Protective antifungal memory CD8+ T cells are maintained in the absence of CD4+ T cell help and cognate antigen in mice

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Individuals who are immunocompromised, including AIDS patients with few CD4+ T cells, are at increased risk for opportunistic fungal infections. The incidence of such infections is increasing worldwide, meaning that the need for antifungal vaccines is increasing. Although CD4+ T cells play a dominant role in resistance to many pathogenic fungal infections, we have previously shown that vaccination can induce protective antifungal CD8+ T cell immunity in the absence of CD4+ T cells. However, it has not been determined whether vaccine-induced antifungal CD8+ T cell memory can be maintained in the absence of CD4+ T cell help. Here, we have shown in a mouse model of vaccination against blastomycosis that antifungal memory CD8+ T cells are maintained in the absence of CD4+ T cells without loss of numbers or function for at least 6 months and that the cells protect against infection. Using a system that enabled us to induce and track antigen-specific, antifungal CD8+ T cells, we found that such cells were maintained for at least 5 months upon transfer into naive mice lacking both CD4+ T cells and persistent fungal antigen. Additionally, fungal vaccination induced a profile of transcription factors functionally linked with persistent memory in CD8+ T cells. Thus, unlike bacteria and viruses, fungi elicit long-term CD8+ T cell memory that is maintained without CD4+ T cell help or persistent antigen. This has implications for the development of novel antifungal vaccine strategies effective in immunocompromised patients.

Introduction

The mounting incidence of opportunistic fungal infections in immunocompromised hosts, including AIDS patients, is of great medical importance (1, 2). Infected patients may receive toxic antifungal drugs over an extended period, often for life (3). To date, there are no commercially available vaccines against fungi. The conundrum is thought to be one of inducing immunity in patients with impaired immunity.

T cells play a vital role against fungal infections (4). CD4+ T cells are the primary immune cells that protect against many pathogenic fungal infections (1). In contrast, CD8+ T cells play a principal role in protection against viruses and tumors. Although the relative contribution of CD8+ T cells in defense against fungal infections has not been studied extensively, we previously reported that vaccine-induced effector CD8+ T cells generated in the absence of CD4+ T cells could mediate resistance against histoplasmosis and blastomycosis (5). Effector cytokines produced by these CD8+ T cells were crucial for fungal defense (6, 7). These findings raised the prospect that immunocompromised hosts lacking CD4+ T cells could be vaccinated against fungal infections by recruiting the CD8+ T cell arm of immunity.

CD4+ T cells are known to provide help for generating CD8+ T cell immunity in vaccination and infection models (8–10). Although the sustenance of memory CD8+ T cells is independent of antigen/MHC-I, CD4+ T cell help was found to be necessary for the maintenance of memory CD8+ T cells directed against bacteria and viruses. In the absence of help, CD8+ T cells failed to survive and mount a protective recall response after challenge (11–15). Of several mechanisms that could contribute to this defect (9, 16, 17), one recently described function is that CD4+ T cells help induce chemokines that are essential for the migration of CXCR3+ CD8+ T cells to the target tissue during genital herpes infection (9, 16, 17). Although induction of antifungal CD8+ T cells against *Blastomyces dermatitidis* and *Histoplasma capsulatum* does not require CD4+ T cells (5), it is not known whether vaccine-induced antifungal effector CD8+ T cells differentiate into long-term memory CD8+ T cells that are maintained and recruited to the appropriate target tissue for protective immunity. Ideally, vaccines should elicit long-lasting immunity to avoid frequent boosting to maintain threshold numbers of effector T cells. Repeated vaccination is not only impractical, but also might pose risks to immunocompromised hosts.

Herein, we studied the durability of vaccine-induced CD8+ T cells that mediate resistance to systemic fungal infection in the absence of CD4+ T cell help. We addressed several questions: (a) Does CD8+ T cell immunity generated in mice lacking CD4+ T cells wane by 60 days as in viral and bacterial immunity? (b) Do long-term memory antifungal CD8+ T cells generated without CD4+ T cell help require persistent vaccine fungal antigen for their maintenance? (c) Do antifungal memory CD8+ T cells that persist still preferentially express CXCR3 chemokine receptor needed for recruitment to target tissue, which is thought to require CD4+ T cell help? (d) Finally, what factor or factors explain the difference in requirement for CD4+ T cell help in maintaining CD8+ T cell memory in response to fungi as compared with other microbes.

We report that durable antifungal memory CD8+ T cells are maintained in the absence of CD4+ T cell help for at least 6 months in the mouse model of vaccination against blastomycosis. These CD8+ T cells fully retained their ability to produce cytokines, recruit to the site of infection, and mediate resistance against lethal

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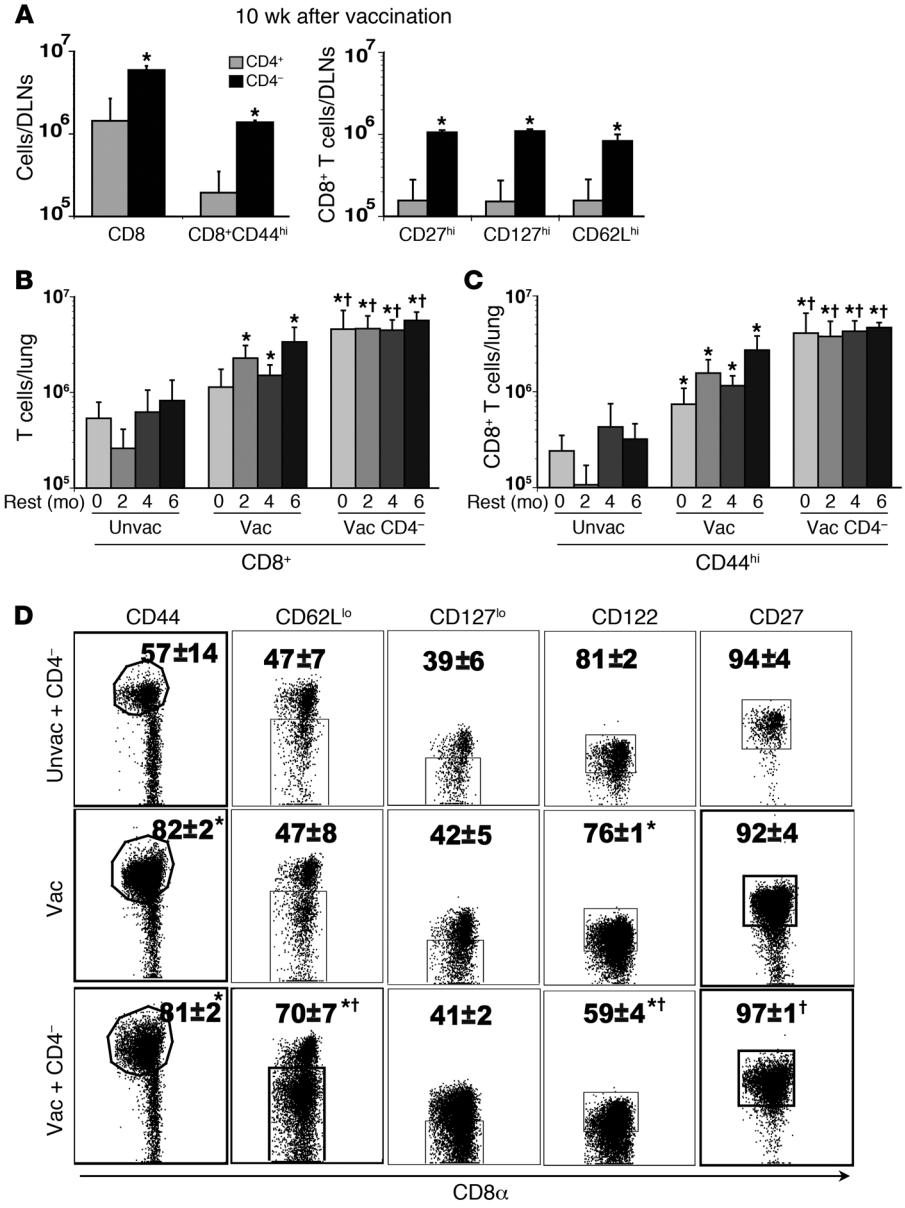

Figure 1
Antifungal memory CD8+ T cells develop in the absence of CD4+ T cell help. Cohorts of C57BL/6 (7 to 8 weeks old) mice were vaccinated s.c. with live attenuated yeast of *B. dermatitis* (10^5 CFU). After 2 weeks, mice were boosted and rested from 2 weeks (0 months) to 6 months. (**A**) Ten weeks after vaccination, DLNs were collected from vaccinated CD4+ T cell–sufficient (CD4+) and CD4+ T cell–depleted (CD4−) mice. DLNs cells were surface stained with antibodies against CD44, CD27, CD127, and CD62L and with anti-CD8 antibody. Cells were analyzed by flow cytometry. *$P < 0.05$. After indicated rest periods, mice were challenged i.t. with a lethal dose of WT *B. dermatitidis*. Lungs were harvested 4 days later for analysis. Total numbers of CD8+ T cells (**B**) and activated (CD44hi) CD8+ T cells (**C**) were quantitated by flow cytometry after surface staining with anti-CD8 and anti-CD44 antibodies. Cells were also surface stained with antibodies against CD62L, CD127, CD122, and CD27. Percentages of CD44hiCD8+ T cells expressing different surface markers were quantitated by flow cytometry and are shown for cells recruited to the lungs after mice were rested for 6 months after vaccination (**D**). *$P \leq 0.05$, vaccinated (vac) versus unvaccinated (unvac) mice; †$P \leq 0.05$, CD4+ T cell–depleted versus CD4+ T cell–sufficient mice (**B**–**D**). Error bars represent mean ± SD.

experimental challenge. Further, by using vaccine yeast engineered to display a model epitope, we tracked antigen-specific antifungal memory CD8+ T cells in the absence of both vaccine antigen and CD4+ T cell help and found no defect in their development, maintenance, and recall response. We found that, while expression of the CXCR3 chemokine receptor on CD8+ T cells is required for the lung recall response of IFN-γ–producing cells, these CD8+ T cells are recalled independently of CD4+ T cell help. Finally, we observed a profile of transcriptional attributes in CD8+ T cells induced by fungi, as opposed to bacteria or virus, which may contribute to the differing requirement for CD4+ T cell help in long term memory.

