentiation was significantly above the isotype group (Figure S9B). These findings point to the successful activation of major effector

T cell subsets present in the vaccinated hosts. In isotype treated mice, the majority of T cells in the BALF were CD4⁺ T cells, with only low levels of CD8⁺ T cells. However, in mice depleted of CD4 T cells, CD8⁺ T cells markedly increased in frequency and numbers (Figure 8F). Furthermore, the frequency of IFN- γ producing CD8⁺ T cells in CD4 depleted mice was higher than that of CD4⁺ T in isotype (Figure 8G and 8H). Similar pattern was also observed in TNF- α and IL-17A production (Figure 8G and 8H), indicating a compensation of effector cytokine produced by CD8 in the absence of CD4⁺ T cells, which is sufficient to confer protection against H99 infection. The production pattern of IL-13 was distinct from that of pro-inflammatory cytokines. IL-13 was not detected in BALF T cells from either isotype control or CD4-depleted mice (Figure 8E and 8F), indicating that neither CD4⁺ nor CD4-independent T-cell subsets produced measurable IL-13 in this setting. Together, these findings demonstrate that in the absence of CD4⁺ T cells, CD8⁺ T cells can functionally compensate by producing key effector cytokines such as IFN- γ , TNF- α , and IL-17A, enabling protective immunity against H99 infection.

ODN dose and boosting define a near-sterilization threshold for intramuscular HK-fbp1-mediated protection.

In our initial experiments, a full protection with complete clearance of fungal burden was readily achieved in C57BL/6 mice but not in the BALB/c mouse strain. In BALB/c mice, the standard two-dose HK-fbp1/ODN regimen yielded ~ 75% survival (Figure 1D). To determine whether the magnitude of CpG adjuvantation imposes a threshold on the efficacy of intramuscular HK-fbp1 vaccination, we further examined whether increasing the ODN amount or adding an additional booster immunization could enhance protection specifically in BALB/c mice. Our data showed that both increasing the ODN amount from 20 μ g to 40 μ g or administering a third HK-fbp1/ODN immunization further enhanced protection and reached 100% survival against lethal H99 challenge in BALB/c (Figure 8A). Consistent with these survival outcomes, increasing the CpG amount in the two-dose regimen resulted in near-complete blockage of fungal dissemination, with brain and spleen CFU reduced to undetectable levels and residual pulmonary burden comparable to that observed in standard two-dose HK-fbp1/ODN-vaccinated mice (Figure 1F). In contrast, adding a third HK-fbp1/ODN dose immunization further decreased lung fungal burden with 7 out of 8 mice reached complete clearance (sterilization) both in the lung and extrapulmonary organs, demonstrating that additional boosting can convert containment into full organ-level sterilization

in BALB/c mice as well. At the effector level, increasing CpG amount in HK-fbp1/ODN vaccine regiment alone did not further elevate BALF CD4⁺ T-cell cytokine production beyond that achieved by the standard two-dose HK-fbp1/ODN regimen, whereas a third immunization markedly amplified IFN- γ , TNF- α and IL-17A while maintaining minimal IL-13 (Figure 8C). These findings indicate that CpG adjuvantation establishes the qualitative Th1/Th17 immune program required for protection, while boosting expands the effector pool necessary to achieve sterilizing immunity in the lung.

Together, our data demonstrate that CpG ODN does not simply enhance intramuscular HK-fbp1 vaccination but instead sets a quantitative immunological threshold that determines whether protection remains partial or transitions to sterilizing immunity.

Discussion

Invasive fungal infections, particularly those caused by *C. neoformans*, pose a serious global health threat. The high rates of morbidity and mortality underscore the urgent need for effective vaccines that can provide long-lasting protective immunity both for the general population and for high-risk groups. An ideal cryptococcal vaccine would not only prevent infection but also enhance host immune responses, particularly by promoting robust T cell-mediated immunity, which plays a central role in fungal pathogen clearance. The HK-fbp1 intranasal vaccine candidate we previously developed provides robust T cell-mediated immunity and is highly effective against both *Cryptococcus* and other fungal pathogens, including *Aspergillus fumigatus*¹⁵. Excitingly, this whole cell-based vaccine candidate provides strong protection not only in immunocompetent but also immunosuppressive mouse models against cryptococcosis, an important indicator for future fungal vaccine development as immune deficiency is one major risk factor for invasive mycoses in general. A recent study from our group has identified a few cryptococcal components contributing to HK-fbp1 vaccine-induced protection³⁵. The cryptococcal capsule was shown to be important for HK-fbp1 vaccine induced protection, particularly the GPI-anchored mannoprotein cytokine-inducing glycoprotein 1 (Cig1)³⁵. In this study, we showed that the deletion of *CIG1* under *fbp1* Δ background resulted in reduced protective profiling. However, the *fbp1* Δ *cig1* Δ double mutant still led to substantial protection, indicating Cig1 is required for HK-fbp1-mediated protection but not the sole immunogenic contributor. Therefore, the repertoire of additional

protective antigens remains to be defined. One potential downside of the intranasal vaccine platform is that the inoculation of large amount yeast cells directly to the lung may complicate the outcome in patients with existing pulmonary conditions. In addition, it is a less established method for large population vaccination compared to other methods, such as intramuscular vaccination. Therefore, we have been exploring other vaccine methods to minimize these side effects and improve the vaccine efficacy to broaden its potential utility. Adjuvants have been widely utilized in vaccine development, including *Cryptococcus* vaccine research^{36,37}. In this study, we developed a highly effective intramuscular (i.m.) vaccine by introducing a novel combination of our HK-fbp1 vaccine with a CpG adjuvant ODN. Comparing to previously established intranasal HK-fbp1 vaccine that directly administrates yeast cells to the lung to induce local protective immunity, this newly developed intramuscular vaccine is easier to administrate and has less side effects without compromising the efficacy and durability. In fact, the i.m. vaccination route is the most commonly used immunization method among FDA-approved vaccines, which may help on further fungal vaccine development³⁸. In addition, intramuscular HK-fbp1/ODN vaccination established a broader and more functionally coordinated priming-effector T-cell axis than local immunization, characterized by strong proliferative priming in distal draining lymph nodes and robust Th1/Th17 effector responses at the site of infection. Strikingly, in all mouse strains tested (C57BL/6, CBA/J and BALB/c), this HK-fbp1/ODN combination vaccine via i.m. injection method provided better protection against both local lung infection and dissemination to cause brain infection than intranasal vaccine. Protection was strongest in C57BL/6 mice, which showed complete fungal clearance in the lung and spleen with minimal brain dissemination, while BALB/c and CBA/J mice exhibited significantly reduced systemic fungal loads. Notably, survivors showed preserved lung architecture with no evident structural damage at the experiment endpoint, suggesting an effective control of pulmonary infection and associated tissue pathology¹⁵. These exciting observations promote us to further decipher the immune mechanism of this improved vaccine protection.

T cells are believed to be the major effector to eliminate fungal infection. We revealed a potent T cell activation and effector cytokine release in protected mice. More importantly, we identified the essentiality of IFN- γ in vaccine-mediated protection and observed high production of IFN- γ across mouse genetic backgrounds. IL-12 may contribute to this IFN- γ response, but delineating its contribution will require further investigation. Although CD4⁺ T cells are a major

source of IFN- γ and IL-17A in protected mice, CD4 depletion did not abrogate vaccine-mediated protection. Instead, CD8⁺ T cells expanded and produced effector cytokines sufficient to maintain survival, indicating that protective immunity can be sustained through alternative T-cell subsets when CD4⁺ T cells are absent. These results align with our previous findings that the compensatory mechanisms in HK-fbp1 i.n. vaccination model ¹⁵. With respect to the activation of early innate effectors, dendritic cells are the principal antigen-presenting cells that process antigen and present it on MHC II to naïve T cells, thereby initiating CD4⁺ T-cell priming and downstream adaptive immunity. Consistent with this role, we observed substantial recruitment of mo-DCs to the lung, along with their precursor-monocytes. We propose that this elevated mo-DC recruitment contributes to enhanced T-cell activation in the lung of HK-fbp1/ODN vaccinated groups post infection, as these DC subsets are known to be mobilized under inflammatory conditions ²². In our previous work on the intranasal HK-fbp1 platform, CCR2⁺ monocytes were shown to be an important early source of IFN- γ , and depletion of CCR2⁺ or CD11c⁺ cells impaired protective Th1 immunity and fungal control ¹⁷. The substantial monocyte recruitment we observed following intramuscular HK-fbp1/ODN vaccination is therefore likely contribute to the protective response both as a direct source of early IFN- γ , and indirectly, through their differentiation into antigen-presenting mo-DCs. Whether CCR2⁺ monocytes play an equally central role in the intramuscular setting remains to be directly tested in future work. Additionally, surviving groups and endpoint survivors were characterized by pronounced neutrophil recruitment accompanied by elevated IL-17A, IFN- γ and TNF- α levels, together with reduced eosinophil infiltration and diminished IL-13 production. This neutrophil-dominant signature aligns with our prior work demonstrating that neutrophils elicited by our vaccine contribute to IFN- γ production ¹⁷. In addition, this coordinated shift toward a neutrophil-dominated, IL-17A and TNF- α -driven inflammatory environment, coupled with suppression of type 2 eosinophilic responses, likely reflects a protective immune profile associated with improved survival. The contribution of neutrophils to cryptococcosis is more context-dependent, but they are well established as rapidly recruited phagocytes that follow complement cues (C3/C5), engulf and kill fungi, and release chemokines that amplify local influx ^{17, 39, 40, 41}. Cell type-specific depletion in this model, for example using anti-Ly6G depleting antibody will be needed to directly validate the contribution of neutrophils to vaccine-induced protection. Eosinophilia in the host with cryptococcosis is a strong manifestation of skewed Th2 immunity and associated with cryptococcal dissemination ^{42, 43, 44}. In our vaccination model, the

