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A TCR Transgenic Mouse Reactive with Multiple Systemic Dimorphic Fungi

Marcel Wüthrich,* Chiung Yu Hung,†‡ Ben H. Gern,* John C. Pick-Jacobs,* Kevin J. Galles,* Hanna I. Filutowicz,* Garry T. Cole,†‡ and Bruce S. Klein*§¶

Dimorphic fungi collectively account for 5–10 million new infections annually worldwide. Ongoing efforts seek to clarify mechanisms of cellular resistance to these agents and develop vaccines. A major limitation in studying the development of protective T cells in this group of organisms is the lack of tools to detect, enumerate, and characterize fungus-specific T cells during vaccination and infection. We generated a TCR transgenic mouse (Bd 1807) whose CD4+ T cells respond to a native epitope in Blastomyces dermatitidis and also in Histoplasma capsulatum. In this study, we characterize the mouse, reveal its applications, and extend our analysis showing that 1807 cells also respond to the related dimorphic fungi Coccidioides posadasii and Paracoccidioides lutzii. On adoptive transfer into vaccinated wild-type mice, 1807 cells become activated, proliferate, and expand in the draining lymph nodes, and they differentiate into T1 effectors after trafficking to the lung upon lethal experimental challenge. Bd 1807 cells confer vaccine-induced resistance against B. dermatitidis, H. capsulatum, and C. posadasii. Transfer of naive 1807 cells at serial intervals postvaccination uncovered the prolonged duration of fungal Ag presentation. Using 1807 cells, we also found that the administration of vaccine only once induced a maximal pool of effector/memory CD4+ cells and protective immunity by 4 wk after vaccination. The autologous adoptive transfer system described in this study reveals novel features of antifungal immunity and offers a powerful approach to study the differentiation of Ag-specific T cells responsive to multiple dimorphic fungi and the development of CD4+ T cell memory needed to protect against fungal infection. The Journal of Immunology, 2011, 187: 1421–1431.

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lthough Ab-based therapies are undergoing a renaissance, the induction of durable T cell memory is the cornerstone of vaccine immunity to fungi. The kinetics of induction and maintenance of Ag-specific CD4+ T cell memory to fungi have not been amenable to study owing to the lack of known protective Ags and clonally restricted T cells with complementary TCRs. We recently created a TCR transgenic (tg) mouse (Bd 1807) to interrogate the Ag-specific CD4+ T cell response to a natural and protective epitope of the fungal pathogen Blastomyces dermatitidis, a systemic fungal pathogen of humans and other mammals (M. Wüthrich, K. Ersland, K.J. Galles, T.D. Sullivan, and B.S. Klein, submitted for publication).

In this study, we report that 1807 cells respond well to four systemic dimorphic fungi, including B. dermatitidis, Histoplasma capsulatum, Coccidioides posadasii, and Paracoccidioides lutzii, implying that the cells recognize a shared epitope. Vaccine priming of 1807 cells protected recipient mice that lack endogenous CD4+ cells from infection with the three major systemic fungi of North America (B. dermatitidis, H. capsulatum, and C. posadasii). Using this tg mouse, we established an adoptive transfer system to follow the activation of T cells that arise in response to protective Ag. TCR tg systems such as this are among the best immunological tools to analyze naive T cell activation in vivo, since the frequency of circulating Ag-specific T cells can be raised above the level of detection for flow cytometric and immunohistological analyses (1–3). The development of this system allowed us to track key stages of activation, differentiation, and memory T cell development during priming and recall phases of protective immunity. Naive 1807 cells were activated, proliferated, and expanded in the skin draining lymph nodes (sdLN)s; however, they required trafficking to the site of vaccine delivery or the lung on experimental challenge to fully differentiate into effector cells. Bd 1807 cells also served as sentinels enabling us to define the duration of Ag persistence after vaccination and the conditions for inducing an optimal pool of effector/memory cells that mediate protective immunity to lethal experimental infection against dimorphic fungi.

Materials and Methods

Generation of the tg mouse

We described generation of the Blastomyces–specific TCR Tg 1807 mouse elsewhere (M. Wüthrich et al., submitted for publication). Briefly, we cloned the α- and β-chains of the TCR from CD4+ T cell clone no. 5, which is specific for a protective epitope of B. dermatitidis (4). Bd 1807 mice were bred to B6.PL (Thy1.1+) to obtain Thy1.1+ 1807 T cells to let us track transferred tg cells in Thy1.2+ recipient C57BL/6 mice.

In vitro-stimulated effector Bd 1807 cells

IFN-γ-producing tg cells were generated by adding naive, magnetic bead-purified CD4 cells from 1807 mice on plate-bound anti-CD3 (5 μg/ml; BD Biosciences, San Jose, CA) with soluble anti-CD28 (5 μg/ml) and human rIL-2 (50 U/ml) in the presence or absence of mouse rIFN-γ (1000 U/ml; Sigma-Aldrich, St. Louis, MO) and anti–IL-4 mAb (10 μg/ml; National Cancer Institute Biological Research Branch, Rockville, MD) for 10–
13 at 37°C and 5% CO₂. Effector T cells (2 × 10⁶) were transferred i.v. into sublethally irradiated (5.5 Gy) C57BL/6 mice. Mice were rested for 5 wk before infection to allow lymphopenia-driven expansion of the transplant (5).

