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Diversity of the T-Cell Response to Pulmonary *Cryptococcus neoformans* Infection

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Cell-mediated immunity plays an important role in immunity to the pathogenic fungus *Cryptococcus neoformans*. However, the antigen specificity of the T-cell response to *C. neoformans* remains largely unknown. In this study, we used two approaches to determine the antigen specificity of the T-cell response to *C. neoformans*. We report here that a diverse T-cell receptor (TCR) Vβ repertoire was maintained throughout the primary response to pulmonary *C. neoformans* infection in immunocompetent mice. $CD4^+$ T-cell deficiency resulted in relative expansion of all $CD8^+$ T-cell subsets. During a secondary immune response, preferential usage of a TCR Vβ subset in $CD4^+$ T cells occurred in single individuals, but the preferences were "private" and not shared between individuals. Both $CD4^+$ and $CD8^+$ T cells from the secondary lymphoid tissues of immunized mice proliferated in response to a variety of *C. neoformans* antigens, including heat-killed whole *C. neoformans*, culture filtrate antigen, *C. neoformans* lysate, and purified cryptococcal mannoprotein. $CD4^+$ and $CD8^+$ T cells from the secondary lymphoid tissues of mice undergoing a primary response to *C. neoformans* proliferated in response to *C. neoformans* lysate. In response to stimulation with *C. neoformans* lysate, lung $CD4^+$ and $CD8^+$ T cells produced the effector cytokines tumor necrosis factor alpha and gamma interferon. These results demonstrate that a diverse T-cell response is generated in response to pulmonary *C. neoformans* infection.

The fungal pathogen *Cryptococcus neoformans* can infect immunocompetent individuals, but this organism causes disease primarily in individuals with defects in cellular immunity (5). T-cell-mediated immunity plays a critical role in clearance of pulmonary *C. neoformans* infection in mice, but the antigen specificity of the responding $CD4^+$ and $CD8^+$ T cells remains largely unknown. In this study, we used two approaches to determine the antigen specificity of the T-cell response to *C. neoformans*.

In αβ T cells, which comprise the majority of T cells, the T-cell receptor (TCR) is expressed as a heterodimeric protein composed of α and β subunits. Somatic recombination of diversity and joining regions in Vα and somatic recombination of variable, diversity, and joining regions in Vβ result in the diversity of the TCR repertoire (3). A number of studies, including studies of the pathogenic fungus *Histoplasma capsulatum*, have demonstrated that there are Vβ preferences during T-cell responses, which correlate to T-cell function or antigen specificity (7–11). Similar to control of *C. neoformans* infection, control of *H. capsulatum* infection is primarily dependent on $CD4^+$ T cells. Based on these results, we studied the Vβ TCR expression by $CD8^+$ and $CD4^+$ T cells during pulmonary *C. neoformans* infection.

Our first objective in this study was to characterize the Vβ TCR usage by $CD4^+$ and $CD8^+$ T cells during primary and secondary responses to pulmonary *C. neoformans* infection. Our goals were to determine (i) whether preferential expansion of specific Vβ subsets by either T-cell subset occurs during primary infection; (ii) whether Vβ skewing, if present, represents superantigen-induced proliferation or oligoclonal expansion of antigen-specific T cells; and (iii) whether tracking a single or limited number of Vβ subsets throughout the course of the T-cell response could be used as a surrogate marker for antigen specificity.

C. neoformans has been shown to have mitogenic activity with human T cells in vitro (20, 21, 25). Superantigens are microbially derived proteins which bind directly to the Vβ region of a limited number of closely related TCR Vβ subsets (17). Neonatal exposure to mouse mammary tumor virus superantigens results in mouse strain-specific deletion of TCR Vβ subsets from the mature TCR repertoire (1). Exposure of mature T cells to superantigens (such as staphylococcal enterotoxin B) results in rapid expansion of T cells bearing Vβ bound by the superantigen, followed by activation-induced cell death. Our second objective was to determine whether *C. neoformans* had superantigen activity in mice (in vitro or in vivo). In addition, we sought to determine the proliferative and effector cytokine responses of $CD4^+$ and $CD8^+$ T cells from the lungs and the secondary lymphoid tissues in response to *C. neoformans* antigens.