reduction in IL-5 and lack of eosinophilia indicates that detrimental Th2 skewing, which often associated with worse outcomes in cryptococcosis, is not a major feature of the protective, adjuvanted response. Although high neutrophil frequencies and elevated IL-17A levels were consistently observed throughout the study, their specific functional roles in this vaccination model remain to be fully elucidated. Previous studies in multiple cryptococcal vaccination models have demonstrated an important contribution of Th17 and IL-17 responses to protective immunity^{45, 46, 47}. Although dispensable after two-dose vaccination, IL-17A/F proved to be important for protection under a single-dose regimen, where increased *Il23a* expression suggests a possible upstream driver, which warrants future studies. Together, our study revealed a balanced act between high Th1/Th17 levels with low Th2 level, leading to the protective effector triggered by this combined vaccine.

Although both HK-fbp1 groups with and without ODN addition mounted CD4⁺ T cell responses in lymph nodes, with ODN group exhibited higher IL-2 production in the vaccine-draining lymph nodes, suggesting more robust T cell priming, which is essential for T cell proliferation and survival (Figure S9). This correlates with greater IFN- γ and IL-17A production by CD4⁺ T cells at the infection site; key effector cytokines required for fungal clearance. In contrast, the lower IL-2 levels in HK-fbp1 group may reflect limited expansion or functionality of effector T cells, resulting in suboptimal IFN- γ /IL-17A responses and failure to protect. IL-2 levels in distal lymph nodes during priming can serve as a surrogate readout of T cell expansion. Elevated IL-2 in protected animals is consistent with greater vaccine-induced T cell expansion, whereas unvaccinated animals exhibited baseline IL-2 following H99 infection. However, in this study we did not directly investigate the trafficking of *Cn*-specific immune cells, particularly CD4⁺ T cells. Central memory-like CD4⁺ T cells (CD44^{hi} CD62L^{hi} CCR7^{hi}) preferentially circulate through the lymphoid organs while retaining proliferative capacity and IL-2 production, whereas effector/effector-memory cells (CD44^{hi} CD62L^{lo} CCR7^{lo}) mainly survey the non-lymphoid tissues and produce effector cytokines^{48, 49, 50, 51}. Based on our T cell recall responses, we infer that after more than one-month of vaccination, the muscle draining inguinal and popliteal lymph nodes are enriched for central memory-like CD4⁺ T cells, while at 7 DPI, the lung draining mediastinal lymph node is an actively responding compartment, in which effector CD4⁺ T cells are generated in response to local antigen. This distribution would be consistent with a model in which vaccination

sustains a long-lived central memory pool in the injected distal site which will give rise to effector cells recruited to infection site upon challenge. Directly addressing this question will require a finely dissected analysis of vaccination versus infection across tissues and timepoints, which will be an important future direction.

The single-cell transcriptomic atlas shows that adjuvanted HK-fbp1 vaccination remodels lymphoid and myeloid compartments, promoting transcriptional programs associated with enhanced activation, antigen presentation, and cellular fitness in both B- and T-cell lineages rather than simply expanding lymphocyte numbers. IFN-responsive B and T lymphocytes were highlighted in HK-fbp1 and HK-fbp1/ODN-vaccinated mice, consistent with our previous reports that the *fbp1*Δ strain strongly induces type-II interferons^{15, 21, 22}. Antigen-presentation signatures in memory B cells in the adjuvanted group reflect transcriptional potential rather than demonstrated APC function in our system and will require functional validation in future studies. Despite the expansion of B-cell populations and their enhanced transcriptional programs, B cells were dispensable under two-dose regimen, however, contributed to single-dose induced protection. One possible explanation is that other APCs, such as TLR-expressing dendritic cells activated by CpG ODN adjuvant, could compensate for the absence of B cells by providing antigen presentation, which remains to be tested.

Overall, we identified a potent combination vaccine (HK-fbp1/ODN) that provided strong protection in mouse models across various administration methods. We also delineated the immune factors required for mobilizing host immune systems to provide robust immune protection in the i.m. model. This novel combination vaccine strategy could pave the way for the development of a highly effective and long-lasting vaccine targeting cryptococcosis. Going forward, there are several questions remaining to be addressed, including the precise role of Th17 responses in vaccine-mediated protection and the mechanisms by which B cells contribute to cellular immunity, etc. In addition, future studies will be required to identify immunodominant antigens within this whole-cell vaccine to enable exploration of multivalent subunit vaccine strategies.

Materials and Methods

Ethics statement on animal use

Mice with an average weight of 20 - 25 g were used throughout these studies. Wild-type BALB/c, WT C57BL/6, $\mu\text{Mt}^{-/-}$ $\text{IFN}\gamma\text{R}^{-/-}$ and $\text{IL-17A/F}^{-/-}$ mice were purchased from the Jackson Laboratories. $\mu\text{Mt}^{-/-}$ mice were also kindly provided by Dr. John Chan (Rutgers University). The genotypes of knockout mice were confirmed by ear punch and/or blood-based genotyping. WT CBA/j mice were purchased from the Inotiv, Inc. $\text{IFN}\alpha\text{R}^{-/-}$, $\text{IFN}\lambda\ 2/3^{-/-}$, $\text{IFN}\alpha\text{R} \ \& \ \lambda 2/3^{-/-}$ mice were kindly provided by Dr. A. Rivera. Animal studies were performed at Rutgers University Newark campus animal facility. All studies were conducted following biosafety level 2 (BSL-2) protocols and procedures approved by the Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee of Rutgers University under protocol 999901066. Animal studies were compliant with all applicable provisions established by the Animal Welfare Act and the Public Health Services (PHS) Policy on the Humane Care and Use of Laboratory Animals.

Vaccination strategies

*C. neoformans fbp1*Δ mutant strain was heat-killed following a previously described procedure¹⁵. Briefly, 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 90 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. Adjuvant CpG ODN 2395 (InvivoGen, CAT# vac-2395-1) was dissolved following manufacturer's instruction. Heat-killed 5×10^7 or 1×10^7 *fbp1*Δ cells (HK-fbp1 vaccines) were prepared in 50 μl 1 x PBS. ODN adjuvanted HK-fbp1 vaccines (HK-fbp1/ODN) were prepared by adding 10 μl (2 mg/ml) ODN 2395 after aspirating the same volume of supernatant from the 50 μl cell suspension in 1 x PBS. ODN 2395 vaccines were prepared in 50 μl (0.4 mg/ml) sterile endotoxin-free physiological water. Adjuvant Addas03 (Invivogen, CAT # vac-as03-10) was provided in a ready-to-use emulsion. AS03 adjuvanted HK-fbp1 vaccines (HK-fbp1/AS03) were prepared by mixing HK-fbp1 cell pellet with AS03 at 1:4 volume ratio in a total of 50 μl suspension/mouse. All vaccines were thoroughly mixed before immunization. Each group of 8~10 mice were vaccinated intranasally, subcutaneously, intraperitoneally, or intramuscularly at day -42 unless otherwise specified. Each group of mice were vaccinated again with the same type and dose at day -12 unless otherwise specified. A group of unvaccinated mice served as a control.

668 **Animal infections with *C. neoformans***

669 To prepare fungal cells for infection, overnight cultures of *C. neoformans* H99 was washed two to
670 three times with 1 x PBS buffer and the concentration of yeast cells was determined by
671 hemocytometer counting. Each mouse was infected intranasally with 1×10^4 (BALB/c and
672 C57BL/6) or 5×10^4 (CBA/J) live H99 cells in a 50 μ l volume after being anesthetized with a mix
673 of Ketamine (12.5 mg/mL) and Xylazine (1 mg/mL). After infection, animals were weighed daily
674 and monitored twice daily for progression of disease, including weight loss, gait changes, labored
675 breathing, and fur ruffling. Over the course of the experiments, animals that appeared moribund
676 or in pain were euthanized by CO₂ inhalation. Survival data from the murine experiments were
677 statistically analyzed between paired groups using the Log Rank (Mantel-Cox) test with PRISM
678 version 10.5 (GraphPad Software) (P values < 0.05 were considered statistically significant). The
679 resulting data were plotted against time. To compare the fungal burdens, infected lungs, brains,
680 and spleens were isolated and homogenized (Fisherbrand, Homogenizer 150) in 3 ml cold 1 x PBS
681 buffer for 1 minute for each type of organ. The tissue suspensions were serially diluted and plated
682 onto YPD agar medium with ampicillin and chloramphenicol, and colonies were counted after 3
683 days of incubation at 30°C.