Fungi

Strains used were ATCC 26199 (6), a wild-type strain of B. dermatitidis, and the isogenic, attenuated mutant lacking BAD1, designated strain 57 (7), as well as H. capsulatum strain G217B, P. latuzii (Pb1) (8), Para-coccidioides brasiliensis (ATCC 60855), and Candida albicans strain 5314. B. dermatitidis was grown as yeast on Middlebrook 7H10 agar with an oleic acid–albumin complex (Sigma-Aldrich) at 39°C. H. capsulatum was grown as yeast at 37°C and 5% CO₂ on Histoplasma macrophage medium. P. latuzii was grown in liquid brain heart infusion (Difco, Detroit, MI) at 37°C and was rotated at 200 rpm. C. albicans was grown on yeast extract/peptone/dextrose plates. The saporiphic phase of C. posadasi (isolate C735) was grown on glucose yeast extract medium (1% glucose, 0.5% yeast extract, 1.5% agar) at 30°C for 3–4 wk to generate a confluent layer of arthroconidia (spores) on the agar surface. Formalin-killed spherules (FKS) of C. posadasi were generated as described (9, 10).

Mouse strains

Inbred mice including C57BL/6, T lymphocyte-specific Thy.1.1 allele carrying congenic B6 strain B6.PL-Thy1.1(C57BL/6) (stock no. 000406) (11), and TCRα⁻/⁻/β⁻/⁻ deficient B6.129S2-Tcra²(TCRα⁻/β⁻/⁻) (stock no. 002116), (12), OT-1 C57BL/6-Tg (Tcrαβ) (1100Mbj) (stock no. 000381) (13), and OT-2 B6.Cg-Tg (Tcrαβ) 425CbuJ mice (stock no. 004194) (14) homozygous for a transgene that encodes a TCR specific for chicken OVA(325-326) and C. posadasii isolate C735 and 2 × 10⁵ isogenic wild-type yeast of H. capsulatum (stock no. 002116) (15, 16) expressing the Thy1.2 allele were provided by Dr. A.Y. Rudensky. TEa cells recognize residues 52–68 of the I-Ea chain (Ea peptide) bound to class II-IA-Eα, OT-1 and TEa tg mice expressing the Thy1.1 allele were made by backcrossing tg mice twice to wild-type C57BL/6 mice expressing the Thy1.1 marker. Male mice were 7–8 wk old at the time of these experiments. Mice were housed and cared for as per guidelines of the University of Wisconsin Animal Care Committee, who approved this work.

Vaccination and infection

Mice were vaccinated twice as described (17), 2 wk apart, s.c. with 10⁵–10⁶ B. dermatitidis strain 55 yeast, 10⁶ H. capsulatum G217B yeast, 10⁶ P. latuzii yeast, 10⁵ C. albicans yeast, 10⁵ FKS C. posadasi, or 200 µg whole cell cytosolic extract of Pneumocystis carinii (18) (gift of Dr. Jay Kolls) emulsified in CFA. Vaccine was injected at each of two sites. Mice were infected intracranially (i.t.) with 2 × 10⁴ isogenic wild-type yeast of B. dermatitidis strain 26199, 2 × 10⁴ H. capsulatum G217B, 2 × 10⁵ P. latuzii, 2 × 10⁴ FKS, or 60 spores of the virulent C. posadasi isolate C735 and 2 × 10⁵ C. albicans as described (17, 19–23). To assess the infiltration of primed CD4 cells into the lungs, lung homogenates of challenged mice were analyzed at day 4 postinfection. To analyze the extent of lung infection, homogenized lungs were plated and yeast CFU enumerated on brain heart infusion agar (Difco), sheep blood-containing Mycosel plates, and on glucose yeast extract plates containing 50 µg/ml chloramphenicol (19).

Adoptive transfer of 1807 cells and experimental challenge. To determine whether 1807 cells mediate protection, we transferred 10⁵ naive 1807 or OT-2 (control) Tg cells into OT-1 mice before vaccination. On the same day, recipients were vaccinated with 10⁵ heat-killed B. dermatitidis and H. capsulatum (strain 55) FKS C. posadasi s.c. Two weeks after vac- cination, the mice were challenged i.t. with 10⁵ B. dermatitidis Bd P. lutzii strain 55 yeast, 10⁶ H. capsulatum G217B yeast, 10⁶ P. latuzii yeast, 10⁵ C. albicans yeast, 10⁵ FKS C. posadasi, or 200 µg whole cell cytosolic extract of Pneumocystis carinii (18) (gift of Dr. Jay Kolls) emulsified in CFA. Vaccine was injected at each of two sites. Mice were infected intracranially (i.t.) with 2 × 10⁴ isogenic wild-type yeast of B. dermatitidis strain 26199, 2 × 10⁴ H. capsulatum G217B, 2 × 10⁵ P. latuzii, 2 × 10⁴ FKS, or 60 spores of the virulent C. posadasi isolate C735 and 2 × 10⁵ C. albicans as described (17, 19–23). To assess the infiltration of primed CD4 cells into the lungs, lung homogenates of challenged mice were analyzed at day 4 postinfection. To analyze the extent of lung infection, homogenized lungs were plated and yeast CFU enumerated on brain heart infusion agar (Difco), sheep blood-containing Mycosel plates, and on glucose yeast extract plates containing 50 µg/ml chloramphenicol (19).