Results

Antifungal memory CD8+ T cells are induced and maintained in the absence of CD4+ T cell help following vaccination. We have previously shown that CD8+ T cells overcome the requirement of CD4+ T cell help during the induction phase of antifungal vaccine immunity (5). Because CD8+ T cell immunity against viral and bacterial pathogens does not persist after 60 days in the absence of CD4+ T cell help (11–15), we tested to determine whether antifungal memory CD8+ T cells shared this property or instead could survive long term without CD4+ T cell help. After mice were vaccinated with attenuated *B. dermatitidis* in the presence or absence of CD4+ T cells, draining LNs (DLNs) were analyzed 10 weeks later for activated (CD44hi) CD8+ T cells. The number of activated CD8+ T cells was significantly higher in the CD4+ T cell–depleted mice than in the CD4+ T cell–sufficient group (Figure 1A). The number of activated CD8+ T cells expressing memory markers CD27hi, CD127hi (IL-7Rα), and CD62Lhi (marks the central memory phenotype) also was much higher in CD4+ T cell–depleted mice than in the CD4+ T cell–sufficient group. Thus, CD4+ T cell help is not essential for the induction of antifungal memory CD8+ T cells.

We next analyzed the longevity of these antifungal memory CD8+ T cells in the absence of CD4+ T cell help. CD4+ T cell–sufficient and

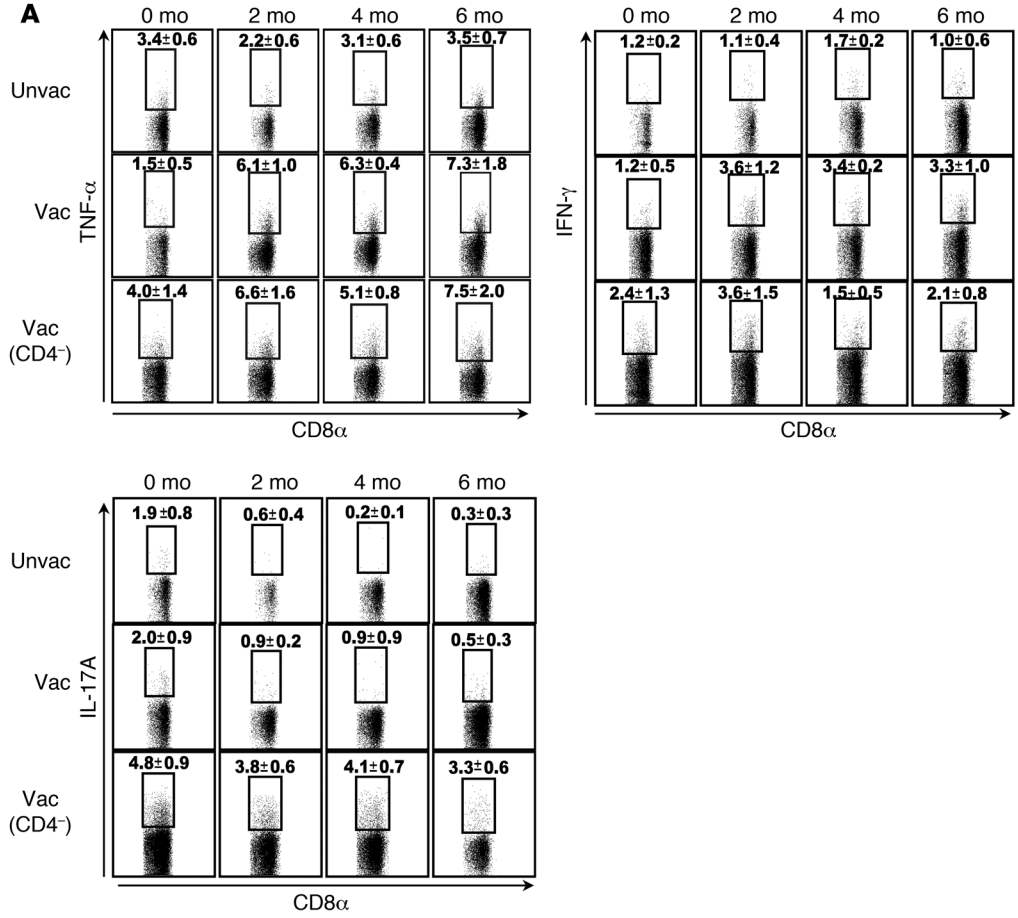

Figure 2

Long-term maintenance of functional antifungal memory CD8+ T cells without CD4+ T cell help. Mice were vaccinated and rested as described in Figure 1. Lungs were harvested 4 days after challenge. Lung CD8+ T cells were restimulated ex vivo with anti-CD3 and anti-CD28 antibodies for 5 hours in the presence of Golgi stop. Cells were first surface stained with anti-CD8 and anti-CD44 antibodies before treating with Fix/Perm buffer (BD Biosciences). Intracellular cytokine staining was done using anti–IFN-γ, anti–IL-17A, and anti–TNF-α. Percentages (**A**) and total numbers (**B**) of cytokine-producing CD8+ T cells were enumerated by flow cytometry and are shown in mice that were rested for different intervals after vaccination. *$P \leq 0.05$, unvaccinated versus vaccinated mice; †$P \leq 0.05$, vaccinated CD4+ T cell–depleted vs. vaccinated CD4+ T cell–sufficient mice. Error bars represent mean ± SD.

–depleted mice were vaccinated and then rested for different time intervals up to 6 months before pulmonary challenge. Four days after challenge, the number of antifungal CD8+ T cells recruited to the lungs was enumerated (Figure 1B). The number of CD8+CD44hi T cells in the lungs of vaccinated, CD4+ T cell–depleted mice was significantly higher than in vaccinated, CD4+ T cell–sufficient mice or in unvaccinated mice at each of the time points, including 6 months (Figure 1C). We also examined the phenotype of recruited CD8+ T cells. The percentage of CD62LloCD127loCD27hi cells (effector phenotype) among CD8+CD44hi cells in vaccinated, CD4+ T cell–depleted mice was similar to or greater than that in unvaccinated mice or vaccinated, CD4+ T cell–sufficient mice even at 6 months after vaccination (Figure 1D). The absolute numbers of effector-phenotype CD8+ T cells recruited to the lung also were significantly higher in vaccinated CD4+ T cell–depleted mice than in CD4+ T cell–sufficient mice or unvaccinated mice ($P < 0.05$; data not shown). Although the percentage of CD122+ CD8+CD44hi T cells recruited to the lungs of the CD4+ T cell–depleted mice was lower than in the CD4+ T cell–sufficient group, the numbers were still elevated in the CD-depleted mice. Thus, antifungal memory

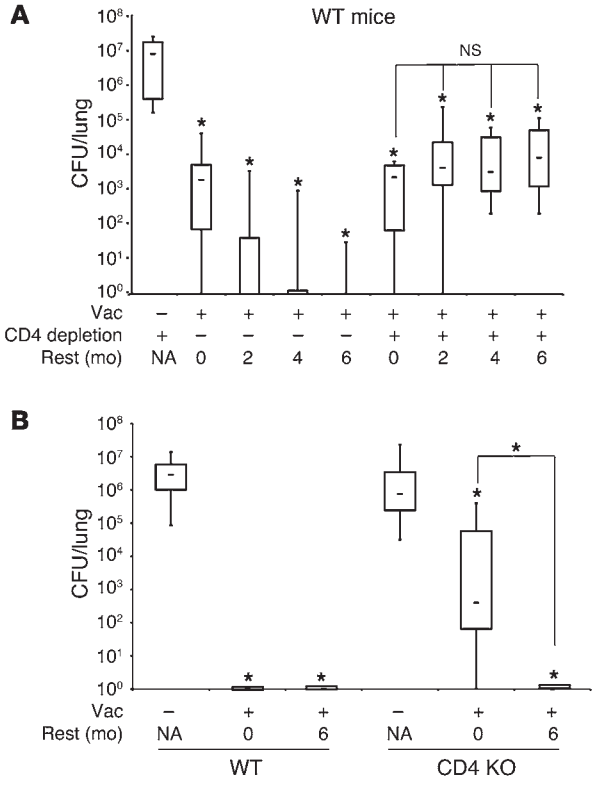

Figure 3
Durable fungal resistance by memory CD8+ T cells in the absence of CD4+ T cell help. Different aged mice were vaccinated and rested for several months as described in Figure 1. Vaccinated mice and unvaccinated controls were challenged i.t. at the same age with a lethal dose of WT *B. dermatitidis*. When unvaccinated controls were moribund (~3 weeks after infection), lungs were harvested to enumerate fungal CFU. (**A**) Lung CFU in nondepleted and CD4+ T cell–depleted mice. Median is shown in box and whisker plots. Data are from 10–13 mice/group. (**B**) Lung CFUs of vaccinated WT and *Cd4*$^{-/-}$ mice and unvaccinated controls. Median is shown in box and whisker plots. Data are from 9–19 mice/group. *$P \leq 0.05$ for vaccinated versus unvaccinated mice.