684 **In vivo CD4⁺ T cell depletion**

685 Mice were depleted of CD4⁺ T cells by intraperitoneal administration of anti-mouse CD4
686 monoclonal antibody (clone GK1.5; Bio X Cell, Cat# BE0003-1). An isotype-matched control
687 antibody (clone LTF-2; Bio X Cell, Cat# BE0090) was administered to control mice. Each mouse
688 received 200 μ g of GK1.5 or LTF-2 in 150 μ L PBS per injection. Antibody administration was
689 initiated 9 days before the first vaccination and continued weekly until the study endpoint. The
690 anti-mouse CD4 (clone RM4-4, FITC; BD, CAT# 553055) antibody was used for depletion
691 confirmation. Depletion efficiency was assessed by flow cytometry using peripheral blood samples
692 collected one day before each vaccination and before infection, and was further confirmed in lung
693 tissues and BALF samples at the study endpoint. Mice receiving LTF-2 underwent the same
694 injection schedule and served as isotype controls at all checkpoints.

695 **Histopathology**

Infected animals were sacrificed at different timepoints of the experiment according to the Rutgers University IACUC approved animal protocol. To compare the fungal burdens and host inflammatory responses, the lungs, brains, and spleens were dissected and fixed in 10% formalin solution for section preparation at Rutgers University Histology Core Facility. Tissue slides were treated with hematoxylin and eosin (H&E) staining.

Lung processing

Single cell suspensions of pulmonary cells were prepared for flow cytometric analysis as previously described^{52, 53}. In brief, lung tissue was minced in 5 ml of 1 x PBS containing 3 mg/ml collagenase type IV (Worthington). Samples were incubated at 37°C for 45 min and washed with 1 x PBS. After digestion, residual RBCs were removed using RBC lysis buffer (155 mM NH₄Cl and 10 mM NaHCO₃, PH 7.2). Lung cell suspensions were used for flow cytometry. Lungs single cell suspensions were stained for monocytes: CD45 (30-F11 BUV395), CD11b (M1/70 PerCP Cy5.5) and Ly6C (AL-21 PE), Mo-DCs: CD45 (30-F11 BUV395), CD11b (M1/70 PerCP Cy5.5), Ly6C (AL-21 PE), CD11c (HL3 BV510) and MHC Class II I-A/I-E (M5/11.415.2 BV711), neutrophils: CD45 (30-F11 APC-Cy7), CD11b (M1/70 PerCP Cy5.5), Ly6C (AL-21 PE) and Ly6G (1A8 APC), CD4 T cells: CD45 (30-F11 BUV395), CD4 (RM4-5 BV421) and/or (RM4-4 FITC) , CD8 T cells: CD45 (30-F11 BUV395), CD8 α (53-6.7 BV711), CD62L (MEL-14 APC), CD44 (IM7 PE). All antibodies used for lung staining were from BD Biosciences. All samples were analyzed using BD LSR FortessaTM Flow Cytometer and FlowJo software (Tree Star, Inc).

Intracellular cytokine staining of T cells harvested in BALF and Flow Cytometry

For analyzing host immune responses, bronchoalveolar lavage fluid (BALF) samples were harvested at different timepoints after inoculation. BALF was collected in 5 ml of 1 x PBS buffer using a catheter inserted into the trachea of animal post-euthanasia, and airway-infiltrating cells were lavaged with ~ 1 mL of 1 x PBS at a time to a total volume of 5 mL. RBCs were removed using RBC lysis buffer. BALF cells were then plated in a 96-well round-bottom plate and restimulated using BD-Leukocyte Activation Cocktail containing BD GolgiPlugTM (BD Biosciences) according to the manufacturer's instructions. Five to six hours after activation, BALF cells were surface-stained with fluorescently labeled antibodies against Thy1.2, CD4 and CD8.

Samples were fixed in 1% paraformaldehyde overnight. Prior to intracellular staining the samples were permeabilized with 1 x BD Perm/Wash buffer according to the manufacturer's instructions. Intracellular cytokine staining (ICCS) was done using fluorescently labeled antibodies against IFN- γ , IL-17A, TNF- α and IL13 diluted in 1 x BD Perm/Wash for 30 minutes on ice. Samples were immediately washed and analyzed by flow cytometry as described below. BALFs were cell surface stained for T cells with Thy1.2 (53-2.1 PE-Cy7), CD4 (RM4-5 BV421), CD8 α (53-6.7 BV711) and ICCS for IFN- γ (XMG1.2 PE), IL-17A (eBio17B7 APC), TNF- α (MP6-XT22 BV711) and IL-13 (eBio13A FITC) following standard procedures. Most antibodies and reagents for cell surface and ICCS were from BD Biosciences, except IL-17A and IL-13 which were obtained from eBioscience, Inc.

CD4⁺ T cell isolation and CD4⁺ T cell recall response

Antigen-presenting cells (APCs) were prepared from the spleens of syngeneic, uninfected donor mice. Splenic cell suspensions were generated by gently pressing the tissue using forested ends of two glass slides to release leukocytes, followed by T-cell depletion via antibody and complement-mediated lysis. Briefly, splenic cells were incubated with anti-Thy1.2 antibodies (Bio X Cell, CAT# BE00066) and rabbit complement (Low-Tox; Cedarlane Labs, CAT# CL3331) at 37 °C for 1 hour. Single-cell suspensions were prepared by passing splenic tissue through a 70- μ m nylon mesh. Lung-draining mediastinal lymph nodes (mLNs), muscle-draining inguinal LN (iLN) and popliteal LN (pLN) were collected and placed in a 10ml of 1 x PBS. Total lymphocyte cell suspensions were prepared by gently releasing the cells into the 1 x PBS by applying pressure to the lymph nodes with the forested ends of two glass slides. Repeated pressure was applied until the tissue was reduced to the smallest size possible. For CD4 T cell isolation, individual samples from each group were pooled. CD4⁺ T cells were purified using a negative-sorting CD4⁺ isolation kit (Miltenyi Biotec, Inc.; CAT# 130-104-454). CD4⁺ T cell isolation was done following the manufacturer's instructions and were consistently found to be > 90% pure, as assessed by flow cytometry. Purified CD4⁺ T cells (2×10^5) were cultured with T cell-depleted antigen-presenting cells (APCs; 3×10^5) in RPMI (Corning, CAT# 15-040-CV) containing 10% fetal bovine serum (FBS, Cytiva, CAT# SH30910.03), Penicillin-Streptomycin (2200 U/ml, Gibco, CAT# 15140-122) and Gentamicin sulfate solution (1 mg/ml). To measure Cryptococcus-specific CD4⁺ T cells responses, CD4-antigen-presenting cell cocultures were incubated with beated (MP FastPrep

Grinder) H99 yeasts as a source of fungal antigens. The amount of antigen used was adjusted to a multiplicity of infection of 1:1.5 (antigen-presenting cell:yeast ratio). The fungal growth inhibitor voriconazole was used at a final concentration of 0.5 mg/ml to prevent any fungal cell outgrowth during the culture period. After 72 hours of culture at 37 °C with 5% CO₂, supernatants were collected for cytokine analysis by ELISA (IL-2, IL-5, IL-17A, IFN- γ ; Mouse Uncoated Elisa Kits; Invitrogen) following manufacturer's instructions.

Sample preparation for single-cell RNA sequencing

Inguinal lymph nodes (iLNs, vaccinated site) were harvested from vaccinated female C57BL/6J mice. Single-cell suspensions were generated by gently dissociating iLNs through a 40 μ m cell strainer into ice-cold 1 $\times$ PBS supplemented with 2% FBS. Cells were pelleted, resuspended, and stained with CD45 and DAPI; live CD45⁺ DAPI⁻ leukocytes were sorted on a BD FACS Aria™ III. Post-sort viability exceeded 90% and was independently confirmed by propidium iodide staining on a Moxi cell counter.

Sequencing and processing of single-cell RNA data

Single-cell RNA sequencing libraries were prepared using the 10x Genomics Chromium platform according to the manufacturer's instructions (Single Cell 3' V4, 10x Genomics). Briefly, single-cell suspensions were loaded onto Chromium microfluidic chips to generate single-cell gel bead-in-emulsions (GEMs), followed by reverse transcription, cDNA amplification, and library construction using the Chromium Single Cell 3' Library & Gel Bead Kit. Libraries were sequenced on an Illumina NovaSeq X Plus platform. Raw sequencing data were processed using Cell Ranger (V9.0.1) for demultiplexing, alignment to the reference genome, barcode processing, and generation of gene-cell count matrices.

For downstream analysis, raw count matrices were imported into Seurat (v4.4.0)⁵⁴ to create individual Seurat objects for each sample. Quality control was performed independently for each sample by filtering cells based on total UMI counts (nCount_RNA), number of detected genes (nFeature_RNA), and mitochondrial gene expression percentage (percent.mt). Robust median absolute deviation (MAD)-based thresholds were applied, retaining cells within ± 3 MADs of the median for nCount_RNA and nFeature_RNA, while excluding cells with elevated mitochondrial

content using an upper MAD-based cutoff. Filtered samples were subsequently merged for downstream analyses. Doublets were identified and removed using scDblFinder. Ambient RNA contamination was corrected using DecontX in a sample-aware manner, followed by a second, more stringent round of doublet detection to minimize false-positive assignments.