MATERIALS AND METHODS

Mice. Female CBA/J mice (weight, 25 ± 4 g; age, 5 to 7 weeks) were obtained from the Jackson Laboratories (Bar Harbor, Maine). The mice were housed under pathogen-free conditions in enclosed filter-topped cages. Clean food and water were provided ad libitum. The mice were handled and maintained using microisolator techniques, and there was daily veterinarian monitoring. Bedding from the mice was transferred weekly to cages of uninfected sentinel mice that were subsequently bled at weekly intervals and were found to be negative for antibodies to mouse hepatitis virus, Sendai virus, and *Mycoplasma pulmonis*. All

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studies involving mice were approved by the University Committee on Use and Care of Animals at the University of Michigan.

***C. neoformans*.** *C. neoformans* strain 52D (= ATCC 24067) was obtained from the American Type Culture Collection (Manassas, VA). For infection, the yeast was grown to the stationary phase (48 to 72 h) at 35°C in Sabouraud dextrose broth (1% neopeptone and 2% dextrose; Difco, Detroit, Mich.) on a shaker. The cultures were then washed in nonpyrogenic saline, counted with a hemacytometer, and diluted to obtain a concentration of 3.3×10^5 CFU/ml in sterile nonpyrogenic saline. The precise number of organisms delivered was determined by counting the CFU in an inoculum plated on Sabaraud dextrose agar (Difco).

Intratracheal inoculation of *C. neoformans*. Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (6.8 mg/kg; Lloyd Laboratories, Shenandoah, IA) and were restrained on a small surgical board. A small incision was made through the skin over the trachea, and the underlying tissue was separated. A 30-gauge needle was attached to a 1-ml tuberculin syringe filled with a diluted *C. neoformans* culture. The needle was inserted into the trachea, and 30 μl of inoculum (10^4 CFU) was dispensed into the lungs. The needle was removed, and the skin closed with cyanoacrylate adhesive. The mice recovered with minimal visible trauma.

Lung, lymph node, and spleen leukocyte isolation. The lungs of each mouse were excised, washed in phosphate-buffered saline (PBS), minced, and digested enzymatically for 30 min in 15 ml/lung of digestion buffer (RPMI, 5% fetal calf serum [FCS], 1 mg/ml collagenase [Boehringer Mannheim Biochemical, Chicago, IL], 30 μg/ml DNase [Sigma Chemical Co., St. Louis, MO]). Following erythrocyte lysis using NH_4Cl buffer, the cells were washed, resuspended in complete medium, and centrifuged for 30 min at $2,000 \times g$ in the presence of 20% Percoll (Sigma) to separate leukocytes from the cell debris and epithelial cells. Total numbers of lung leukocytes were determined in the presence of trypan blue using a hemocytometer; the viability was >85%. Lung-associated lymph nodes (LALN) and spleens were excised, and the cells dispersed with the plunger of a 3-ml syringe. Erythrocytes were lysed using NH_4Cl buffer, and the cells were resuspended in complete medium (RPMI, 5% FCS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate).