Intracellular cytokine stain

Skin tissue-derived cells were collected serially after vaccination and cells from lung homogenates were harvested at day 4 postinfection. Cells (0.5 × 10⁶ cells/ml) were stimulated for 4 h with anti-CD3 (clone 145-2C11: 0.1 µg/ml) and anti-CD28 (clone 37.51: 1 µg/ml) in the presence of GolgiStop (BD Biosciences). Stimulation with fungal ligands yielded comparable cytokine production by transgenic T cells compared with CD3/CD28 stimulation (data not shown). After cells were washed and stained for surface CD4 and CD8 using anti-CD4-PerCP, anti-CD8-PE-Cy7, anti-CD44-allophycocyanin, anti–CD62L-PE, and B220 Alexa 700 (to gate out non-T cell background) mAbs (BD Pharmingen), they were fixed and permeabilized in Cytofix/Cytoperm at 4°C overnight. Permeabilized cells were stained with anti–IFN-γ (clone 10G1) and anti–IL-2 (clone 7D11) (BD Pharmingen). Cells were analyzed by FACS. Gated data were collected on a FACSCalibur for at least 10,000 events per sample. Cytokine expression in each gate was analyzed. The number of cytokine-positive CD4+ and CD8+ T cells per lung was calculated by multiplying the percentage of cytokine-producing cells by the number of CD4+ and CD8+ T cells in the lung.

Statistical analysis

The number and percentage of activated, proliferating, or cytokine-producing T cells and the differences in number of CFU were compared among groups by using ANOVA models (26). A two-sided p value <0.05 was considered statistically significant.

Results

Bd 1807 cells recognize a shared Ag in systemic dimorphic fungi

B. dermatitidis, P. brasiliensis (and P. latuzii), H. capsulatum, and Coccidioides immitis (and C. posadasi) are closely related at the level of 18S ribosomal DNA sequences (27, 28). We recently found that Bd 1807 cells respond to a shared immunodominant Ag in B. dermatitidis and H. capsulatum (M. Wuthrich et al., submitted for publication). In this study, we extended this analysis and tested whether Bd 1807 cells may also respond to this shared Ag in C. posadasi and P. latuzii. After transferring 1807 cells into mice, we vaccinated them with C. posadasi, P. latuzii, H. capsulatum, and B. dermatitidis. We also vaccinated mice with C. albicans and P. carinii as controls for agents that are not closely related to dimorphic fungi (http://timm.main.teikyo-u.ac.jp/pfdb/cover/taxonomic_list_eng.html). Bd 1807 cells were activated in response to the four systemic dimorphic fungi, but they were not activated in unvaccinated mice or mice vaccinated with C. albicans or P. carinii (Fig. 1A and data not shown). More than 98% of 1807 cells proliferated and became CFSElow in response to P. latuzii, C. posadasi, H. capsulatum, and B. dermatitidis, and the pattern for CD44 and CD62L was also comparable for the groups. Vaccinated recipients of Bd 1807 cells were challenged i.t. with the homologous (vaccinating) fungus to determine whether the cells acquired memory, differentiated, and recruited back to the lung. Bd 1807 Th1 effectors were recruited to lung in response to all four dimorphic fungi and 5–25% produced IFN-γ (Fig. 1B). Bd 1807 responses were similar to endogenous, polyclonal CD4+ T cell responses in each case, implying that the Tg cells portray the endogenous response. Bd 1807 cells failed to recall in response to Candida, although endogenous CD4+ T cells produced a robust IFN-γ response. These results imply that Bd 1807 cells recognize a shared Ag in four dimorphic fungi and can be used to study and compare T cell development in response to these organisms.

Precursor frequency and vaccine dose dictate proliferation and activation of 1807 cells