The filtered data were normalized, highly variable genes were identified, and principal component analysis (PCA) was performed. Batch effects between samples were corrected using Harmony, and cells were embedded using uniform manifold approximation and projection (UMAP) for visualization. Graph-based clustering was performed using the Harmony-corrected low-dimensional space. Cell type annotation was carried out using SingleR with the MouseRNAseqData reference and further refined based on canonical marker gene expression. Cluster-specific marker genes were identified by differential expression analysis. Gene Ontology biological process (GO:BP) enrichment analysis was conducted using pre-ranked gene set enrichment analysis (GSEA) implemented in the clusterProfiler package, with genes ranked by average log₂ fold change within each cell type and enrichment assessed using MSigDB C5:GO:BP gene sets. Enriched GO terms were defined at a nominal P-value < 0.05 and visualized using dot plots.

Detection of H99-protein specific serum IgG

H99 protein-specific serum IgG was measured by enzyme-linked immunosorbent assay (ELISA). *C. neoformans* H99 protein was extracted as previously described⁵⁵. High-binding 96-well plates (Corning, CAT # CLS9018) were coated with 2 µg of H99 protein in 100 µl 1× PBS per well and incubated overnight at 4°C. The following day, plates were washed twice with PBS containing 0.05% Tween-20 (PBS-T) and blocked with 200 µL of 5% nonfat milk in PBS for 2 h at room temperature or 1 h at 37°C. Plates were then washed three times with PBS-T. Serum samples were tested in blocking buffer and incubated for 2 h at room temperature. Blocking buffer alone was included as a technical negative control, and serum from naïve mice and purified mouse IgG were included as experimental negative controls. Plates were subsequently washed and incubated with HRP-conjugated anti-mouse IgG antibody at 1:2000 dilution for 1-2 h at room temperature (Cytiva, Cat. No. NA931V). After four washes with PBS-T, 100 µl of TMB substrate was added to each

well for 15 min at room temperature. The reaction was stopped with 100 μ l 1 M H₂SO₄, and absorbance was measured at 450 nm.

Quantitative Real-Time PCR (qPCR)

Mouse lung tissues were collected and immediately stored at -80°C . Total RNA was extracted by homogenizing lung tissue in 3 mL of TRIzolTM Reagent (Invitrogen, CAT# 15596026). The crude RNA preparations were subsequently purified using the NucleoSpin RNA Clean-up Kit (MACHEREY-NAGEL, CAT# 740948) according to the manufacturer's instructions. First-strand cDNA was synthesized using SMARTScribeTM Reverse Transcriptase (Takara, CAT# 639536) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed using TB Green[®] Advantage[®] qPCR Premix (Takara, CAT# 639676) on an Agilent AriaMx Real-Time PCR System with AriaMx Software v2.0. Relative gene expression was normalized to Gapdh and calculated using $2^{-\Delta\Delta C_t}$ method. The primers used for qRT-PCR were listed in Supplementary Table 1.

Data Availability

The raw and processed single-cell RNA-seq data are deposited in the NCBI Gene Expression Omnibus (GEO), under accession number: GSE319728. All other source data supporting the findings of this study are provided in the Supplementary Data.

Statistics and Reproducibility

Statistical analyses were performed using GraphPad Prism version 10.5.0 and R version 4.5.0 (2025-04-11) using RStudio/Posit (version 2025.25.0+496). GraphPad Prism was used for statistical analyses of survival, fungal burden, flow cytometry, and other experimental datasets, whereas R was used for single-cell RNA-sequencing data processing, analysis, and visualization. Data are presented as mean $\pm$ SD unless otherwise indicated. Each data point represents an independent biological sample or experimental replicate, unless otherwise stated. Technical replicates, when performed, were averaged and were not treated as independent observations. Exact sample sizes (N) and statistical tests are provided in the corresponding figure or figure legends. All statistical tests were two-sided, and $P < 0.05$ was considered statistically significant.

No data were excluded unless otherwise stated. Experiments were independently repeated at least two to three times unless otherwise specified.

Acknowledgments

We appreciate Dr. Sukhwinder Singh and Tammy Galenkamp from the Rutgers Flow Core for helping with flow cytometry and cell sorting experiments. We acknowledge Lu Gan and Aaron Lemmer for suggestive discussion of this manuscript. We acknowledge Drs. Veera D'Mello and Alexander Lemenze from the Rutgers Genomic Center for their support in scRNA-seq library preparation and sequencing, and for providing and delivering and processing the raw processed data. We thank Dr. John Chan for providing muMt-/- mice for our study. We thank Yu Zhang's thesis committee members for helpful discussion to improve the study design and data interpretation. We also thank Dr. Vaishnavi Kaipilyawar for guidance on single-cell RNA-seq data analysis. This study is supported by NIH grants AI169769 and AI141368 to CX and AR. The Xue lab is also supported by NIH grants AI123315 and AI155647. AR holds an Investigators in the Pathogenesis of Infectious Disease Award from the Burroughs Wellcome Fund.

Author contributions

Y.Z., A.R. and C.X. developed hypothesis and study design. Y.Z. and C.X. wrote the first and successive drafts of the manuscript. Y.Z., W.Y., K.W., A.R. and C.X. contributed to interpretation of data. Y.Z., Y.W., and K.W. performed experiments. A.R. and C.X. obtained funding.

Competing interests

The authors declare no competing interests.

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990

Figure 1

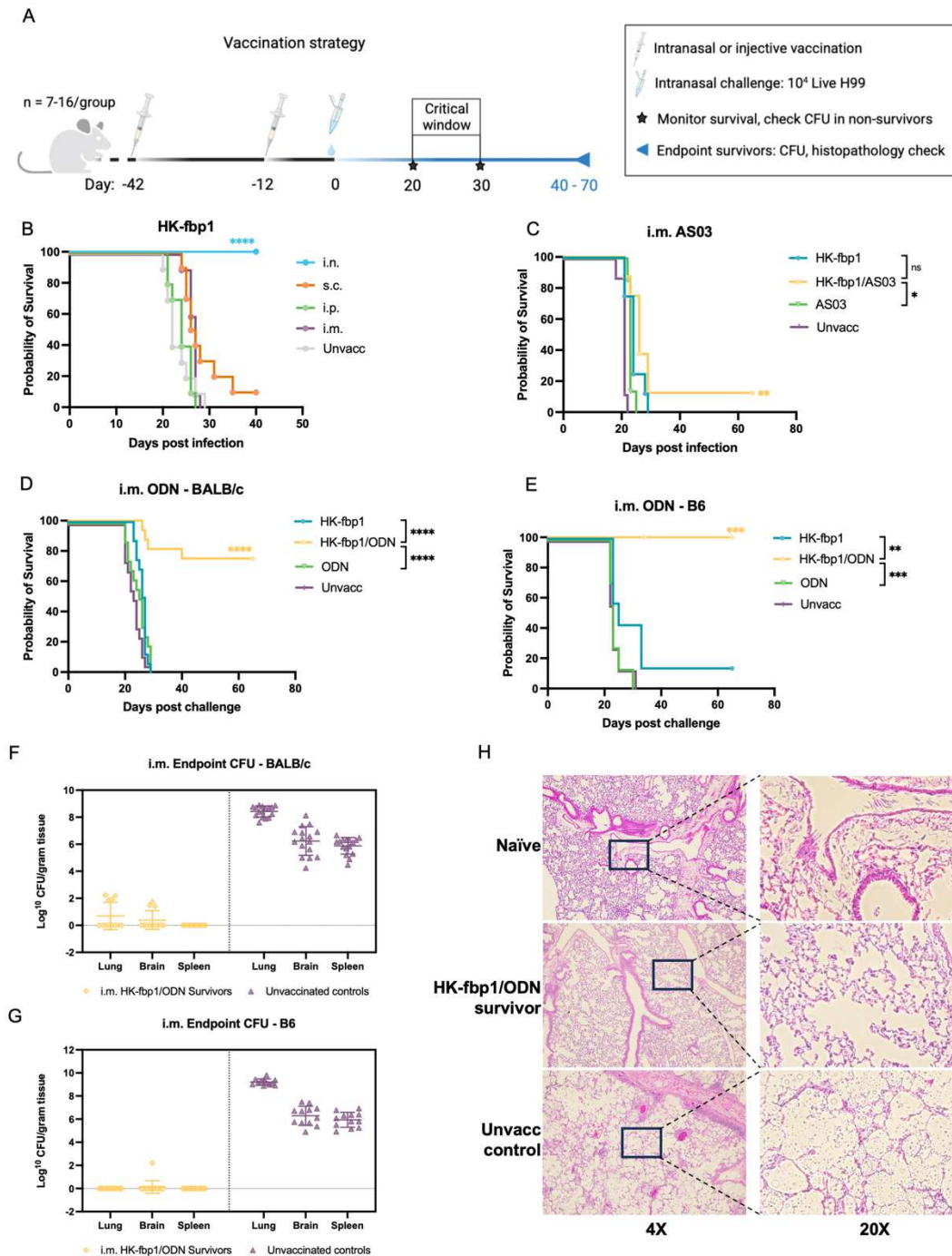
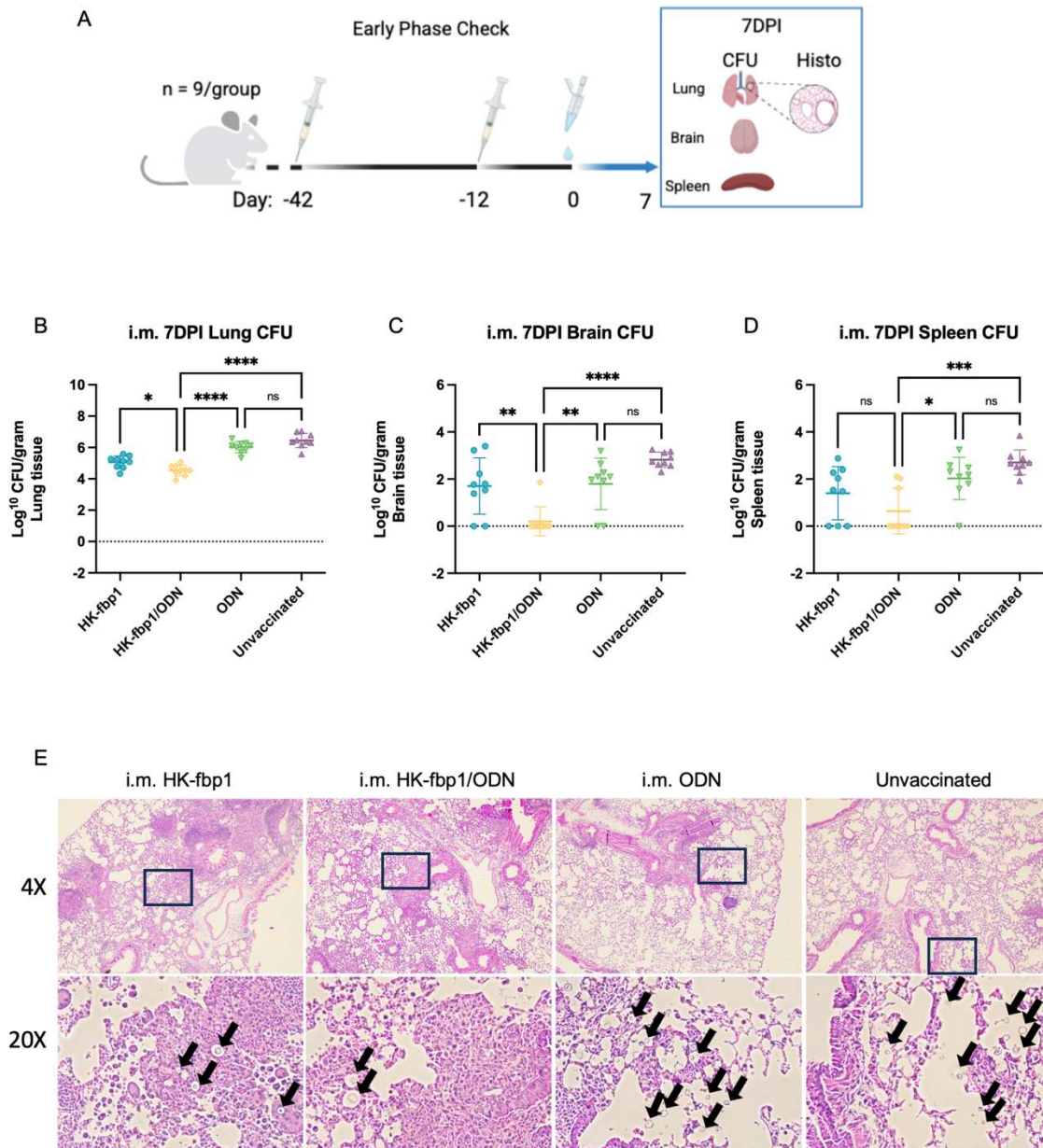