CD8+ T cells persisted without CD4+ T cell help for at least 6 months and showed an effector phenotype when they were recalled to the lung after lethal pulmonary challenge.

Antifungal memory CD8+ T cells retain the ability to produce cytokines in the absence of CD4+ T cell help. We have shown that effector cytokines TNF-α, IFN-γ, GM-CSF, and IL-17 produced by CD8+ T cells are vital for fungal resistance (ref. 5 and our unpublished observations). Thus, antifungal memory CD8+ T cells that are maintained in the absence of CD4+ T cells must retain the ability to produce cytokines. To explore this issue, mice were vaccinated and challenged as above and lungs were analyzed for cytokine-producing T cells. The percentages of TNF-α–, IFN-γ–, and IL-17A–producing cells among CD8+ T cells did not wane significantly in the CD4+ T cell–depleted group compared with the other groups (Figure 2A). In fact, the numbers of TNF-α–, IFN-γ–, and IL-17A–producing CD8+ T cells were each significantly higher in the CD4+ T cell–depleted group compared with the 2 other groups, and this difference was sharpest for IL-17A–producing CD8+ T cells in mice that were vaccinated in the absence of CD4+ T cells (Figure 2B). Thus, in the absence of CD4+ T cell help, antifungal memory CD8+ T cells retained the ability to make cytokines even 6 months after vaccination and rest.

Protective memory CD8+ T cells endure and protect against lethal pulmonary challenge in the absence of CD4+ T cell help. We next asked whether the antifungal memory CD8+ T cells that are induced and maintained without CD4+ T cell help would mediate resistance to lethal challenge. Because age can independently affect resistance, we vaccinated mice at different ages so that they were the same age at the time of challenge. Vaccinated mice in which CD4+ T cells were depleted mounted robust CD8+ T cell responses and had nearly 3 to 4 logs fewer CFUs in their lungs (672- to 14,542-fold) versus unvaccinated controls (Figure 3A). CD4-knockout mice also evinced a 4- to 6-log reduction versus controls over this 6-month rest period (Figure 3B). Vaccine resistance did not wane significantly over time in any CD4+ T cell–depleted group rested from 0 to 6 months after vaccination (Figure 3, A and B), nor did it wane in the CD4-knockout mice.

These results suggested that even though mice were different ages at the time of vaccine administration, protective memory and numbers and function of memory CD8+ T cells were retained in the absence of CD4+ T cell help (similar to mice vaccinated at the same age in Figure 2B). To confirm that the resistance phenotype reflected a CD8+ T cell recall response with robust cytokine production in this setting (vaccination at different ages), we again analyzed the surface and intracellular cytokine phenotype of CD8+ T cells recalled to the lungs 4 days after lethal pulmonary challenge. Memory CD8+ T cells that were induced and recruited in the absence of CD4+ T cells were similar in number and function to the CD4+ T cell–sufficient group throughout the entire period (Supplemental Figure 2, A–C; supplemental material available online with this article; doi:10.1172/JCI58762DS1). These results demonstrate that antifungal memory CD8+ T cells are induced and also maintained without CD4+ T cell help for an extended time period of up to 6 months and that they also retain their ability to mediate protective immunity. Thus, our results with antifungal CD8+ T cell immunity in the absence of CD4+ T cells appear to be different than those in models of viral and bacterial infection.

Establishment of a vaccine model to interrogate fungal "antigen-specific" CD8+ T cell responses following vaccination. In our studies above, we investigated the *polyclonal* antifungal CD8+ T cell response. This is because no CD8+ T cell epitopes have been identified for pathogenic fungi, including dimorphic fungi such as *B. dermatitidis*. Nevertheless, we sought to track fungal antigen–specific CD8+ T cell responses after vaccination and to monitor the fate of formed memory CD8+ T cells. To do so, we engineered attenuated *B. dermatitidis* to express the model CD8 epitope of Tg OT-I mice — OVA SIINFEKL — on its surface as a translational fusion with truncated BAD-1 and mCherry (Figure 4A). Yeast SIINFEKL display was confirmed by visual and microscopic inspection of mCherry expression (Figure 4B). Following adoptive transfer of OT-I cells into WT recipients depleted of CD4+ T cells, we vaccinated them with SIINFEKL yeast. We found that OT-I Tg responses accurately portrayed the polyclonal, endogenous antifungal CD8+ T cell response. For example, at 14 days after vaccination, the percentages of IFN-γ– and TNF-α–producing OT-I cells in the DLNs were similar to those observed for polyclonal CD8+ T cells (Figure 4C). We also compared activated (CD44^{hi}) OT-I cells and polyclonal CD8+ T cells for their surface phenotypes. The surface expression of effector and memory markers CD62L, KLRG-1, CD127, CD43, and

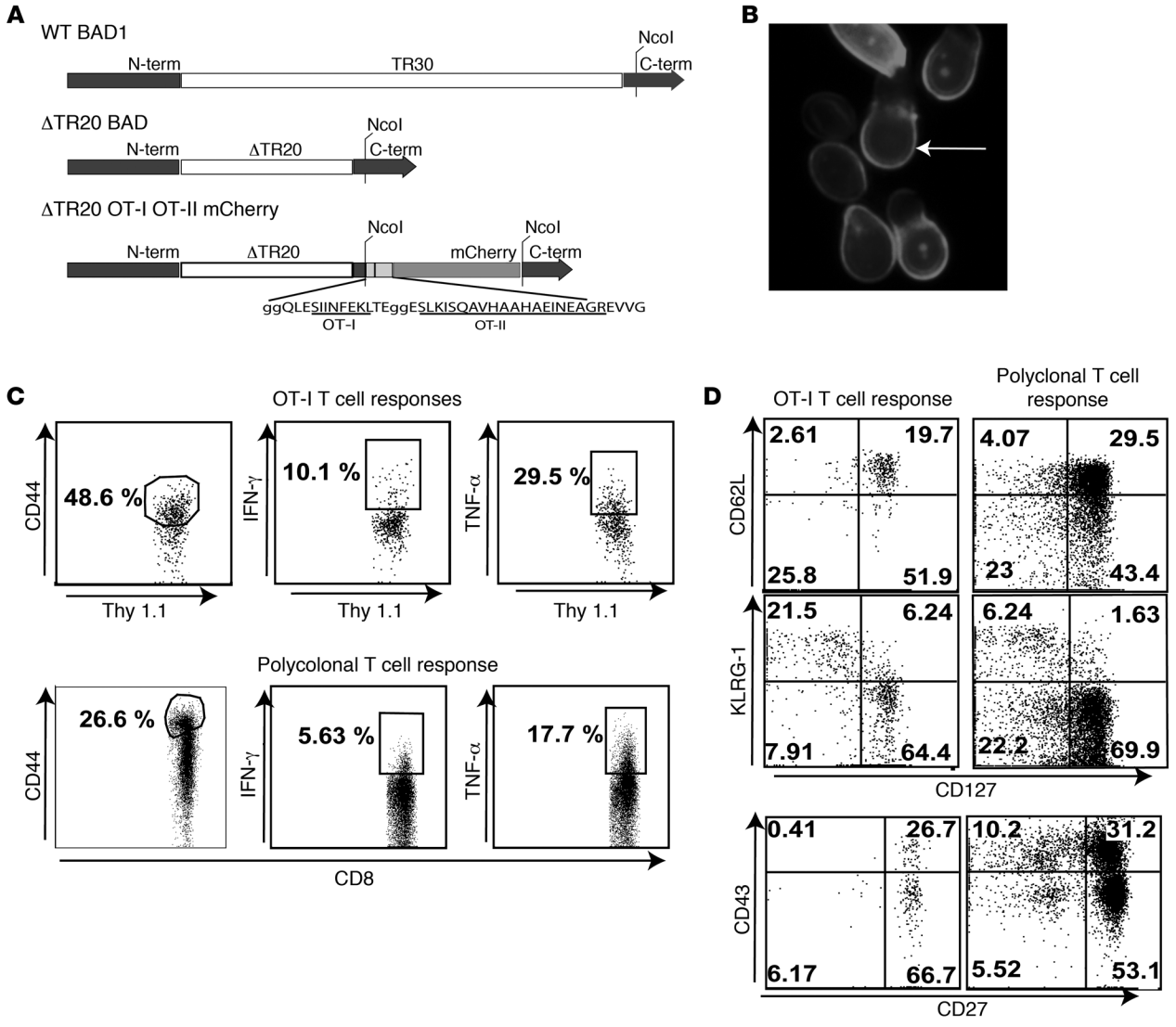

Figure 4

Antigen-specific antifungal CD8$^+$ T cell responses to SIINFEKL-expressing vaccine yeast. (**A**) Schematic diagram illustrating generation of recombinant yeast carrying the OT-I epitope SIINFEKL fused to truncated BAD-1 and fluorescent mCherry. N-term, N terminus; C-term, C terminus. (**B**) Microscopic visualization of recombinant vaccine yeast. Surface localization of model epitope-mCherry fusion (white arrow). Original magnification, ×60. (**C** and **D**) Primary response of OT-I cells after vaccination. Approximately 1×10^6 Thy1.1$^+$ OT-I cells were adoptively transferred to naive Thy1.2 mice. A day later, mice were vaccinated s.c. with SIINFEKL yeast (10^6 CFU). After 14 days, DLN cells were harvested and incubated at 37°C with SIINFEKL peptide or anti-CD3 and anti-CD28 antibodies for 5 hours. Cells were then surface stained with anti-Thy1.1, anti-CD8, and anti-CD44 before staining for intracellular cytokines with anti–IFN-γ and anti–TNF-α antibodies. The percentage of activated (CD44^{hi}) and cytokine-producing OT-I T cells and polyclonal T cells was enumerated by flow cytometry (**C**). DLN cells were also directly surface stained using anti-CD8, anti-Thy1.1, anti-CD44, anti-CD62L, anti-CD127, anti–KLRG-1, anti-CD43, and anti-CD27 antibodies and analyzed by flow cytometry (**D**). Numbers in dot plots indicate the percentage of cells gated on activated (CD44^{hi}) OT-I and polyclonal T cells. Plots are representative of data collected from 4 mice per group.