Flow cytometry. For surface staining, leukocytes were washed and resuspended at a concentration of 10^7 cells/ml in FA buffer (Difco, Detroit, MI) containing 0.1% NaN_3 (Sigma), and Fc receptors were blocked by addition of anti-CD16/32 (Fc block; BD Pharmingen, San Diego, CA). Following Fc receptor blocking, 10^6 cells were stained in 120-μl (final volume) mixtures in polystyrene tubes (12 by 75 mm; BD Pharmingen) for 20 min at 4°C. Leukocytes were stained with the following antibodies, according to the manufacturer's instructions: CD4 (RM4-4 and H129.19), CD8α (5H10-1), CD8β (53-5.8), and a mouse TCR Vβ screening kit (BD Pharmingen). Preliminary experiments confirmed that CBA/J mice did not express Vβ 3, 5, 6, 7, 8.1, 9, 12, and 17 due to expression of endogenous proviruses and mouse mammary tumor viruses and were thus were not included in the Vβ analysis. Cells were washed twice with FA buffer and resuspended, and 200 μl of 4% formalin (Fisher Chemical, Pittsburgh, PA) was added to fix the cells. A minimum of 20,000 events were acquired with a FACScaliber flow cytometer (BD Pharmingen) using the Cell-Quest software (BD Pharmingen).

Intracellular flow cytometry. Leukocytes (2×10^6 cells/ml) were cultured for 12 h in 12-well plates in the presence of 0.1 μg/ml soluble anti-CD3 (145-2C11; BD Pharmingen) with or without 0.1 μg/ml anti-CD28 (37.51; BD Pharmingen). Brefeldin A and/or monensin (in the form of Golgi-stop or Golgi-block) were added for the last 4 h of incubation according to the manufacturer's instructions (BD Pharmingen). Nonadherent cells were harvested and washed twice with FA buffer, and staining for cell surface molecules was performed as described above. For intracellular staining, cells were washed to remove excess surface stains, fixed and permeabilized using Cytofix/Cytoperm (BD Pharmingen), and stained using anti-gamma interferon (IFN-γ) (XMG1.2) or anti-tumor necrosis factor alpha (TNF-α) (MP6-XT22; BD Pharmingen) in permeabilization buffer (FA buffer containing 0.1% saponin [Sigma]) at 4°C for 30 min. Flow cytometry was performed as described above for surface staining, except that >50,000 events per sample were routinely collected. The specificity of anticytokine antibodies was tested by comparing staining of experimental samples to at least two of the following three negative controls: (i) isotype control, (ii) excess unlabeled antibody, and (iii) preincubation of antibody with recombinant cytokine.

In some experiments, T cells were enriched from the lungs by fluorescence-activated cell sorting (FACS) and from lymph nodes and spleens by magnetism-activated cell sorting (MACS). For FACS, lung leukocytes were stained using anti-CD4 (RM4-4) and anti-CD8 (5H10-1). Cell sorting was performed at the University of Michigan Cancer Center Flow Cytometry Core with a FACSVantage SE cell sorter (BD Immunocytometry Systems, San Jose, CA). The purity of the sorted population was >99%, as determined by postsorting analysis. For MACS, cell suspensions from secondary lymphoid tissues were stained using a panel of biotinylated antibodies, including anti-CD19 (1D3), anti-CD49b (DX5), anti-Gr-1 (RB6-8C5), and anti-erythroid cells (TER-119) (all obtained from BD Pharmingen), as well as anti-mouse F4/80 (CI:A3-1; Caltag Laboratories, Burlingame, CA), and T cells were enriched by negative selection using antibiotin microbeads with a SuperMACS separator (Miltenyi Biotec, Auburn, CA). A total of 10^6 enriched T cells were cocultured with 10^6 adherent lung cells from uninfected mice and stimulated with either (i) anti-CD3 and anti-CD28 or (ii) *C. neoformans* lysate.

Proliferation assay. Cells were assayed for proliferation using an in vitro fluorescence-based assay. Briefly, 2×10^6 cells from the various organs were stained with 5 μM 5-(and 6-)carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) in PBS containing 5% FCS for 7 min at room temperature. The cells were washed three times to remove the excess CFSE and cultured for 3 days in the presence or absence of anti-CD3 antibodies (0.1 μg/ml). A minimum of 20,000 events were acquired with a FACScaliber flow cytometer (BD Pharmingen) using the Cell-Quest software (BD Pharmingen).