To establish an adoptive transfer system, we titrated precursor frequency of naive 1807 cells and vaccine dose of B. dermatitidis. The frequency of transferred T cells can affect proliferative ex-panision of responding T cells (24, 29). We determined the mini- mum number of transferred 1807 cells, enabling us to observe
FIGURE 1. *Bd* 1807 cells recognize a common Ag shared with *P. lutzii*, *C. posadasii*, and *H. capsulatum*. A, Thy1.2+ wild-type mice received 10^6 naive Thy1.1+ 1807 cells and were vaccinated s.c. with 10^7 *P. lutzii*, 10^7 *B. dermatitidis* (strain no. 55), 10^7 *H. capsulatum* G217B, and 10^8 *C. albicans* yeast and 10^6 FKS *C. posadasii*. Seven days after vaccination, sdLN s were removed and Thy1.1+ CD4+ cells assayed for proliferation (CFSE) and activation (CD44 and CD62L expression) by FACS. B, Recall responses of 1807 cells to the lung. Four weeks after recipients of 10^6 *Bd* 1807 cells were vaccinated as in A, they were challenged with the homologous fungus via the respiratory route: *P. lutzii*, wild-type *B. dermatitidis*, *H. capsulatum*, *C. albicans*, and FKS. Four days later, lung T cells were harvested and analyzed for intracellular cytokine. The dot plots show IFN-γ–producing T cells from a representative mouse of three mice per group in one of three experiments performed.
in vivo responses to the vaccine. Varied numbers of 1807 cells were transferred into Thy1.2<sup>+</sup> recipients on the day of vaccination and the expansion of tg cells was assessed in the sdLNs 4 d postvaccination (peak of expansion; data not shown) using a saturating dose of 10<sup>5</sup> vaccine yeast. Transfer of 10<sup>3</sup>, 10<sup>5</sup>, 10<sup>6</sup>, and 10<sup>8</sup> naive tg precursors yielded an expansion of 10-, 55-, 287- and 63-fold, respectively, as measured by the total number of Thy1.1<sup>+</sup>CD4<sup>+</sup> T cells in vaccinated versus unvaccinated recipient mice. The greatest in vivo expansion of 1807 cells in the sdLNs was seen when 10<sup>5</sup> precursors were transferred. The relative expansion of 1807 cells in vaccinated mice was 4, 25, 10, and 4, respectively, for the following comparisons: 10<sup>6</sup> versus 10<sup>5</sup>, 10<sup>5</sup> versus 10<sup>3</sup>, 10<sup>6</sup> versus 10<sup>3</sup>, and 10<sup>8</sup> versus 10<sup>3</sup>, again implying that the relative expansion was maximal using 10<sup>5</sup> precursors for adoptive transfer.

To determine whether the vaccine dose affects proliferation and activation of naive tg precursors, we adoptively transferred 1807 cells and then vaccinated recipient mice with 10<sup>5</sup>, 10<sup>6</sup>, or 10<sup>7</sup> yeast. Immunization with increasing numbers of vaccine yeast increased expansion of 1807 cells in the sdLNs, according to the precursors transferred. This was evident in the number of calculated 1807 cells and then vaccinated recipient mice with 10<sup>5</sup>, 10<sup>6</sup>, or 10<sup>7</sup> yeast. The induction of effector function and the generation of memory are dependent on the number of transferred cells and the amount of Ag available per naive precursor (24, 30, 31). Decreasing the input number of naive CD4<sup>+</sup> T cells requires a higher Ag level threshold per naive T cell precursor than does proliferation (30, 31). For example, survival and memory generation of adoptively transferred TCR tg cells are dependent on the number of transferred cells and the amount of Ag available per naive precursor (24, 30, 31). Decreasing the input number of naive CD4<sup>+</sup> T cells can promote memory development; conversely, limiting the availability of Ag can impair memory development.

To determine whether Bd 1807 tg cells develop memory, we tested a precursor frequency of 10<sup>5</sup> transferred cells and a vaccine dose ranging from 10<sup>3</sup> to 10<sup>7</sup> yeast. At 31 d after vaccination, the number of transferred 1807 cells in the sdLNs was significantly higher in vaccinated mice versus controls (Fig. 3A). The pool of primed CD4<sup>+</sup>tg cells in all groups of vaccinated mice was 115- to 349-fold larger than in the unvaccinated controls. In contrast, the increase in the number of endogenous, polyclonal CD4<sup>+</sup> CD44<sup>hi</sup> T cells was <2.4-fold higher in vaccinated versus unvaccinated mice, and the percentage of CD44<sup>hi</sup> T cells was <15%. In vaccinated mice, 80–92% of the transferred 1807 cells expressed high levels of CD44 versus 11% in unvaccinated control mice. Thus, the analysis of polyclonal cells did not permit detection or tracking of a memory pool, whereas transfer of 10<sup>5</sup> Bd 1807 precursors and ensuing vaccination induced a significant population of memory cells. This discrepancy between polyclonal and 1807 cells underscores the utility of this mouse and adoptive transfer system for interrogating Ag-specific T cell memory immunity to fungi.

To determine whether memory 1807 cells migrate to the lung during pulmonary recall, we analyzed lung T cells during the first 4 d postinfection when memory T cells arrive and peak in the lung (20, 32). In vaccinated mice, 1807 cells that produced IFN-γ migrated into the lungs by day 2 and increased sharply by day 3 postinfection (Fig. 3B). Unvaccinated mice showed no influx of IFN-γ-producing tg cells during this interval. Polyclonal IFN-γ–producing CD4<sup>+</sup> cells from vaccinated mice showed a similar kinetics of influx, indicating that 1807 cells accurately report the behavior of endogenous Ag-specific CD4<sup>+</sup> T cells. Vaccine-induced expansion of transferred 1807 cells did not affect the recruitment of IFN-γ–expressing CD4<sup>+</sup> cells into the lung. In unvaccinated mice infected i.t. with wild-type yeast, polyclonal CD4<sup>+</sup> cells that produced IFN-γ were detected by day 1 of infection, whereas 1807 cells were not detected. It is unclear whether these polyclonal CD4<sup>+</sup> cells are fungus-specific. Thus, Bd 1807 cells acquired memory, were recalled to the lung upon experimental challenge, and showed fidelity with endogenous CD4<sup>+</sup> T cells.