CD27 was comparable between activated OT-I cells and polyclonal CD8$^+$ T cells (Figure 4D). Thus, we established a system to track antifungal CD8$^+$ T cell responses to monitor the long-term fate of antigen-specific effector and memory CD8$^+$ T cells.

To further characterize and validate this transfer system, we studied the impact of precursor frequency on expansion of OT-I cells. Transfer of 10^5 or 10^6 cells was found be optimal (Supplemental Figure 3A). We also asked whether SIINFEKL-expressing yeast would elicit endogenous OVA-specific T cell responses. Naive mice were vaccinated with recombinant yeast without adoptive transfer of OT-I cells, and expansion of endogenous OVA-specific T cell responses was assessed by ex vivo restimulation of DLN cells with the SIINFEKL peptide. The endogenous response was weak, but cytokine-producing OVA-specific CD8$^+$ T cells were detected (Supplemental Figure 3B).

Long-term maintenance of antigen-specific, antifungal memory CD8$^+$ T cells in the absence of CD4$^+$ T cell help and persistent fungal vaccine antigen. A cardinal feature of true memory CD8$^+$ T cells is their homeo-

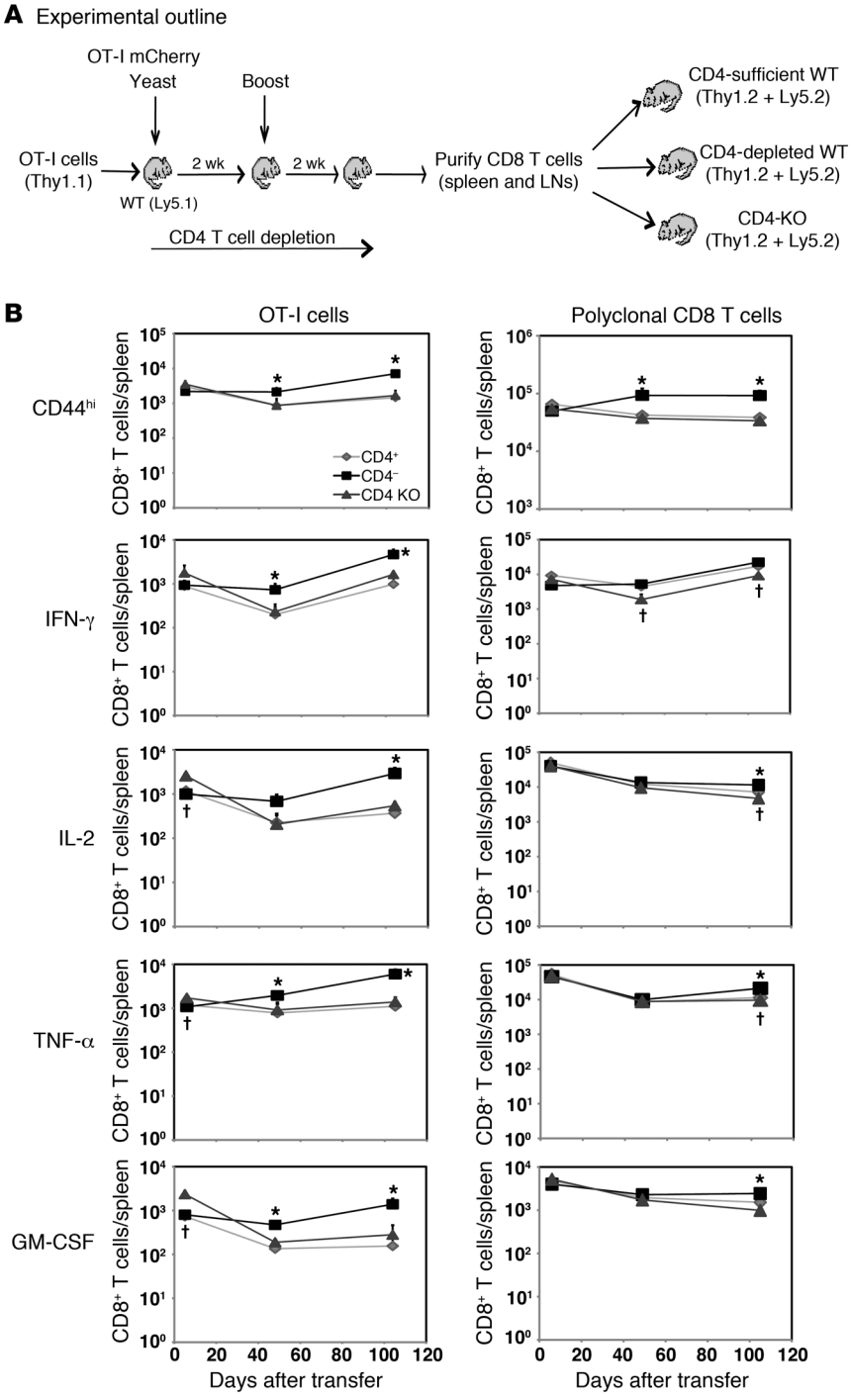

Figure 5

Long-term maintenance of antigen-specific antifungal memory CD8$^+$ T cells in the absence of vaccine antigen and CD4$^+$ T cell help. (**A**) Flow diagram of experimental design. Thy1.1$^+$ OT-I Tg T cells (~1 × 10^6) were adoptively transferred into naive Ly5.1$^+$ congenic mice. A day later, mice were vaccinated s.c. with SIINFEKL expressing *B. dermatitidis* yeast (10^6 CFU), as shown. CD4$^+$ T cells were depleted using GK1.5 antibody during the induction phase. On day 28 after vaccination, spleens and DLNs were collected and CD8$^+$ T cells were purified using autoMACS by negative enrichment. A total of 0.88 × 10^5 OT-I T cells along with endogenous Ly5.1$^+$CD44^{hi} (1.42 × 10^6) T cells were transferred into groups of naive Ly5.2$^+$Thy1.2$^+$ congenic mice: CD4$^+$ T cell–sufficient, CD4$^+$ T cell–depleted, and *Cd4*$^{-/-}$ (CD4 KO). (**B**) Persistence of antifungal memory CD8$^+$ T cells in spleen. At the times shown (1, 44, and 100 days after transfer), spleens were collected to enumerate activated (CD44^{hi}) and cytokine-producing OT-I and polyclonal CD8$^+$ T cells by flow cytometry. Data are shown as mean ± SD from 4 mice/group at each time point. *$P \leq 0.05$, CD4$^-$ versus CD4$^+$; †$P \leq 0.05$, CD4-KO versus CD4$^+$.

stasis, using IL-7 and IL-15, which is independent of cognate antigen (14, 18–21). In our model, live-attenuated yeast can persist at the skin vaccine site for 14 to 16 weeks (Supplemental Figure 4A) and in the DLNs for up to 8 weeks (Supplemental Figure 4B). Moreover, free nonviable antigen might persist longer. Therefore, we sought to exclude the possibility that persistent fungal vaccine antigen is responsible for driving the maintenance of memory CD8$^+$ T cells in the absence of CD4$^+$ T cells.