T-cell depletion using monoclonal antibodies. Depletion of $CD4^+$ T cells was accomplished by intraperitoneal administration of monoclonal antibodies. Anti-CD4 (GK1.5, rat immunoglobulin G2b) was prepared from ascites by dilution in nonpyrogenic saline and filtration through a 0.45-μm syringe filter. Mice received 200 μg of GK1.5 or a nonpyrogenic saline control in 200 μl nonpyrogenic saline at zero time and on day 1 of infection, followed by 200 μg every 8 days. The efficiency of T-cell depletion was assessed by flow cytometric analysis using anti-CD4 (RM 4-4), which binds a region of CD4 distinct from the region bound by GK1.5. The efficiencies of $CD4^+$ T-cell depletion in the lungs (>98%) and spleens (>99%) of mice were calculated by comparing the numbers of T cells in treated mice with the numbers of T cells in controls.

Preparation of *C. neoformans* antigens. (i) Heat-killed *C. neoformans*. *C. neoformans* strain 52D (= ATCC 24067) was obtained from the American Type Culture Collection. This yeast was grown to the stationary phase (72 h) at 34°C in Sabouraud dextrose broth (1% neopeptone and 2% dextrose; Difco, Detroit, Mich.) on a shaker platform. The yeast was washed three times in phosphate-buffered saline, counted with a hemacytometer, resuspended at a concentration of 10^9 cells/ml, and incubated in a 60°C water bath for 2 h with periodic mixing. The absence of viable organisms was confirmed by plating an aliquot containing 10^8 organisms on Sabouraud dextrose agar (Difco, Detroit, MI).

(ii) *C. neoformans* culture filtrate. *C. neoformans* culture filtrate (CneF) was prepared from *C. neoformans* grown to the stationary phase in asparagine broth. The yeast cells were pelleted by centrifugation at $1,500 \times g$ for 30 min. The supernatant was filtered though a 0.22-μm bottle top filter (Corning Incorporated, Corning, NY). The filtered culture supernatant was concentrated 10-fold using an Amicon concentration apparatus with a 50-kDa-cutoff filter (Millipore, Billerica, MA) and then was washed with five times the original culture volume of nonpyrogenic saline. The protein concentration of CneF (622 μg/ml) was determined by the bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL).

(iii) Purified *C. neoformans* mannoprotein. Mannoprotein purified from culture supernatants of acapsular *C. neoformans* strain ATCC 52817 (19, 22) was a generous gift from Stuart Levitz, Boston University.

(iv) *C. neoformans* lysate. Lysate was prepared from stationary-phase organisms as follows. A 30-ml yeast culture was centrifuged at $1,500 \times g$ for 20 min and washed three times with PBS, and the cells were resuspended in a minimal volume of PBS. Then 0.5-mm glass beads were added to cover the yeast (Biospec Products, Bartlesville, OK). The yeast cells were lysed by 25 cycles consisting of 30 s of vortexing and 30 s in an ice bath. The yeast lysate was diluted with 10 ml PBS and separated from the cellular debris by low-speed centrifugation ($750 \times g$), and the supernatant was filtered through a 0.22-μm bottle top filter. The sterile lysate was frozen at −70°C until it was used.

Secondary pulmonary *C. neoformans* infection in mice. CBA/J mice were infected intratracheally with 10^3 CFU of *C. neoformans* strain 52D. The mice were housed in specific-pathogen-free conditions with food and water provided ad libitum for 16 weeks to allow clearance of the primary infection. For secondary infections, mice were then infected intratracheally with 10^4 CFU *C. neoformans* (a dose that was 10-fold higher than the primary dose).

Statistics. For preferential Vβ usage during primary infection in *C. neoformans*-infected CBA/J mice, an analysis of variance was used to compare fold increases in each $V\beta^+$ T-cell population. A P value of <0.05 was considered statistically significant.