Figure 1. The ODN adjuvanted HK-fbp1 vaccine (HK-fbp1/ODN) provides strong protection against *C. neoformans* challenge in an intramuscular vaccination model. A. Schematic of the vaccination and challenge strategy. Wild-type BALB/c or C57BL/6 mice received two doses of

vaccine via different routes on days –42 and –12, followed by an intranasal challenge with 10^4 C. neoformans strain H99 on day 0. Mice were monitored after infection and euthanized upon reaching humane endpoints to collect lung, brain, and spleen tissues for CFU enumeration. Endpoint survivors were euthanized for both CFU and histopathological analyses. **B.** Survival curves of BALB/c mice vaccinated with 5×10^7 HK-fbp1 via different routes and subsequently challenged with H99. i.n., intranasal; s.c., subcutaneous; i.m., intramuscular; i.p., intraperitoneal; unvacc, unvaccinated controls. $N = 10$ –11 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 AddaS03, or AddaS03 alone via intramuscular injection, followed by intranasal challenge with H99. $N = 8$ per group. **D.** 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 intramuscular injection, followed by intranasal challenge with H99. $N = 16$ per group. **E.** Survival curves of wild-type C57BL/6 mice vaccinated with 5×10^7 HK-fbp1, 5×10^7 HK-fbp1 formulated with ODN2395, or ODN2395 alone via intramuscular injection, followed by intranasal challenge with H99. $N = 7$ –16 per group. **F.** 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 (1D). $N = 12$ –16 per group. **G.** Fungal burdens in the lungs, brains, and spleens of vaccinated surviving C57BL/6 mice at the study endpoint and of non-survivor unvaccinated controls shown in (1E). $N = 12$ –16 per group. **H.** Representative H&E-stained lung sections from surviving vaccinated BALB/c mice at the study endpoint, visualized under light microscopy at $4\times$ and $20\times$ magnification. Black boxes in the $4\times$ panels indicate the corresponding magnified areas shown at $20\times$. Lung tissues from naïve and unvaccinated mice are shown as controls.

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–E). ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. CFU data are presented as mean $\pm$ SD (F, G). Each symbol represents an individual mouse.

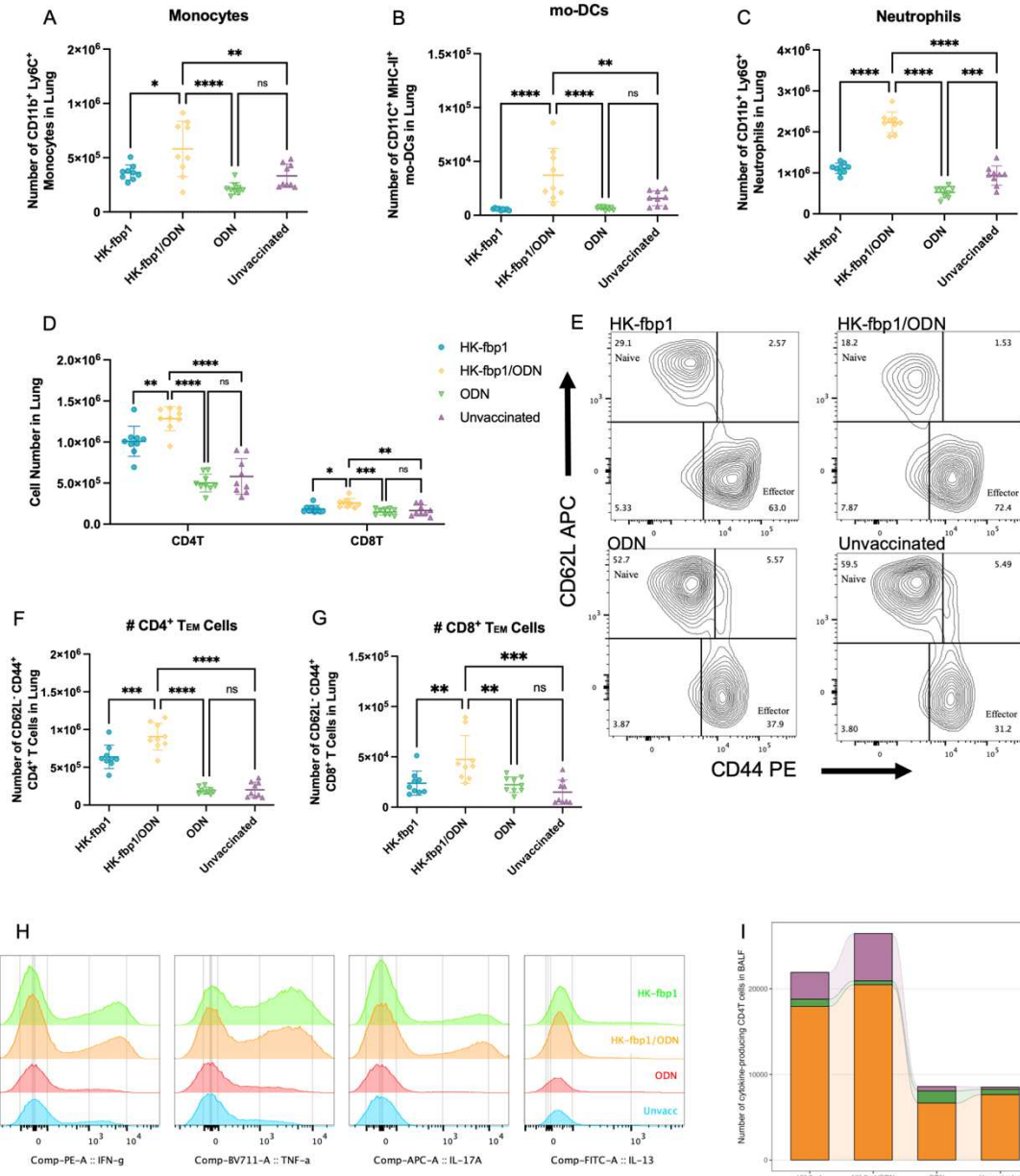
Figure 2



analyses. **B–D.** Fungal burdens in the lungs (B), brains (C), and spleens (D) at 7 days post-infection in intramuscularly vaccinated and unvaccinated mice. **E.** Representative H&E-stained lung sections from infected mice at 7 days post-infection, visualized by light microscopy at 4× and 20× magnification. Black boxes in the 4× panels indicate the corresponding magnified areas shown at 20×. Black arrows indicate *C. neoformans* yeast cells.