Persistence of fungus-derived Ag presentation

The kinetics of presentation of fungal Ag and its impact on the differentiation of CD4<sup>+</sup> T cell effectors and generation of memory

![FIGURE 2. Impact of precursor frequency and vaccine dose on T cell expansion and proliferation. Mice received 10<sup>5</sup>–10<sup>6</sup> Bd 1807 cells and were vaccinated with 10<sup>5</sup>–10<sup>7</sup> B. dermatitidis vaccine yeast. Four days later, the draining lymph nodes were harvested and analyzed for the presence of Thy1.1<sup>+</sup>CD4<sup>+</sup> T cells. The averaged number of total (A) and percentage of CFSE<sup>lo</sup> (B) 1807 cells are depicted. The fold change in the number of total and CFSE<sup>lo</sup> 1807 cells in vaccinated versus unvaccinated control was calculated and is depicted in white in the filled bars. *p < 0.05 versus nonvaccinated mice receiving the same precursor frequency as vaccinated mice; **p < 0.05 versus mice vaccinated with 10-fold less yeast.](https://www.jimmunol.org/content/jimmunol/178/6/3024/F2.large.jpg)
remain undefined. During an experimental influenza infection, live virus is cleared within the first 10 d of infection, but adoptive transfer of naive influenza-specific CD4 T cells indicated Ag persistence for 3 wk after virus clearance (33). To assess the duration and relative amount of persistent vaccine Ag in this study, we used 1807 cells. We transferred the tg cells into mice at various times after Blastomyces vaccination and analyzed the donor cells for cell division and activation 7 da after transfer (33). This approach also enabled us to compare persistence of Ag using heat-killed versus live vaccine. To confirm that 1807 cells report proliferation and activation in an Ag-specific manner, we analyzed 1807 phenotypes in vaccinated and unvaccinated recipients. Naive 1807 cells transferred into unvaccinated mice remained largely CFSEhi, CD44lo, and CD62Lhi for most time points, but there were minor fluctuations of the phenotypic markers for individual batches of donor cells. Consequently, this unvaccinated control group was used as a baseline. To establish that the division of donor 1807 cells was Ag-specific, Eα-specific T Ea cells were transferred into vaccinated controls. T Ea cells did not divide or become activated, establishing that 1807 cells behaved in an Ag-specific manner (data not shown).

In response to live vaccine, 105 Bd 1807 cells became activated within the first 3 wk after vaccination, as noted by loss of CFSE (>90%) and expression of CD44 (>80%) (Fig. 4A and data not shown). Thereafter, 1807 T cell activation dwindled but was still evident by day 89 postvaccination (Fig. 4B). At day 28 postvaccination and thereafter, T cell activation was hardly detectable with 103 precursors, and the lower frequency of 104 cells plus Thy1.1-based enrichment (24, 25) was required to detect T cell activation. Thus, the amount of vaccine Ag became limiting by 4 wk after vaccination. In mice vaccinated with heat-killed yeast, T cell priming during the first 3 wk after vaccination was comparable to that in mice with live vaccine; it was reduced thereafter and not detected 35 d after vaccination. Thus, fungal Ag persisted longer with live versus dead vaccine, and 1807 cells reported extended persistence of vaccine Ag.

We measured Ag persistence using another approach. We vaccinated mice with live yeast and measured CFU at the vaccine site (skin). Fungal CFU persisted during the first 3 wk postvaccination, and they fell consistently thereafter and were cleared completely by day 104 postvaccination (Fig. 4C). Thus, the findings revealed with 1807 cells are consistent with these results.

Prolonged Ag exposure drives optimal T cell development

Because fungal Ag persists up to 3 mo after vaccination, we wondered what parameters are needed to drive optimal T cell priming and protection. We have used an empirical schedule of two vaccinations during 4 wk, which induces sterilizing immunity in most vaccinated mice (17, 32). The generation of the 1807 tg
**FIGURE 4.** *Bd* 1807 cells are a sentinel of persistent fungal Ag. A. C57BL/6 wild-type mice were vaccinated with $10^4$ and $10^5$ live or $10^6$ heat-killed attenuated *B. dermatitidis* and proliferation of sentinel 1807 cells was measured. Beginning at day 0 and biweekly thereafter, naive, CFSE-labeled 1807 cells were transferred into vaccinated mice or unvaccinated controls. Then, $10^5$ cells per mouse were transferred at days 0 and 14 postvaccination and $10^4$ cells per mouse at the later time points. Seven days after transfer, Thy1.1+ CD4+ T cells from the sdLNs were analyzed for proliferation. The numbers depict the percentages of CFSE<sup>lo</sup> donor CD4+ T cells in vaccinated mice (bold number and histogram) and unvaccinated controls (gray number and histogram).