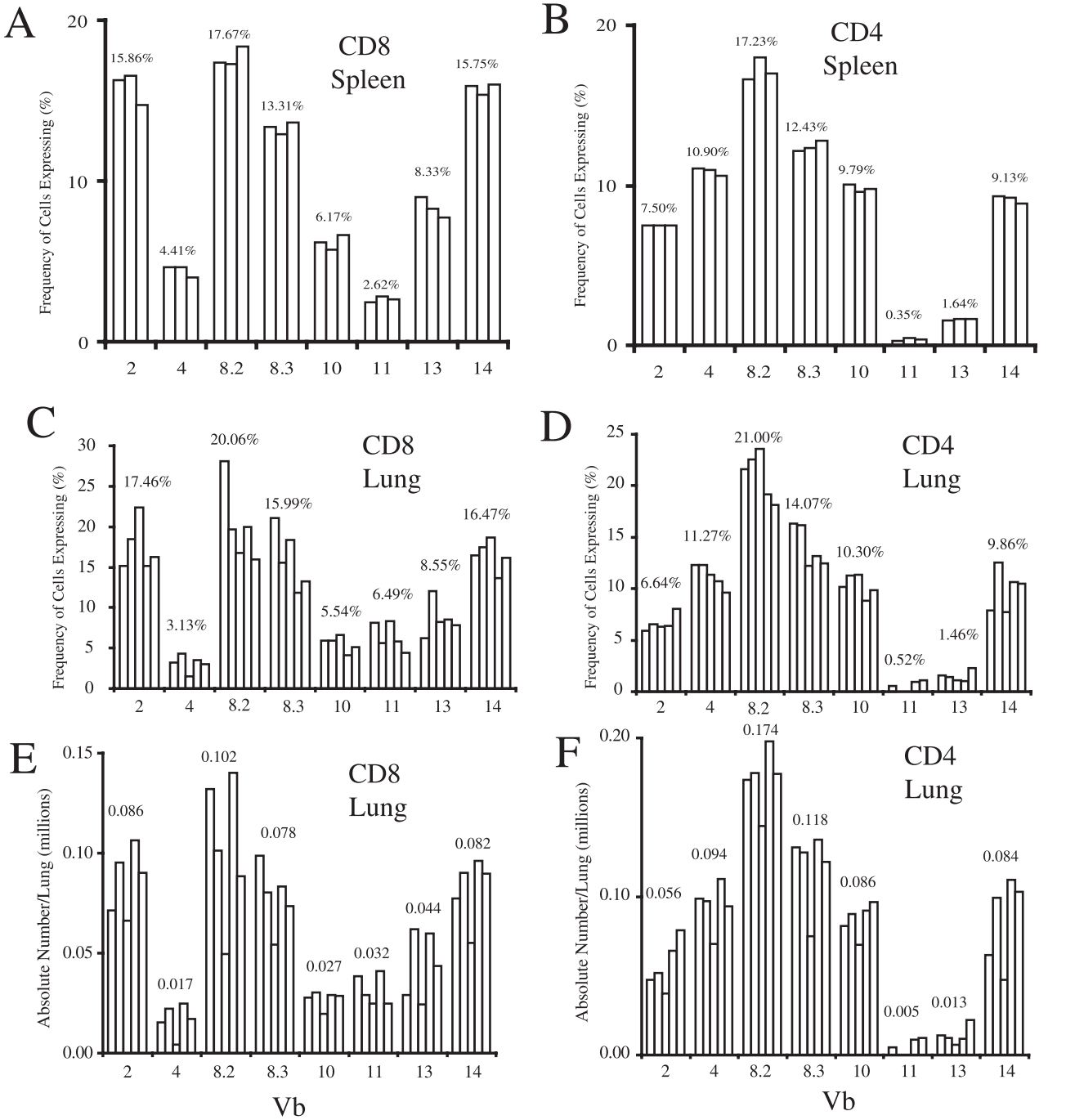

FIG. 1. Vβ TCR repertoire of $CD4^+$ and $CD8^+$ T cells in uninfected CBA/J mice. The frequencies of splenic $CD8^+$ (A) and $CD4^+$ (B) T cells expressing each Vβ subset were determined as described in Materials and Methods by flow cytometric analysis. Each bar represents a single animal. The frequencies (C and D) and absolute numbers (E and F) of each Vβ subset in lung $CD4^+$ and $CD8^+$ T cells were determined by flow cytometric analysis of lymphocytes recovered from enzymatic digests of whole lungs, as described in Materials and Methods. Each bar represents a single animal. The numbers indicate the mean for each group.

RESULTS

Vβ TCR repertoires in the spleens and lungs of naive CBA/J mice. In order to establish a baseline to determine the relative enrichment of any particular Vβ subset, it was necessary to first determine the naive Vβ repertoire in uninfected control mice. To do this, uninfected specific-pathogen-free mice were sacrificed, and leukocytes were recovered from spleens as described in Materials and Methods. The expression of various Vβ subsets was determined by flow cytometry using a commercially available screening kit. The Vβ repertoires in the spleens were different for $CD4^+$ and $CD8^+$ T cells. While some Vβ subsets were expressed at higher frequencies in both $CD4^+$ and $CD8^+$ T cells (Vβ 8.2 and Vβ 8.3) (Fig. 1A and B), other Vβ subsets were expressed differentially in the populations. Vβ 2, 11, 13,