We tracked antigen-specific CD8$^+$ T cell memory in the absence of persistent fungal antigen. After mice received SIINFEKL yeast, vaccine-induced OT-I cells were adoptively transferred into fresh, naive recipient mice, as shown in Figure 5. OT-I cells (and polyclonal T cells) were purified and equal numbers of cells transferred to congenically disparate naive Thy1.2$^+$Ly5.2$^+$ recipient mice (*Cd4*$^{+/+}$, *Cd4*$^{-/-}$, and CD4$^+$ T cell–depleted WT). At serial times after transfer (days 1, 44, and 100), we enumerated the numbers of transferred/donor (OT-I; Thy1.1 and polyclonal; Ly5.1) CD8$^+$ T cells in the recipients. Figure 5 shows the number of engrafted activated (CD44^{hi}) and cytokine-producing antifungal memory CD8$^+$ T cells in the spleens of recipients. As expected, transferred cells ini-

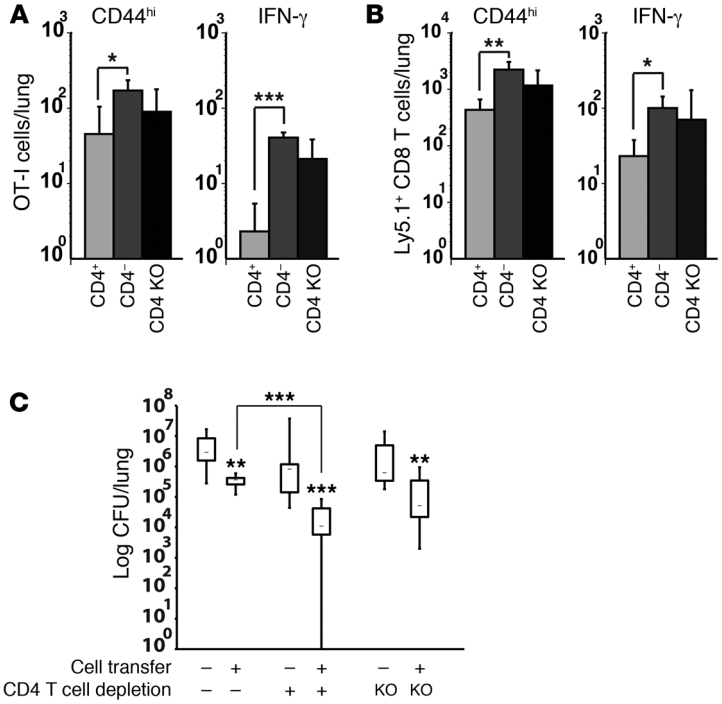

Figure 6
Maintenance of protective antifungal memory CD8+ T cells without vaccine antigen or CD4+ T cell help. Recall to the lung of antifungal memory OT-I T cells (**A**) and polyclonal endogenous CD8+ T cells (**B**). Cell transfer and vaccination were done as described in Figure 5. The groups of recipient mice were challenged i.t. with 1×10^4 SIINFEKL-expressing yeast. On day 4 after challenge, lungs were harvested to enumerate activated and IFN-γ–producing antifungal CD8+ T cells by flow cytometry. Data are from 4 mice/group. Error bars represent mean ± SD. (**C**) Resistance to infection. CD4+ T cells were depleted from vaccinated mice using GK1.5. Two weeks after vaccination was complete, CD8+ T cells from spleens and DLNs were collected and pooled. Equal numbers of CD8+ T cells were transferred into naive CD4+ T cell–sufficient, CD4+ T cell–depleted and *Cd4*$^{-/-}$ mice. Approximately 75 days later, these mice and naive controls that received no cell transfer were lethally challenged with WT yeast. 15 days later, lung CFUs were assessed. Data are shown as box and whisker plots. n = 9–16 mice per group. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. In **C**, the comparisons are between groups that did or did not receive cell transfer, unless indicated.

tially declined in all 3 groups of recipients, reflecting the contraction phase from days 1 to 44 after transfer. Importantly, in CD4+ T cell–depleted recipients, donor cells did not undergo marked contraction and in fact increased over time in the spleen. Donor OT-I and polyclonal CD8+ T cells persisted and produced cytokines in both *Cd4*$^{-/-}$ and CD4+ T cell–depleted groups (Figure 5). The numbers of cytokine-producing OT-I cells in the spleens of CD4+ T cell–depleted and *Cd4*$^{-/-}$ recipients were similar to or greater than those in the CD4+ T cell–sufficient group. The polyclonal pool showed similar trends, except that numbers of IL-2–producing cells fell in all groups. In the DLNs (Supplemental Figure 5), donor cells producing IFN-γ persisted or increased, while those making IL-2 and GM-CSF progressively declined in all groups of recipient mice. The percentages of OT-I and polyclonal CD8+ T cells with effector (CD127^{hi}CD62L^{lo}) or central memory (CD127^{hi}CD62L^{hi}) phenotype were similar in the CD4+ T cell–sufficient and –deficient groups (Supplemental Figure 6). The results showed similar trends at 142 days after transfer (data not shown).

It is possible that the duration or amount of vaccine antigen exposure over 4 weeks during our prime and boost schedule could alter the differentiation of memory CD8+ T cells and reduce their dependence on CD4+ T cell help. We assessed this possibility by examining memory CD8+ T cells that were exposed to vaccine antigen for different durations. At 2 and 4 weeks after a single vaccination, we adoptively transferred purified OT-I cells and endogenous CD8+ T cells from vaccinated mice into naive, congenically disparate recipients and monitored memory persistence. In each setting, independent of the initial length of antigen exposure, antifungal memory CD8+ T cells persisted without CD4+ T cell help (Supplemental Figure 7, A–C). Memory persistence was similar to that shown in Figure 5, for which a prime and boost vaccine schedule was used. Thus, fungal antigen–specific memory CD8+ T cells were maintained for an extended period approaching 5 months in the absence of either CD4+ T cell help or persistent fungal antigen, without the loss of their function, phenotypic attributes, or quantum.

Protective antifungal memory CD8+ T cells maintained in the absence of fungal antigen and CD4+ T cell help are efficiently recalled after lethal rechallenge. Studies have shown that "helpless" CD8+ T cells do not recruit or protect well when compared with those that receive help (12, 13, 16, 22). These studies prompted us to assess the recall response of helpless fungal antigen–specific memory CD8+ T cells upon challenge. Using the vaccination and adoptive transfer scheme outlined above, we found that memory (CD44^{hi}) OT-I cells were recalled to the lung after challenge similarly in CD4+ T cell–depleted and *Cd4*$^{-/-}$ mice compared with CD4+ T cell–sufficient mice. The numbers of IFN-γ–producing OT-I cells in this pool were significantly higher in the CD4+ T cell–deficient groups compared with the CD4+ T cell–sufficient group (Figure 6A). Similar results were found with polyclonal, endogenous antifungal CD8+ T cells (Figure 6B). To test the protective role of these memory precursors, naive recipients that had received vaccine-induced CD8+ T cells, were experimentally challenged 75 days after the cell transfer. Compared with controls, recipients of vaccine-induced helpless CD8+ T cells had approximately 1 to 2 logs fewer CFUs (Figure 6C). Importantly, the protective efficacy of antifungal memory CD8+ T cells maintained in the absence of CD4+ T cell help (*Cd4*$^{-/-}$ and CD4+ T cell–depleted) (Figure 6C) was as good as that observed for CD8+ T cells maintained with CD4+ T cell help (19- to 99-fold reduction vs. 9-fold, respectively). Thus, fungal antigen–specific memory CD8+ T cells that were generated and maintained in the absence of CD4+ T cell help, and without persistent fungal antigen, were able to be recalled to the lung and mediate resistance following the lethal challenge.

Recruitment of antifungal IFN-γ–producing CD8+ T cells into lungs after challenge requires the chemokine receptor CXCR3, but not CD4+ T cell, help. Expression of chemokine receptors on T cells is crucial for their migration into target tissues (23–25). Recent work sug-