B. The average numbers of CFSE<sup>lo</sup> 1807 cells were calculated for each group and time point. The adoptive transfer, harvest, and analysis of 1807 cells were identical as described in A. The data represent averages ± SEM of four to five mice per group. *p < 0.05 versus 1807 cells from all three groups of vaccinated mice, **p < 0.05 versus 1807 cells from unvaccinated control mice.

C. Skin CFU correlate with T cell proliferation and activation. Skin tissue from the site of vaccination was harvested, homogenated, and plated for CFU.
mouse and an adoptive transfer system enables us to study the parameters for optimal priming of protective T cells. We studied the time interval after vaccination needed to induce maximal protection and T cell memory. In view of the persistence of vaccine Ag, we postulated that vaccine boosting might not be required. We vaccinated mice during the course of 2, 4, or 8 wk and boosted them or not to measure the size of the effector/memory T cell pool in the sdLN. The number of primed (CD44hi) 1807 cells was significantly greater in all groups of vaccinated mice versus unvaccinated controls (Fig. 5A). Mice vaccinated during 4 wk, independent of a boosting dose, harbored a significantly larger pool of effector/memory T cells than did the groups vaccinated for 2 or 8 wk. Remarkably, the number of polyclonal CD4+CD44hi T cells evinced only subtle increases in vaccinated mice versus unvaccinated controls. Thus, Bd 1807 cells let us monitor and quantify the emergence of Ag-specific effector/memory T cells in the sdLN in a setting where the analysis of polyclonal T cells was uninformative.

After we challenged vaccinated mice i.t., we enumerated 1807 cells that migrated to the lung and measured lung CFU in unvaccinated and vaccinated mice. Mice vaccinated during 4 wk, whether they were boosted or not, harbored 22-fold more primed 1807 cells in their lungs than mice vaccinated during 2 wk (Fig. 5B). Whereas the number of primed polyclonal CD4+ T cells in the lung was increased in all groups of vaccinated and unvaccinated mice, the analysis of the polyclonal pool again revealed only small differences between groups. Thus, polyclonal and 1807 tg cells migrated to the lung in an Ag-specific manner, but only 1807 tg cells offered the resolving power needed to determine the vaccine conditions that optimally induce and recall memory T cells to the lung.

The number of primed 1807 cells recruited to the lung correlated with the lung burden. Mice challenged 4 wk postvaccination cleared yeast from their lungs, whereas mice that had a shorter 2-wk interval of vaccination yielded 2–3 logs higher lung CFU (Fig. 5C). When vaccinated mice were challenged 8 wk after vaccination, they still cleared the yeast from their lungs, despite the fact that the influx of primed 1807 cells was smaller than in mice challenged 4 wk after vaccination. By 8 wk the pool of primed 1807 cell had likely contracted but retained the functional capacity of a long-term memory pool. Thus, a 4-wk interval after vaccination, independent of boosting, is necessary and sufficient to
FIGURE 6. *Bd* 1807 cells mediate protection against experimental infection with *B. dermatitidis*, *C. posadasi*, and *H. capsulatum*. A. In vitro-activated, adoptively transferred effector T cells mediate protection. Ten weeks after adoptive transfer, mice were challenged with *B. dermatitidis* and 21 d later analyzed for lung CFU. Data represent the geometric mean CFU ± SEM; n = 9–13 mice/group. *p < 0.001 versus no transfer control group. Bold numbers indicate the n-fold reduction in lung CFU versus control mice that received no T cells.

B and C. In vivo-primed 1807 cells engender resistance to *B. dermatitidis* infection. B. TCR-α-deficient mice received naive 1807 or TEa cells as a control and were vaccinated with 10^6 heat-killed *B. dermatitidis* yeast twice 2 wk apart. Four weeks after the boost, mice were challenged with 26199 wild-type yeast (10^3) and 25 d later, when unvaccinated mice were moribund, they were analyzed for lung CFU. Data represent the geometric mean CFU ± SEM; n = 8 mice/group. *p < 0.001 versus unvaccinated OT-1 and wild-type mice that received no TCR tg cells, **p < 0.005 versus unvaccinated mice that received 1807 or TEa cells. Bold numbers indicate the n-fold reduction in lung CFU versus control mice that received no T cells or vaccine.

C. OT-1 mice received TCR tg 1807 or OT-2 cells as a control and were vaccinated (or not) with 10^6 heat-killed yeast on the day of transfer and boosted 2 wk later. Two weeks after the boost, mice were challenged with 26199 yeast (2 × 10^3). Nineteen and 25 d postinfection when the unvaccinated wild-type and OT-1 mice, respectively, were moribund, the lungs were harvested and plated for CFU. *p < 0.05 versus unvaccinated wild-type and OT-1 mice that received no

D. Histoplasmosis

E. Coccioidiomycosis

n-fold vs. OT-2 controls

Lung CFU

Spleen

Wild-type

OT-1 mice

Wild-type

OT-1 mice

Transfer

Vaccine

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OT-1 mice

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maximally induce a pool of effector/memory T cells in the sDLNs that can efficiently migrate to the lung upon challenge to mediate sterilizing immunity against a lethal pulmonary infection.