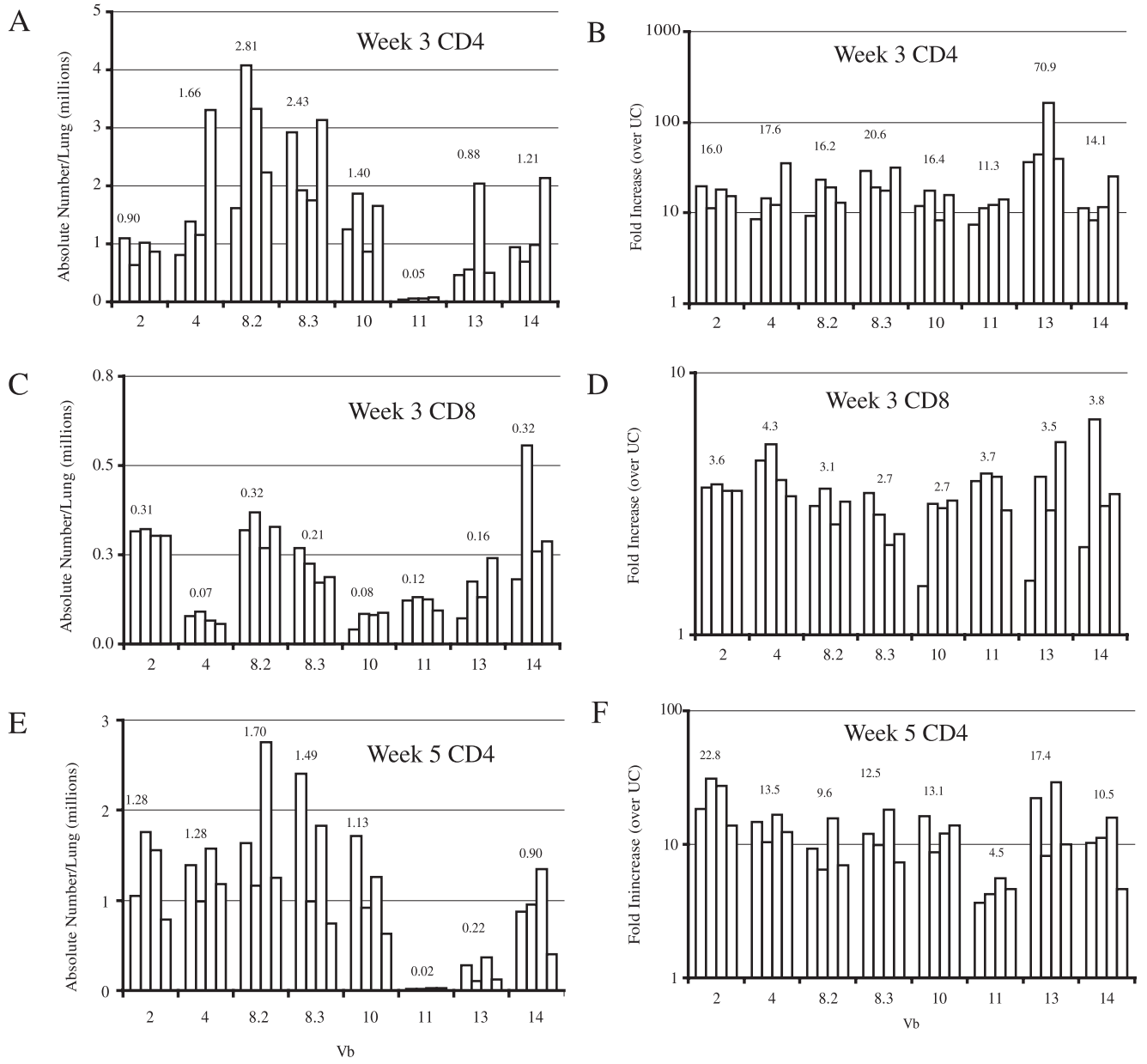

FIG. 2. Vβ TCR repertoire of lung T cells in CBA/J mice after *C. neoformans* infection. The absolute number (A, C, and E) of T cells expressing each Vβ subset and the increase compared to uninfected control lungs (B, D, and F) are shown. Mice were infected intratracheally with 10^4 CFU of *C. neoformans*. $CD4^+$ T cells were recovered from the lungs of infected mice at weeks 3 (A and B) and 5 (E and F) postinfection, and CD8 T cells were recovered from the lungs of infected mice at week 3 postinfection (C and D). Each bar represents a single animal, and the numbers indicate the mean for each group.

and 14 were expressed at higher frequency in $CD8^+$ T cells, and Vβ 4 and 10 were expressed at higher frequencies in $CD4^+$ T cells (Fig. 1A and B). Although Vβ 11-expressing T-cell subsets would be expected to be eliminated from CBA/J mice, based on their expression of mammary tumor virus 9, incomplete deletion of the Vβ 11^+ $CD8^+$ subset has been described previously (16). In the $CD4^+$ or $CD8^+$ compartment, however, the relative frequency of each Vβ subset was relatively conserved (Fig. 1A and B). Thus, the splenic TCR Vβ repertoire was conserved from one animal to another in both $CD4^+$ and $CD8^+$ T cells.