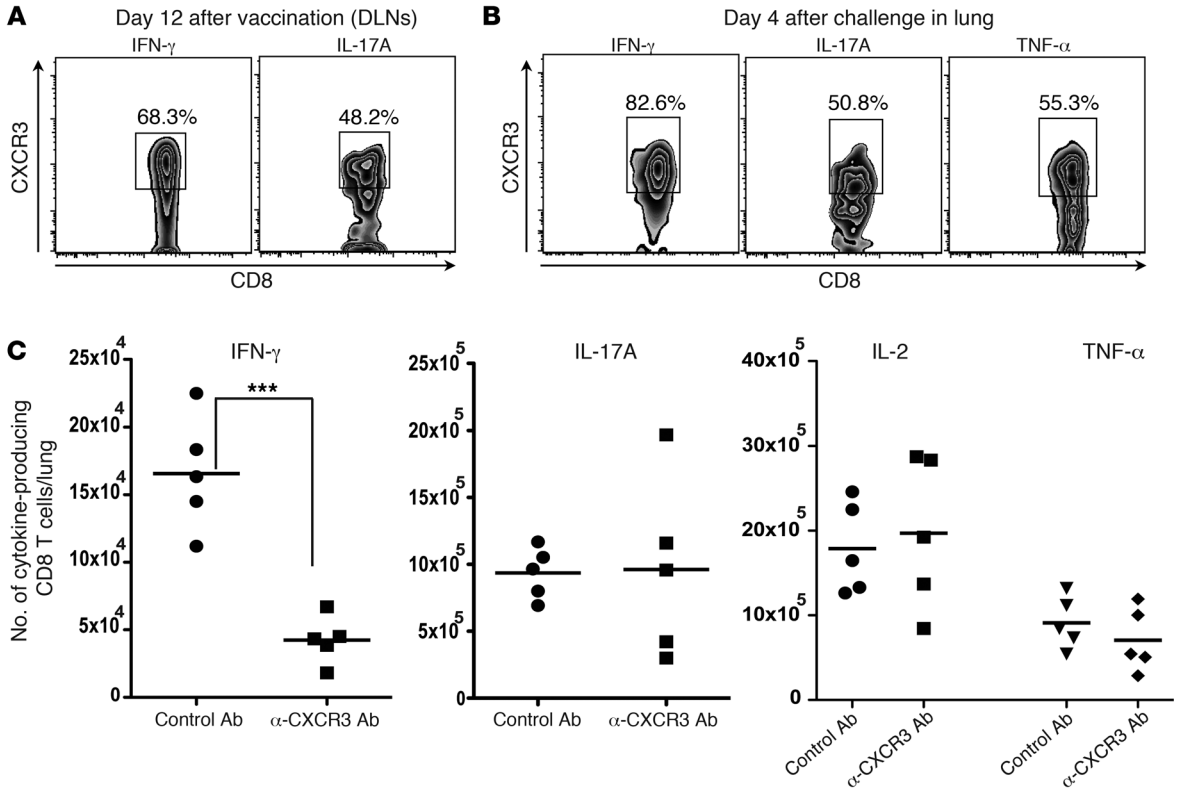

Figure 7

Antifungal IFN-γ$^+$ CD8$^+$ T cells preferentially express CXCR3, which is required for recruitment into lungs, but is independent of CD4$^+$ T cell help. (**A**) Expression of CXCR3. Naive 7- to 8-week-old mice with CD4$^+$ T cell depletion were vaccinated. Cells from DLNs (**A**) and lungs (**B**) were collected and restimulated ex vivo using anti-CD3 and anti-CD28 for 5 hours. Cells were surface stained with anti-CD8, anti-CD44, and anti-CXCR3 antibodies and permeabilized to stain intracellular IFN-γ, TNF-α, and IL-17A. The percentage of cytokine-producing CD8$^+$ T cells that expressed CXCR3 was quantitated by flow cytometry. For CXCR3-blocking experiments (**C**), 100 μg of LEAF purified hamster anti-mouse CXCR3 was given i.v. at days 0, +1, and +2 after challenge. On day 4 after challenge, lungs were harvested to enumerate the number of cytokine-producing CD8$^+$ T cells by flow cytometry. Each symbol represents 1 mouse. n = 5 mice/group. Data are representative of 2 independent experiments. ***$P < 0.001$.

gests that migration of IFN-γ$^+$CD8$^+$ T cells into infected vaginal tissue requires CXCR3 expression, which is cued by the resident cells through chemokines (16). However, secretion of chemokines by infected tissue cells requires CD4$^+$ T cell help, without which migration of antiviral CD8$^+$ T cells is impaired (16). Here, we assessed (a) whether IFN-γ$^+$ antifungal CD8$^+$ T cells express CXCR3, and if so, (b) whether CXCR3-dependent recall of IFN$^+$ antifungal memory CD8$^+$ T cells to the lung required CD4$^+$ T cells as in viral infection. Following vaccination of CD4$^+$ T cell–depleted mice, CXCR3 was preferentially expressed on IFN-γ$^+$CD8$^+$ T cells as compared with IL-17A$^+$CD8$^+$ T cells in the DLNs, whereas the latter population preferentially expressed CCR6 (Figure 7A and data not shown). After challenge, IFN-γ$^+$CD8$^+$ T cells expressing CXCR3 were preferentially recruited into lungs (Figure 7B). Anti-CXCR3 blocking mAb sharply reduced recruitment of IFN-γ$^+$CD8$^+$ T cells into the lungs of challenged mice; it blunted their recall by nearly 4-fold vs. isotype control antibody (P = 0.0003), but did not affect recruitment of other cytokine-producing CD8$^+$ T cells elaborating IL-17A, TNF-α, or IL-2 (Figure 7C). Thus, CXCR3 expression is critical for the recruitment of IFN-γ$^+$CD8$^+$ T cells into the lungs of challenged mice. However, in contrast to models of viral infection, CD4$^+$ T cell help is not required for recall responses of antifungal CXCR3$^+$IFN-γ$^+$ memory CD8$^+$ T cells.

Antifungal effector CD8$^+$ T cells are programmed to commit to a memory T cell lineage independently of CD4$^+$ T cell help, in contrast with antiviral and antibacterial CD8$^+$ T cells. Several transcription factors are linked with the ability to acquire CD8$^+$ T cell memory. Eomes and T cell factor 1 (TCF-1) positively regulate memory cell development (26, 27), while T-bet impedes CD8$^+$ T cell memory in the absence of CD4$^+$ T cell help (28). We analyzed effector CD8$^+$ T cells for phenotypic and transcriptional attributes that typify memory cells. To gain more insight into the differences among fungi, bacteria, and viruses in memory requirements for CD4$^+$ T cell help, we analyzed these attributes in OT-I cells after vaccination/infection with recombinant Vaccinia virus *Listeria monocytogenes* or yeast, each expressing SIINFEKL. Vaccination with yeast sharply induced CD127^{hi}KLRG-1^{lo} and TCF-1^{hi}KLRG^{lo} OT-I cells compared with receipt of Vaccinia or *Listeria* (Figure 8A; $P \leq 0.001$). We also examined the relative expression of Eomes versus T-bet in OT-I cells. The ratio of Eomes to T-bet was substantially higher in OT-I cells after vaccination with yeast as compared with Vaccinia or *Listeria* (Figure 8B). Similar results were found with polyclonal CD8$^+$ T cells (data not shown). These data suggest that fungal vaccination selectively induces a profile of transcription factors and superior effector cells that are fit for survival as memory CD8$^+$ T cells in the absence of CD4$^+$ T cell help.

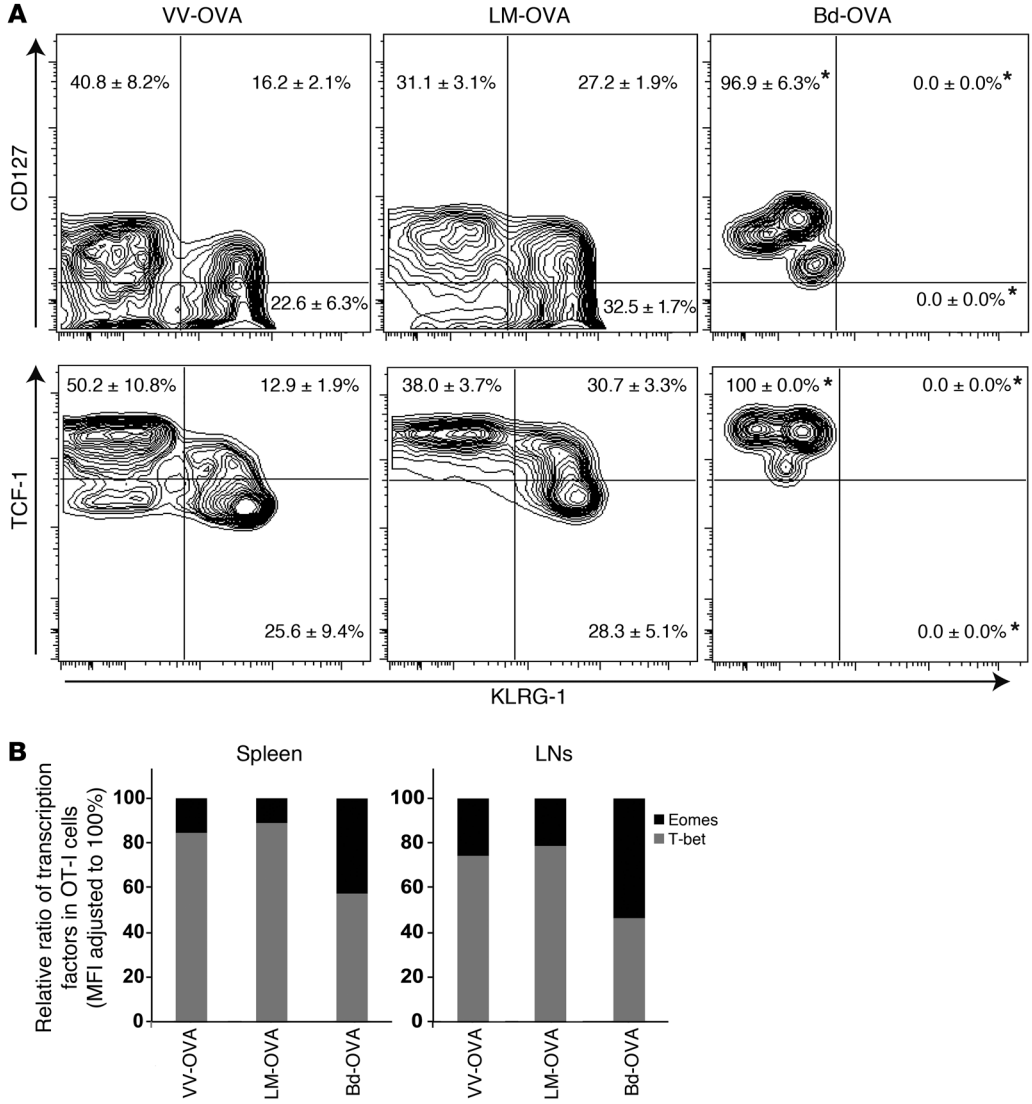

Figure 8

Attributes of memory cells elicited by fungi, bacteria, and virus. Thy1.1$^+$ OT-I T cells (~10^5) were transferred into naive 7- to 8-week-old Thy1.2 mice who were infected or vaccinated with SIINFEKL expressing *L. monocytogenes* (~3.5×10^5 CFU i.v.), Vaccinia (~5×10^5 PFU i.p.), and yeast (~1×10^6 CFU s.c.). At the peak of response (day 7 for *Listeria*- and Vaccinia-infected mice and day 14 for yeast-vaccinated mice), spleens, and LNs were harvested for analysis. Lymphocytes were surface stained with anti-CD8, anti–KLRG-1, anti–CD127, and anti-CD44 antibodies, fixed and permeabilized, and then stained with anti–TCF-1, anti–T-bet, and anti-Eomes antibodies. Cells were analyzed by flow cytometry. (**A**) Contour plots depict expression of CD127, TCF-1, and KLRG-1 in OT-I cells from spleen. Percentages of activated (CD44^{hi}) OT-I cells for each quadrant are indicated. (**B**) Relative levels of T-bet and Eomes expression in OT-I cells from spleen and LNs. Expression, based on MFI, was adjusted to total 100% to determine the ratio between T-bet and Eomes. Data are shown as mean ± SD of n = 4–5 mice per group. *$P \leq 0.001$ for the yeast group (Bd-OVA) vs. Vaccinia group (VV-OVA) and *Listeria* group (LM-OVA).