CFU data are presented as mean ± SD. Each symbol represents one mouse. $N = 9$ per group. Statistical analysis of CFU data was performed using one-way ANOVA with Tukey's multiple-comparison test. Each symbol represents an individual mouse. ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

Figure 3.



monocyte-derived DCs (mo-DCs) as CD11b⁺ Ly6C⁺ CD11c⁺ MHC-II⁺; neutrophils as CD11b⁺ Ly6C^{int} Ly6G⁺; and CD4⁺ or CD8⁺ T cells as CD11b⁻ CD4⁺ or CD11b⁻ CD8⁺, respectively. **E.** Representative FACS plot showing activation of lung CD4⁺ T cells in intramuscularly vaccinated and unvaccinated BALB/c mice at 7 DPI. **F–G.** Numbers of effector memory CD4⁺ T cells (F) and CD8⁺ T cells (G) in the lungs of vaccinated and unvaccinated BALB/c mice at 7 DPI. Each symbol represents one mouse. *N* = 9 per group. **H.** Histogram plots showing cytokine-producing CD4⁺ T cells in the bronchoalveolar lavage fluid (BALF) of vaccinated and unvaccinated BALB/c mice at 7 DPI. mouse. *N* = 8–9 per group. CD4⁺ T cells were identified as Thy1.2⁺ CD4⁺ CD8⁻. **I.** Alluvial ribbons depict the numbers of cytokine-producing CD4⁺ T-cell subsets in BALF across vaccination groups (values represent mean cell counts from *N* = 8–9 mice per group). Colors denote Th1 (IFN- γ ⁺ and TNF- α ⁺, orange), Th2 (IL-13⁺, green), and Th17 (IL-17A⁺, purple) CD4⁺ T-cell populations.

Data are presented as mean $\pm$ SD (A-D, F-G). Statistical significance was determined using one-way ANOVA with Tukey's multiple-comparison test. Each symbol represents an individual mouse. ****, *P* < 0.0001; ***, *P* < 0.001; **, *P* < 0.01; *, *P* < 0.05.

Figure 4

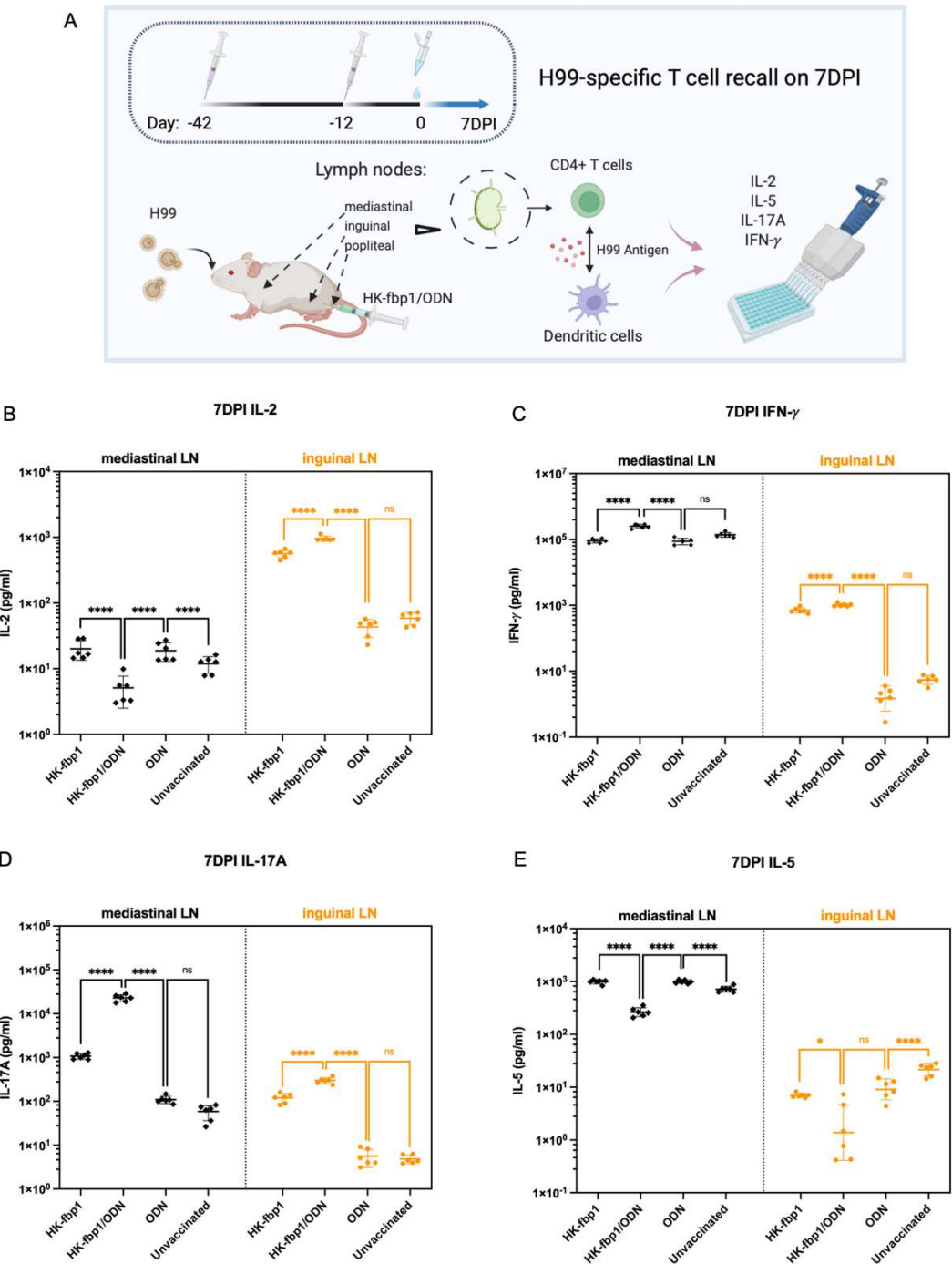
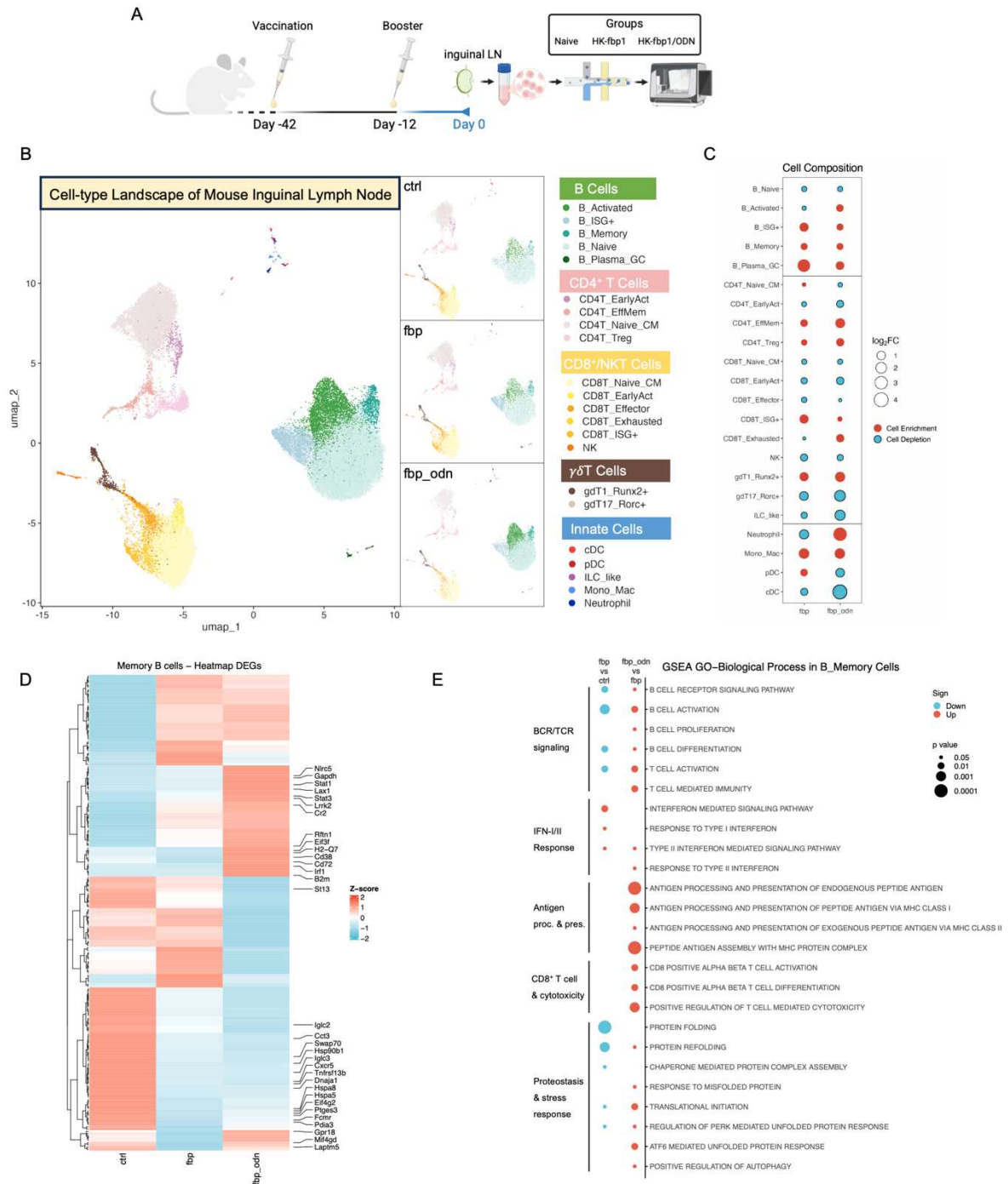