Our ultimate goal was to identify populations of T cells that were enriched during pulmonary *C. neoformans* infection. To determine whether the population of T cells in the lungs mirrored the population in the spleens of uninfected mice, we determined the frequency of each Vβ subset expressed by $CD4^+$ and $CD8^+$ T cells from the lungs of uninfected mice. In the $CD8^+$ T-cell compartment, there was slightly more variability in the TCR Vβ usage of $CD8^+$ T cells from the lungs than in the TCR Vβ usage of $CD8^+$ T cells from the spleens (Fig. 1A and C). Lung $CD4^+$ T cells had a pattern of Vβ usage similar to that of $CD4^+$ T cells from the spleen (Fig. 1B and

D). The TCR Vβ usage of $CD4^+$ T cells from the lungs was conserved, similar to the results found for lung $CD8^+$ T cells, but the frequencies of some $CD4^+$ TCR Vβ subsets were more variable in the lungs than in the spleen (Fig. 1B and D). The total numbers of $CD4^+$ and $CD8^+$ T cells in the lungs were consistent for five uninfected mice, and so the absolute number of each Vβ subset mirrored the frequencies (Fig. 1E and F). Thus, although the Vβ TCR repertoire in lung T cells was similar to that in the splenic compartment and was conserved, there was some heterogeneity in the Vβ TCR repertoire in lung T cells.