Discussion

Immunocompromised hosts, such as AIDS patients with few or no CD4$^+$ T cells, are prone to opportunistic infections. The development of suitable vaccines against opportunistic microbes including fungi must therefore exploit alternative arms of immunity, for example, CD8$^+$ T cells. Although CD4$^+$ T cells play a dominant role in resistance to fungal infections (5, 6, 29–32), we have shown that antifungal effector CD8$^+$ T cells that mediate resistance can be induced in the absence of CD4$^+$ T cells (5, 6, 29–32). The current immunological dogma holds that the maintenance of memory CD8$^+$ T cells requires CD4$^+$ T cell help (9). According to this view, it would be futile to vaccinate immunocompromised patients when seeking the generation of competent memory CD8$^+$ T cells without CD4$^+$ T cell help. This premise is based largely on data from models of viral and bacterial infection. We investigated the differentiation, maintenance, and recall response of memory CD8$^+$ T cells in a model of lethal pulmonary fungal infection following vaccination in the absence of CD4$^+$ T cell help.

Herein, we show that helpless antifungal effector CD8$^+$ T cells efficiently differentiate into long-term memory CD8$^+$ T cells and mediate resistance after lethal challenge. First, we show that in CD4$^+$ T cell–depleted mice, CD8$^+$ T cells with a memory phenotype

were induced following vaccination and they maintained their numbers, phenotype and, cytokine-producing ability for at least 6 months following vaccination. Second, we also show that protective memory CD8$^+$ T cells were preserved over this time interval in both CD4 T cell–depleted and *Cd4*$^{-/-}$ mice. In experimental *Listeria* and LCMV infections, although the primary CD8$^+$ T cell response was optimal, secondary responses were defective in the absence of CD4$^+$ T cells (12, 13). CD4$^+$ T cell help was indispensable for maintenance of memory CD8$^+$ T cells and in its absence, secondary expansion and effector function of CD8$^+$ T cells were feeble and lethargic (11). Defective secondary expansion of helpless memory CD8$^+$ T cells might be due to activation-induced cell death via TNF-related apoptosis-inducing ligand (TRAIL) (17), although it was not the sole mechanism (33). Our earlier findings suggested that primary antifungal CD8$^+$ T cell responses and generation of protective effector CD8$^+$ T cells were independent of CD4$^+$ T cell help (5), at least in the short term. However, we did not study animals beyond 60 days after vaccination, as was done in models of viral and bacterial infection. Here, using varied models of CD4$^+$ T cell deficiency, we now show that antifungal memory CD8$^+$ T cells are both induced and maintained in the absence of CD4$^+$ T cell help for an extended period without apparent loss of their quality or quantity and that their recall to the lung and ability to confer resistance is unabated for at least 6 months.

We observed that CD4$^+$ T cell–depleted and *Cd4*$^{-/-}$ mice exhibited different levels of vaccine resistance even though both groups showed long-term persistence of CD8 T cell memory and resistance. This difference between the groups could be due to the persistence of MHC class II–restricted T cells in *Cd4*$^{-/-}$ mice (34), which may have enhanced resistance. Nevertheless, we observed that memory CD8$^+$ T cells persisted even in the absence of MHC class II in adoptive transfer studies in which OT-I cells and polyclonal cells were monitored in *MHC-II*$^{-/-}$ recipients.

Homeostasis of naive CD8$^+$ T cells depends chiefly on MHC-I and IL-7. Once activated through the TCR and appropriate costimuli, naive CD8$^+$ T cells rapidly undergo clonal expansion and convert into effectors. After clonal expansion, about 5%–10% of the activated CD8$^+$ T cells differentiate to become memory CD8$^+$ T cells. Upon antigen reencounter, memory CD8$^+$ T cells have the ability to respond swiftly. They proliferate rapidly and robustly, differentiate into cytotoxic T cells, and produce antimicrobial cytokines. These features impart CD8$^+$ T cell immunity against microbes (35). Unlike naive T cells, homeostasis of memory CD8$^+$ T cells occurs independently of antigen and MHC-I and utilizes cytokines IL- 7 and IL-15 (14, 15, 18–21). It has been suggested that persisting antigen might help sustenance of effector and memory CD8$^+$ T cells (36, 37). Thus, it was possible that homeostatic maintenance of helpless antifungal memory CD8$^+$ T cells benefited from the presence of persisting vaccine antigen in our model. However, we rigorously excluded antigen-dependent maintenance of memory CD8$^+$ T cells here by engineering vaccine yeast to express SIINFEKL. After recombinant vaccine-induced priming of OT-I cells and adoptive transfer of antifungal OT-I memory CD8$^+$ T cell precursors into fresh naive mice, we showed that these fungal antigen–specific memory CD8$^+$ T cells (and endogenous, polyclonal memory CD8$^+$ T cells) removed from residual vaccine antigen persisted in number and function and were recalled to the lung where they mediated protective immunity.

Chemokines guide the migration of T cells to a target location (24). Upregulation of CCR5 on naive CD8$^+$ T cells permits their migration to DC-CD4$^+$ T cell interaction sites after immunization, enhancing their prompt activation (23). Although resting memory CD8$^+$ T cells express CXCR3 and migrate in response to CXCR3 ligands, upregulation of CCR5 after lung infection was critical for their rapid influx and production of antiviral molecules in lungs (38). In contrast, CXCR3 rather than CCR5 regulated the migration of CD4$^+$ T cells after parainfluenza infection (39). Migration of IFN-γ–producing CXCR3$^+$ CD8$^+$ T cells required CD4$^+$ T cell help for their migration to infected vaginal tissues (16). These studies indicate that chemokine receptors play a role in guiding the migration of CD8$^+$ T cells into infected tissues. Here, we show that after vaccination and challenge, IFN-γ$^+$ antifungal memory CD8$^+$ T cells recruited to the lungs preferentially expressed CXCR3. Blocking CXCR3 during the recall response prevented their influx into lungs, indicating that CXCR3 expression is essential for recruiting IFN-γ$^+$ antifungal memory CD8$^+$ T cells. However, the recruitment of antifungal CXCR3$^+$IFN-γ$^+$CD8$^+$ T cells was unaffected in CD4$^+$ T cell–depleted mice, suggesting that the fungal infection–induced inflammatory environment was sufficient to recall these cells to the lung. These findings underscore differences in the long-term memory requirements between antifungal and antiviral memory CD8$^+$ T cells. McAllister et al. (40) also have reported that CXCR3, and the monokine IP-10, enhance recruitment of Tc1 CD8 cells to the lung and control of primary *Pneumocystis* pneumonia in CD4$^+$ T cell–depleted mice. Tc1 CD8$^+$ T cells recalled to the lung also are a source of GM-CSF (41), which enhances fungal clearance (5). Although we did not study the role of CCR6$^+$ Tc17 cells, they too promote vaccine resistance (our unpublished observations).

How do antifungal memory CD8$^+$ T cells persist without antigen or CD4$^+$ T cell help? Fungal cell wall components that trigger immunity are complex (42). Several pattern recognition receptors (PRRs) of immune cells recognize fungal cell wall moieties that could lead to activation of immune cells and host defense mechanisms (42–44). We hypothesize that vaccine yeast components (pathogen-associated molecular patterns [PAMPs]) elicit a strong inflammatory milieu during the induction of antifungal CD8$^+$ T cell immunity, such that effector CD8$^+$ T cells efficiently differentiate into competent memory cells (45). In support of this idea, we have shown that the antifungal CD8$^+$ T cell response was intact even in the absence of CD40 and CD40L (9, 10, 46), which are involved in CD4$^+$ T cell help (9, 10, 46). Second, IL-2 produced by primed CD4$^+$ T cells seems to be important for generating good memory CD8$^+$ T cells (47). Following fungal vaccination, we found that approximately 25% of fungal antigen–specific CD8$^+$ T cells produce IL-2 (data not shown). Hence, it is possible that antifungal CD8$^+$ T cells receive IL-2 signals in both autocrine and paracrine modes. Third, the cytolytic function of CD8$^+$ T cells is essential for clearing viral and intracellular bacterial infections, whereas effector cytokines are required for fungal resistance (6, 48, 49). It is possible that the requirement for CD4$^+$ T cell help might differ for CD8$^+$ T cells with these distinct functions. Fourth, rapid expansion and expression of effector function is the signature feature of CD8$^+$ T cells during secondary response (35). During an anamnestic antifungal immune response, CD8$^+$ T cell recruitment and effector cytokine production promoting the rapid infiltration of inflammatory cells, including neutrophils and macrophages, might be sufficient for resistance (6, 7). Fifth, in contrast with viral genital infection (16), recruitment of antifungal CXCR3$^+$IFN-γ$^+$ memory CD8$^+$ T cells did not require CD4$^+$ T cell help during the recall

response, although it did require CXCR3 expression. These findings together support the hypothesis that complex fungal cell wall components might promote an inflammatory milieu for inducing and maintaining memory CD8$^+$ T cell recruitment and function independently of CD4$^+$ T cell help.