Figure 4. CD4⁺ T cells are highly proliferative in the muscle draining LNs, but only produce high effector Th1/Th17 cytokines in the lung draining LN. A. Schematic illustration

of *Cryptococcus neoformans* H99-specific cytokine detection by CD4⁺ T cells in lymph nodes (LNs). Mediastinal, inguinal, and popliteal LNs were collected from BALB/c mice 7 days post-infection (7 DPI). CD4⁺ T cells were purified from LNs and co-cultured with antigen-presenting cells in the presence of *C. neoformans* H99 antigens. Cytokine production in the supernatant was measured after 72 h of in vitro restimulation. **B–E.** Cytokine levels produced by CD4⁺ T cells in the mediastinal and inguinal LNs.

Each group contained six technical replicates. Data are presented as mean $\pm$ SD. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple-comparison test within each LN. ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

Figure 5.



1094

1095 **Figure 5. Single-cell transcriptomic analysis of immune responses following intramuscular**

1096 **vaccination. A.** Schematic representation of the intramuscular vaccination strategy and single-cell

1097 RNA sequencing (scRNA-seq) sampling time point on Day 0. **B.** UMAP visualization of major

immune cell clusters identified across the three experimental groups (naïve, HK-fbp1, and HK-fbp1/ODN). Cells are color-coded by annotated cluster identity, showing conserved cell-type distributions across samples with treatment-dependent shifts in population abundance. **C.** Variation in cellular composition across immune cell clusters in HK-fbp1– and HK-fbp1/ODN–vaccinated mice relative to controls. Each dot represents a cell subset, with dot size indicating the magnitude of log₂ fold change and fill color indicating enrichment (red) or depletion (blue) compared with ctrl. **D.** Heatmap showing scaled expression (Z-score) of differentially expressed genes (DEGs) in memory B cells across naïve control, HK-fbp1, and HK-fbp1/ODN groups. **E.** Gene set enrichment analysis of memory B cells showing selected GO biological processes enriched in HK-fbp1 vs. control and HK-fbp1/ODN vs. HK-fbp1 comparisons. Dot color indicates enrichment direction (red, up; blue, down), and dot size reflects nominal p value.

Figure 6.

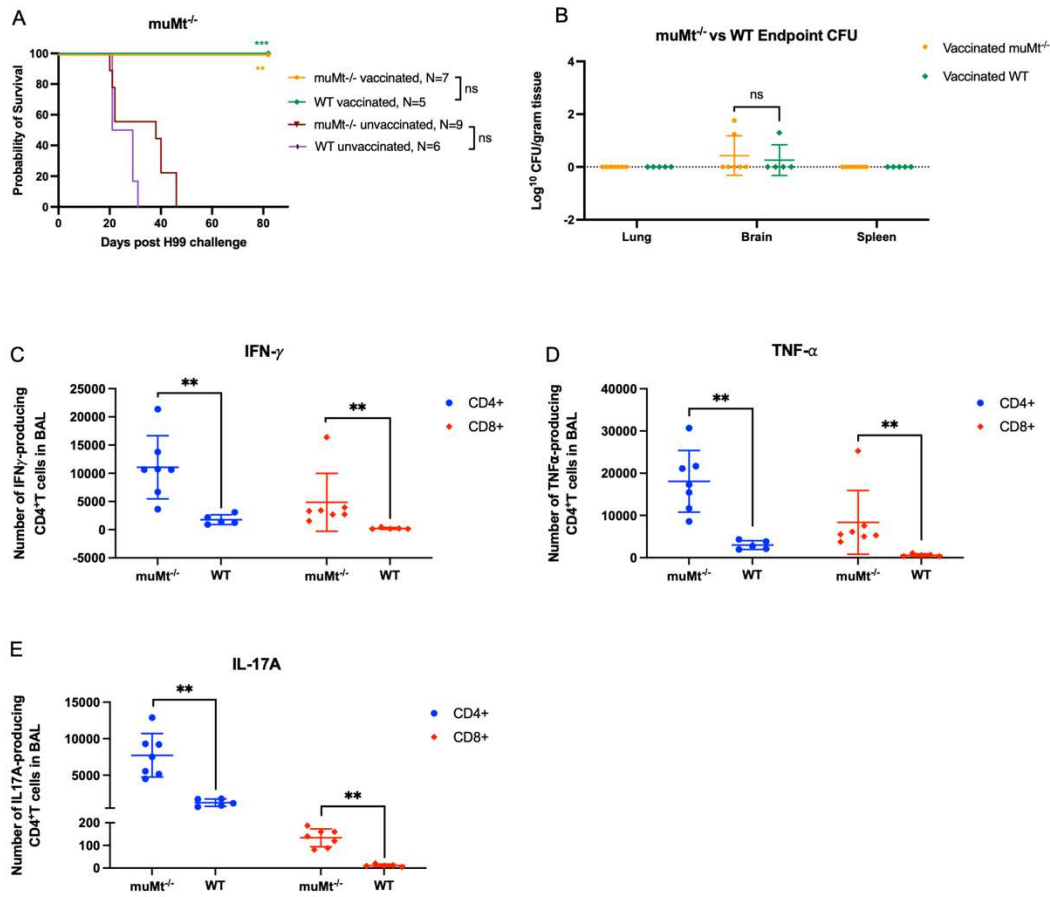


Figure 6. B cell is dispensable for HK-fbp1/ODN mediated vaccine protection in mice.
A. Survival curves of WT C57BL/6 and muMT^{-/-} mice, unvaccinated or vaccinated with HK-fbp1/ODN intramuscularly, following intranasal infection with H99 cells. Numbers of mice in each group are indicated in legend. **B.** Fungal burdens in the lungs, brains, and spleens of surviving mice from each group at the study endpoint, *N* = 5–7 per group. **C–E.** Numbers of cytokine-producing CD4⁺ and CD8⁺ T cells in the bronchoalveolar lavage fluid (BALF) of vaccinated WT and muMT^{-/-} mice at the study endpoint, *N* = 5–7 per group.