**Bd 1807 cells confer protection against multiple dimorphic fungi**

Because the 1807 mouse was made from a TCR that mediates resistance to infection with *B. dermatitidis* (4), we sought to determine whether 1807 cells confer protection against infection. We used two approaches. First, we generated T1-polarized effector cells in vitro and transferred them into naive wild-type mice before challenge. In vitro-primed TCR tg CD4+ effector cells confer protection against lethal influenza infection (34). Naive 1807 cells were stimulated in vitro with anti-CD3 and anti-CD28 mAbs in the presence of recombinant IFN-γ and anti–IL-4 mAb to polarize them into a T1 phenotype (35). After 10 d culture, 1807 cells were polarized and produced chiefly IFN-γ in cell culture supernatants, whereas unpolarized 1807 cells produced both Th1 and T2 cytokines. Upon adoptive transfer and challenge, all three effector T cell populations reduced lung CFU significantly compared with mice that did not receive any T cells (Fig. 6A). Thus, 1807 effector cells confer protection.

Second, we tested whether in vivo priming of 1807 cells could promote vaccine immunity. We transferred naive 1807 and TEa (irrelevant control) cells into TCR-α/− mice and vaccinated them with heat-killed yeast. Heat-killed yeasts were used to avoid dissemination in immune-deficient mice (36). Vaccination engendered robust immunity (Fig. 6B). Transfer of naive 1807 cells into vaccinated or unvaccinated TCR-α/− mice reduced lung CFU by 6 and 3 logs, respectively. Because transfer of naive T cells into lymphopenic hosts can activate T cells and promote memory (37), 1807 cells transferred into unvaccinated TCR-α/− mice could have promoted immunity due to nonspecific activation. To demonstrate that vaccine priming and resistance of 1807 cells was Ag-specific, we transferred naive TEa cells specific for E. coli Ag (16), which was not present in the vaccine strain. Vaccinated mice that received TEa cells before vaccination had 3 logs less lung CFU than did unvaccinated controls that did not receive TEa cells. Thus, vaccine-primed 1807 cells reduced lung CFU by 3 logs more than did TEa cells, indicating that primed Ag-specific 1807 cells contributed significantly to vaccine resistance.

Why did transferred CD4+ T cells from TEa mice reduce lung CFU? The frequency of Tg cells in TEa mice (and 1807 mice) is 90–95% of the total CD4+ T cell pool, indicating that the mice harbor endogenous non-TEa CD4+ cells. Those cells may have become activated during vaccination or due to lymphopenia-induced expansion and contributed to vaccine immunity.

To circumvent the limitation above, we used another design. To avoid lymphopenia-induced activation of transferred T cells, we transferred Tg cells into OT-1 mice that harbor chiefly SIINFEKL-specific CD8 T cells and few endogenous CD4+ T cells (13). Because OT-1 mice are not lymphopenic, transferred Tg cells should not expand and become nonspecifically activated. To determine whether OT-1 mice that received nonspecific OT-2 cells acquire vaccine immunity, we vaccinated these mice with *B. dermatitidis* yeast and analyzed lung CFU after challenge. Vaccinated and nonvaccinated mice showed similar lung CFU, indicating that any endogenous cells in OT-1 mice or OT-2 donor cells do not confer immunity (Fig. 6C). This transfer system was thus deemed suitable to assess in vivo protection by 1807 cells. OT-1 mice that received naive 1807 cells before vaccination and challenge reduced lung CFU by ≈3 logs versus control mice that received OT-2 cells. Transfer of naive 1807 cells without vaccination reduced lung CFU 33-fold, whereas OT-2 cells had no effect. Thus, in vivo-primed 1807 cells confer vaccine protection to *B. dermatitidis* and naive 1807 cells confer resistance to primary infection, but much less so. Because 1807 cells recognize a protective Ag in *Blastomyces* yeast that is shared in other dimorphic fungi, we tested whether 1807 cells protect against *Histoplasma* or *Coccidioides* infection. Transfer of 1807 cells into OT-1 mice vaccinated with *H. capsulatum* or *C. posadasi* reduced lung CFU significantly by 8- and 31–fold, respectively, versus vaccinated OT-1 controls (Fig. 6D, 6E). Thus, vaccine-induced 1807 cells are sufficient to protect against experimental coccidioidomycosis and histoplasmosis.