We explored features that distinguish helpless CD8$^+$ T cell responses to fungi from those directed against bacteria or virus. We found that yeast induced transcriptional features in CD8$^+$ T cells, fostering their competence to survive as long-lasting memory cells. The fate of CD8$^+$ T cell lineages is dictated early in the response (45, 50). The transcription factor T-bet regulates the ratio of short-lived effectors (KLRG-1^{hi}CD127^{lo}) and memory precursor effector cells (KLRG-1^{lo}CD127^{hi}) (51). The higher the level of T-bet, the lower the chance that effectors survive into a memory pool (28). Conversely, memory CD8$^+$ T cells express higher levels of Eomes and lower levels of T-bet. Eomes allows effector CD8$^+$ T cells to compete for a memory niche (26). The transcription factor TCF-1 in turn regulates Eomes and is essential for effector cells to survive as memory T cells (27). We found that yeast vaccine–induced effector CD8$^+$ T cells preferentially expressed promemory Eomes, CD127, and TCF-1 and reduced levels of antimemory KLRG-1 and T-bet, as compared with *Listeria* and Vaccinia virus. Thus, programming of effector CD8$^+$ T cells after fungal vaccination differs from that induced by bacteria or virus.

Interestingly, our study showed that mice that were depleted of CD4$^+$ T cells harbored more CD8$^+$ T cells after adoptive transfer than did the *Cd4*$^{-/-}$ and *Cd4*$^{+/+}$ groups. This may be due to increased CD8$^+$ T cell proliferation (52) in CD4$^+$ T cell–depleted mice compared with the other groups (53). It is not clear why depletion of CD4$^+$ T cells would augment proliferation of memory CD8$^+$ T cells. It is possible that transient lymphopenia due to loss of CD4$^+$ T cells might favor the increased turnover of naive and memory CD8$^+$ T cells (18). Alternatively, elimination of Tregs may have contributed. Further studies are needed to address specific alterations of memory CD8$^+$ T cell homeostasis induced by CD4$^+$ T cell depletion. Importantly, in our adoptive transfer studies, resistance mediated by helpless CD8$^+$ T cells against lethal challenge was equal or better in both CD4$^+$ T cell–depleted and *Cd4*$^{-/-}$ mice when compared with CD4$^+$ T cell–sufficient mice. Thus, protective antifungal memory CD8$^+$ T cells persisted without CD4$^+$ T cell help.

In summary, our study demonstrates that antifungal memory CD8$^+$ T cells could be generated, maintained, and recalled for resistance over an extended period of at least 6 months in the absence of CD4$^+$ T cell help. Our findings have practical implications for the design of suitable fungal vaccines targeting CD8$^+$ T cells in immunocompromised patients.

Methods

Mice. Seven- to eight-week-old C57BL/6 mice were purchased from the National Cancer Institute. OT-I Tg mice carrying Thy1.1 allele were generated from backcrossing C57BL/6-Tg (TcrαTcrβ) 1100Mjb/JB mice (stock #003831) with B6.PL-Thy1^a/Cy mice (stock #000406) that were obtained from Jackson Laboratories. B6.129S2-*Cd4*^{tm1Mak}/J (stock #002663) lacking the CD4 allele and B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ carrying the congenic allele Ly5.1 were purchased from Jackson Laboratories. Seven- to eight-week-old mice were used for all experiments except age-synchronized resistance experiments. All mice were maintained under specific pathogen–free conditions at the University of Wisconsin–Madison.

Fungi. WT virulent *B. dermatitidis* strain 26199 was obtained from ATCC. An isogenic attenuated mutant strain lacking BAD-1 (strain #55) was used for vaccination as described elsewhere (5). Isolates were maintained as yeast on Middlebrook 7H10 agar with oleic acid–albumin complex (Sigma-Aldrich) at 39°C.

Engineering yeast expressing the model antigenic epitope for OT-I T cells. Yeast cells expressing the OVA T cell epitopes for OT-I (and OT-II) cells were generated by *Agrobacterium*-mediated transformation using a binary plasmid expressing a 4-way fusion of the 2 epitopes, the red fluorescent protein mCherry (54) and a truncated version of the *BAD1* gene (TRΔ20; ref. 55 and Figure 4) as described in ref. 56. Splicing by overlap extension (SOE) PCR (57) was used to generate the coding sequence for both OT-I & OT-II epitopes, which were fused with the mCherry coding sequence and inserted into the truncated *BAD1* gene. The sequence encoding the 2 epitopes included 2 or 3 flanking amino acids from the region next to the epitopes in the *OVA* gene, and glycine residue was also added as spacers, so that the amino acid sequence encoded by the insertion was GGQLESIINFEKLTEGGESLKISQAVHAAHAEINEAGREVVG (underlined sequences are OT-I and OT-II epitopes). Primers were obtained from Integrated DNA Technologies. PCR for cloning was done using Elongase (Invitrogen). The *E. coli* strain DH10β was used for the initial cloning, and the sequence of the added epitope-coding region was confirmed before electroporating the plasmid into *Agrobacterium tumefaciens* strain LBA1100, harboring the Ti plasmid pAL1100 (58, 59). Confirmed *A. tumefaciens* strains were used to transform yeast-phase cells of *B. dermatitidis* strain ATCC26199 and strain 55, the BAD1-knockout derivative of 26199 (55, 60). Transformants were screened for production of the BAD1 fusion protein and/or red fluorescence, and colonies of high-expressing strains were noted to have visibly pink color when grown on Middlebrook 7H10 agar.

Vaccination, infection, and CD4$^+$ T cell depletion. Mice were vaccinated with specific strains of *B. dermatitidis* as described (5). Briefly, 10^5 yeast of the attenuated strain #55 was inoculated s.c. at each of 2 sites, dorsally and at the base of the tail. For challenge experiments, mice were infected intratracheally (i.t.) with 2×10^3 isogenic WT strain 26199 as described (30). For OT-I experiments, 10^6 heat-inactivated yeast cells that express OVA epitopes were used for vaccination as above. For comparison, we studied *L. monocytogenes* that expresses OVA (~3.5×10^5 CFU i.v.) and Vaccinia virus that expresses OVA (~5×10^5 PFU i.p.), which were provided by Jack Bennink (NIH, Bethesda, Maryland, USA) and M. Suresh (University of Wisconsin–Madison).

To deplete CD4$^+$ T cells, 100 μg GK1.5 mAb (Biovest International Inc.) was given once weekly by the i.v. route. We verified that CD4$^+$ T cells were efficiently depleted in both the spleen and LNs using this regimen (Supplemental Figure 1).

Adoptive transfer. Approximately 10^6 OT-I Tg cells were transferred i.v. into naive Ly5.1 congenic mice a day before vaccination. For memory cell transfer experiments, OT-I cells and endogenous Ly5.1$^+$ were collected from spleens and DLNs and purified by CD8$^+$ T cell–negative selection using Miltenyi beads and autoMACS. Enriched cells were pooled and transferred into naive mice.

Flow cytometry. Single-cell suspensions of spleen and LNs were stained with anti-CD44, anti-CD8α, anti-Thy1.1, anti-CD62L, anti-CD127, anti-CD122, anti-CD27, anti–KLRG-1, anti-CD43 (clone 1B11), anti-CXCR3, anti-Eomes, anti–T-bet, anti–TCF-1, and anti-Ly5.1 antibodies using FACS buffer (1.5% BSA in PBS) for 30 minutes at 4°C. Cells were washed and fixed in 2% paraformaldehyde. Stained cells were analyzed by flow cytometry using an LSR II (BD Biosciences). All antibodies were obtained from BD Biosciences except for anti-CD43 and anti-CXCR3 (BioLegend); anti-CD127, anti–KLRG-1, and anti-Eomes (eBiosciences); and anti–TCF-1 (Cell Signaling Technology).

Intracellular cytokine staining. Single-cell suspensions from spleen and LNs were restimulated ex vivo with either anti-CD3 (clone 145-2C11; 0.1 μg/ml) and anti-CD28 (clone 37.51; 1 μg/ml) or with SIINFEKL peptide (OT-I cells) (0.1 μg/ml) in the presence of Golgi-stop (BD Biosciences) for 5 hours

at 37°C. Cells were first stained with anti-CD44 and anti-CD8α antibodies and then for intracellular cytokine with anti–IFN-γ, anti–TNF-α, anti–IL-2, anti–GM-CSF, or anti–IL-17A antibodies using the BD Cytoperm/Cytofix Kit (BD Biosciences). In some experiments, anti-CXCR3 antibody (BioLegend) was also used during surface staining. Stained and fixed cells were analyzed by flow cytometry.

CXCR3 blocking experiments. LEAF purified Armenian hamster anti-CXCR3 (clone CXCR3-173) was obtained from BioLegend, and 100 μg/mouse was injected i.v. daily on day 0, day 1, and day 2 after challenge.

Study approval. All animal studies were approved by the University of Wisconsin–Madison Institutional Animal Care Committee.

Statistics. Statistical significance of differences in fungal CFUs was assessed by the nonparametric Mann-Whitney *U* test. All other statistical analysis was performed using a 2-tailed unpaired Student *t* test. A 2-tailed *P* value of ≤ 0.05 was considered statistically significant.

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