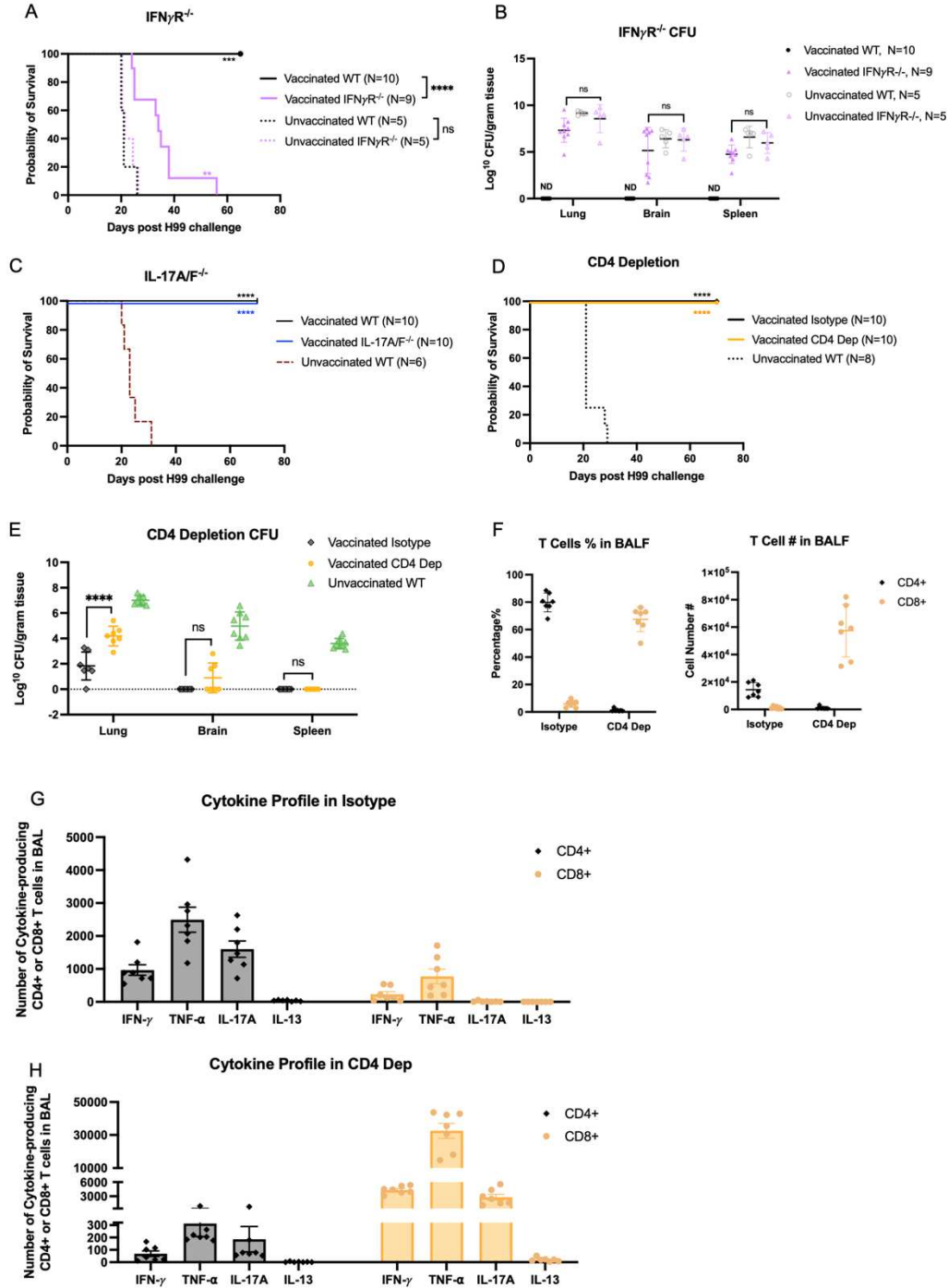
Survival data were analyzed using the Kaplan–Meier method, and statistical significance was determined by the log-rank (Mantel–Cox) test with Holm–Šidák correction for multiple comparisons (A). CFU and cytokine data are presented as mean ± SD, and statistical significance was assessed using the Mann–Whitney U test (non-parametric) (B–F). **, *P* < 0.01. Statistical

1129 significance of IgG data was determined using one-way ANOVA followed by Tukey's multiple-
1130 comparison test (C). ****, $P < 0.0001$. Each symbol represents an individual mouse.

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1132

Figure 7



intramuscular HK-fbp1/ODN vaccine and subsequently challenged intranasally with H99. The number of mice in each group is indicated in the legend. **B.** Fungal burdens in the lungs, brains, and spleens of vaccinated mice at the humane or study endpoint shown in (A). **C.** Survival curves of WT C57BL/6 and IL-17A/F^{-/-} mice that were twice vaccinated or left unvaccinated with the intramuscular HK-fbp1/ODN vaccine and subsequently challenged intranasally with H99. **D.** Survival curves of CBA/J mice weekly treated with isotype control or anti-CD4 (GK1.5) antibodies that were vaccinated or left unvaccinated with the intramuscular HK-fbp1 vaccine and subsequently challenged intranasally with 5x10⁴ H99 cells. **E.** Fungal burdens in the lungs, brains, and spleens of surviving vaccinated mice at the endpoint of the study shown in (D). Unvaccinated mice are shown as controls. *N* = 7–8 per group. **F.** Comparison of the percentages (left) and the absolute numbers (right) of CD4⁺ and CD8⁺ T cells among total Thy1.2⁺ cells in the bronchoalveolar lavage fluid (BALF), and the absolute numbers (right) of CD4⁺ and CD8⁺ T cells in the BALF of isotype control– and anti-CD4 antibody–treated surviving mice at the study endpoint. *N* = 7 per group. **G–H.** Cytokine levels in BALF of isotype control– (G) and anti-CD4 antibody–treated (H) CBA/J mice at the study endpoint. *N* = 7 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 (A, C–D). The number of mice in each group is indicated in the legend. CFU and flow cytometry data are presented as mean ± SD. CFU statistical significance was determined using Mann–Whitney U test within each tissue (non-parametric) (B). Each symbol represents an individual mouse. *****, *P* < 0.0001; ***, *P* < 0.001; **, *P* < 0.01.

Figure 8

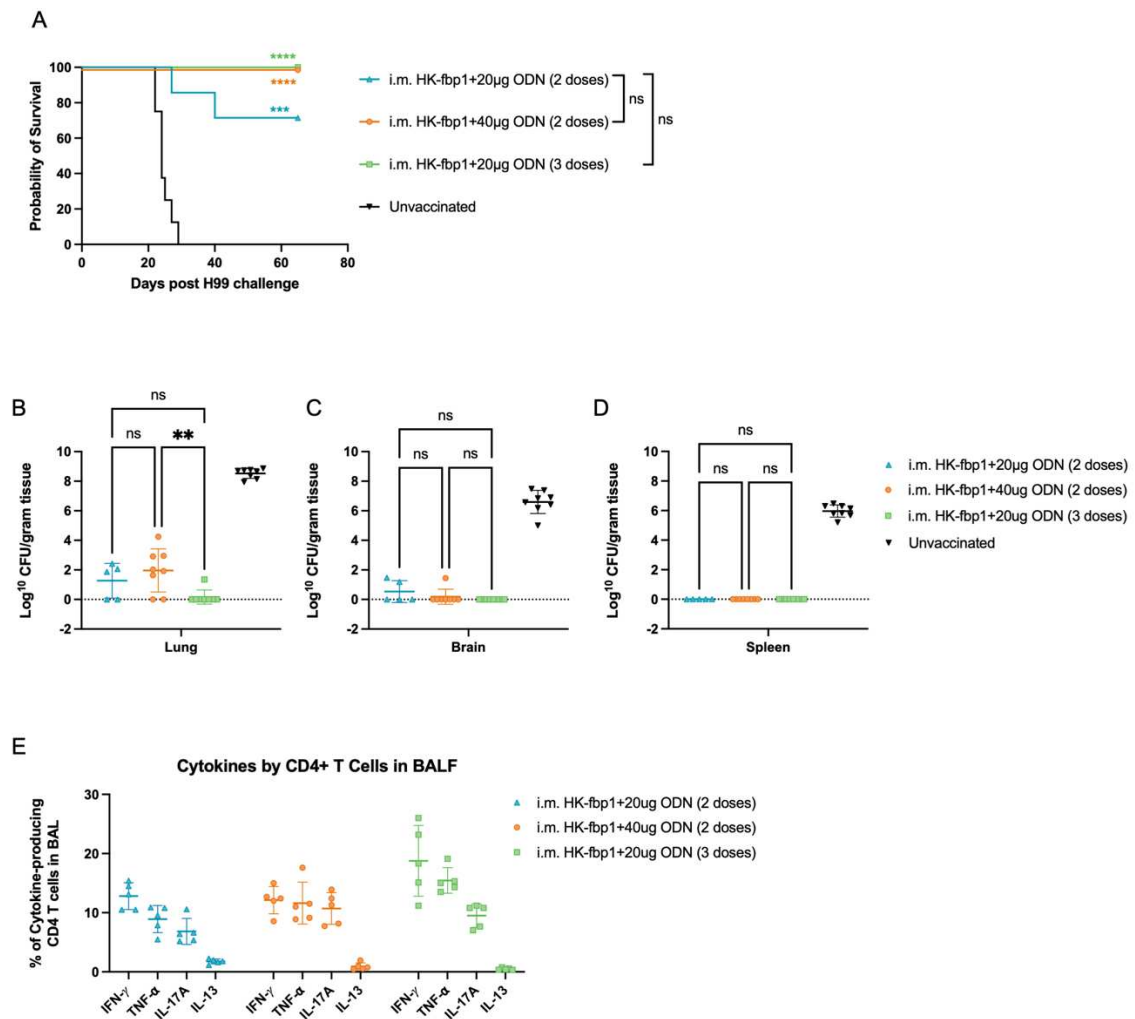


Figure 8. ODN dose and boosting can define a near-sterilization threshold for intramuscular HK-fbp1-mediated protection. **A.** Survival curves of WT BALB/c mice that were vaccinated or left unvaccinated with the intramuscular HK-fbp1/ODN vaccine and subsequently challenged intranasally with H99. *N* = 7-8 per group. **B-D.** Fungal burdens in the lungs (B), brains (C), and spleens (D) of surviving vaccinated mice in vaccinated group or sick mice in unvaccinated group at the humane or study endpoint shown in (A). *N* = 5-8 per group. **E.** Cytokine levels in BALF of vaccinated mice at the study endpoint. Each symbol represents an individual mouse. *N* = 5-8 per group.

1171 Survival data were analyzed using the Kaplan–Meier method, and statistical significance was
1172 determined by the log-rank (Mantel–Cox) test with Holm–Šidák correction for multiple
1173 comparisons (A). ****, $P < 0.0001$; ***, $P < 0.001$. CFU and flow cytometry data are presented
1174 as mean $\pm$ SD (B-E). CFU statistical significance was determined using one-way ANOVA followed
1175 by Tukey’s multiple-comparison test (B-D). Each symbol represents an individual mouse.

1176

Supplementary Files

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- [SupplementaryTable.xlsx](#)
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