**Discussion**

We describe a TCR tg mouse and its use in studying the developmental progression of fungal Ag-specific T cells. This mouse is unique and will benefit the field of medical mycologists studying cellular mechanisms of CD4 T cell immunity and memory to dimorphic fungi. First, *Bd 1807 cells recognize a shared Ag in *B. dermatitidis*, *H. capsulatum*, *P. brasiliensis*, and *C. posadasi*. Because T cell responses are requisite for vaccine immunity against dimorphic fungi, investigators studying them will now have a tool to track and precisely enumerate Ag-specific T cells during development. Second, *Bd 1807 cells report a biologically vital endpoint, as they harbor a TCR from cells that protects against experimental infection. *Bd 1807 cells themselves protect vaccinated mice against infection. Thus, *Bd 1807 cells and the adoptive transfer system we developed produce functional T cells that differentiate, develop memory, and protect against experimental pulmonary blastomycosis, histoplasmosis, and coccidiomycosis. To our knowledge, this is the first anti-fungal TCR tg mouse that harbors protective T cells. Although Afl3.16 TCR tg T cells are specific for *A. fumigatus* and migrate, proliferate, and differentiate in an Ag-specific manner, their protective role was not investigated (3). The ability of *Bd 1807 cells to confer resistance to infection with the three major endemic, dimorphic fungi of North America should allow us and others to study the role of CD4+ T cell differentiation down varied T helper pathways in generation of long-term memory and immunity to fungal disease.

The TCR tg mouse and adoptive transfer system in this study enabled us to analyze T cells at a level that has not been readily attainable in fungal immunology (3). We used 1807 Tg cells to address two simple questions about Ag persistence and optimal conditions for vaccine immunity. First, we studied how long Ag persists after immunization with live or heat-killed vaccine. We used naive 1807 cells as a sentinel, transferring them into vaccinated mice at serial time points postvaccination to assess T cell activation and proliferation 1 wk later. T cells proliferated and
became activated for up to 2 or 3 mo postvaccination using heat-killed or live vaccine, respectively. In the case of the live vaccine, CFU plating of skin tissue harvested from the vaccine site confirmed the prolonged presence of Ag reported by sentinel 1807 cells. The persistence of fungal Ag contrasts with influenza where viral Ag persisted for several weeks after viral clearance as detected by HNT-specific TCR tg T cells (33). Bd 1807 cells did not report persistent fungal Ag after CFU clearance. The Ag load might be higher after systemic viral infection as opposed to a local fungal vaccine administration. If so, the amount of Ag available per naive TCR tg precursor would be lower in our fungal model. This hypothesis was supported by the fact that T cell proliferation and activation after 21 d postvaccination was only detectable when we transferred fewer (10^3) naive 1807 cells and enriched them with beads (24).

We asked what vaccine schedule drives the largest effector/ memory pool of Ag-specific T cells that mediates protective immunity. The increase in number of primed Ag-specific T cells in vaccinated versus unvaccinated mice was more than one magnitude larger for the 1807 cells than for polyclonal CD4^+ cells. The number of primed 1807 cells detected in the sdLNs and lung after recall was the largest in mice vaccinated during a period of 4 wk, demonstrating that this interval induces a maximal pool of effector/memory cells. A 2-wk interval was too short and by 8 wk effector T cells likely contracted. The analysis of polyclonal CD4^+ T cells did not reveal a difference in the number of primed T cells in the sdLNs or the lung upon challenge over the course of 4 wk vaccination. Thus, only 1807 cells offered the resolution to track the development and recruitment of protective T cells upon vaccination, experimental challenge, and recall to the lung.

To evaluate the utility and fidelity of the mouse in monitoring Ag-specific T cells, we compared 1807 tg cells and polyclonal CD4^+ T cells during sequential stages after vaccination: activation, differentiation, memory, and protection. We established an adoptive transfer system and defined the conditions that induce optimal activation and expansion of the TCR tg T cells. Bd 1807 tg cells expanded maximally using a precursor frequency of 10^5 tg cells and a vaccine dose of 10^7–10^9 yeast. Therein, >90% of 1807 cells were activated, and they proliferated and expanded at a maximum by 2000- to 5800-fold. At either higher or lower precursor frequencies, the maximal expansion of T cells and their activation and proliferation, as denoted by the number and percentage of CD44^hi cells or CFSE^lo cells, were all reduced. Considering that ~10–15% of the transferred precursors (~10^5 cells) survive transplantation (24, 29, 38), we could have created a nonphysiological situation wherein transferred 1807 tg cells exceeded the pool size of endogenous naive Ag-specific T cells. The largest pool of such cells known so far is exceeded the pool size of endogenous naive Ag-specific T cells.

We transferred fewer (10^3) naive 1807 cells and enriched them with beads (24). At either higher or lower precursor frequencies to portray Ag-specific T cell responses, but we found that a frequency of 10^5 precursors enabled optimal in vivo expansion. We also found that this frequency was needed to analyze recall responses in the lung and preserve key functions such as IFN-γ production in vivo and resistance to infection. We expect that the better resolving power of 1807 cells over polyclonal analysis will enable us to explore and answer critical questions in fungal immunology, including: 1) the occurrence and importance of Ag-specific T cell differentiation down various T helper pathways in establishing protective immunity; 2) the contribution of distinct APC populations in vaccine priming of Ag-specific T cell immunity to fungi; 3) the tissue specific influences on priming T cell immunity, for example in lung versus skin; and 4) the presence of shared Ags in the fungal kingdom that are recognized by protective T cells, such as those used to generate the Bd 1807 mouse described in this study.

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Disclosures
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References