Lung TCR Vβ repertoire during pulmonary *C. neoformans* infection. Our next objective was to determine whether particular Vβ subsets were enriched in either the $CD4^+$ or $CD8^+$ T-cell populations during pulmonary *C. neoformans* infection. In response to pulmonary *C. neoformans* infection, resistant mice recruit both $CD4^+$ and $CD8^+$ T cells to the lungs (13). $CD4^+$ T cells are relatively enriched, particularly late in the response (13). To control for differences in the number of T cells in the lungs of infected mice, we converted data to absolute numbers of each Vβ subset per lung and determined the relative increase compared to uninfected controls for each subset. The absolute number and fold increase for $CD4^+$ and $CD8^+$ T cells expressing each of the Vβ subsets were determined for mice at weeks 1, 3, and 5 postinfection. At week 1 postinfection, no Vβ subsets were increased by more than onefold (data not shown), and there was no difference between subsets. At the peak of the T-cell response (week 3 postinfection), a modest increase in each of the $CD8^+$ T-cell Vβ subsets was observed, which represented 2.7- to 4.3-fold expansion compared to uninfected controls (Fig. 2D). Similarly, in $CD4^+$ T cells, expansion of all Vβ subsets occurred in response to infection (Fig. 2B). In contrast to the data for $CD8^+$ T cells, the $CD4^+$ Vβ subsets were all expanded more than 11-fold compared to the $CD4^+$ Vβ subsets for uninfected control lungs (Fig. 2B). The number of $V\beta13^+$ $CD4^+$ T cells was significantly expanded compared to the controls (>70-fold) (Fig. 2B); however, $V\beta13^+$ $CD4^+$ T cells comprised a minority of the total $CD4^+$ T-cell population in the lungs (6.3%) (Fig. 2A). This preferential expansion of $V\beta13^+$ $CD4^+$ T cells was not observed at week 5 postinfection (Fig. 2F), and $V\beta13^+$ T cells were an even smaller proportion of the total lung $CD4^+$ T cells (1.9%). Thus, during primary pulmonary *C. neoformans* infection, a diverse TCR Vβ repertoire was maintained by both $CD4^+$ and $CD8^+$ T cells infiltrating the lungs.

Lung $CD8^+$ TCR Vβ repertoire during pulmonary *C. neoformans* infection in $CD4^+$ T-cell-deficient mice. Pulmonary *C. neoformans* infection primarily affects individuals with compromised $CD4^+$ T-cell immunity (5). To determine the effect of $CD4^+$ T-cell deficiency on the resulting $CD8^+$ TCR Vβ repertoire, mice were made $CD4^+$ T cell deficient by systemic administration of anti-CD4 depleting monoclonal antibodies as described in Materials and Methods. The expansion of $CD8^+$ T cells belonging to the various TCR Vβ subsets was assayed as described above. At week 3 postinfection, higher numbers of $CD8^+$ T cells in each of the Vβ subsets were present in the lungs compared to the numbers in $CD4^+$ mice (Fig. 3A and 2D). The numbers represented a much greater expansion compared to uninfected controls (range, 6.9- to 42.0-fold), and Vβ 2, 4, 10, and 14 all expanded more than 25-fold (Fig. 3A). At week 5 postinfection, Vβ 2, 4, and 10 were expanded more than 35-fold (Fig. 3B). These numbers reflect total $CD8^+$ T-cell expansion, which was exaggerated in the absence of $CD4^+$ T cells. Thus, $CD4^+$ T-cell deficiency resulted in expansion of all $CD8^+$ Vβ subsets compared to $CD4^+$ mice, but the expansion was particularly pronounced for Vβ subsets 2, 4, and 10.

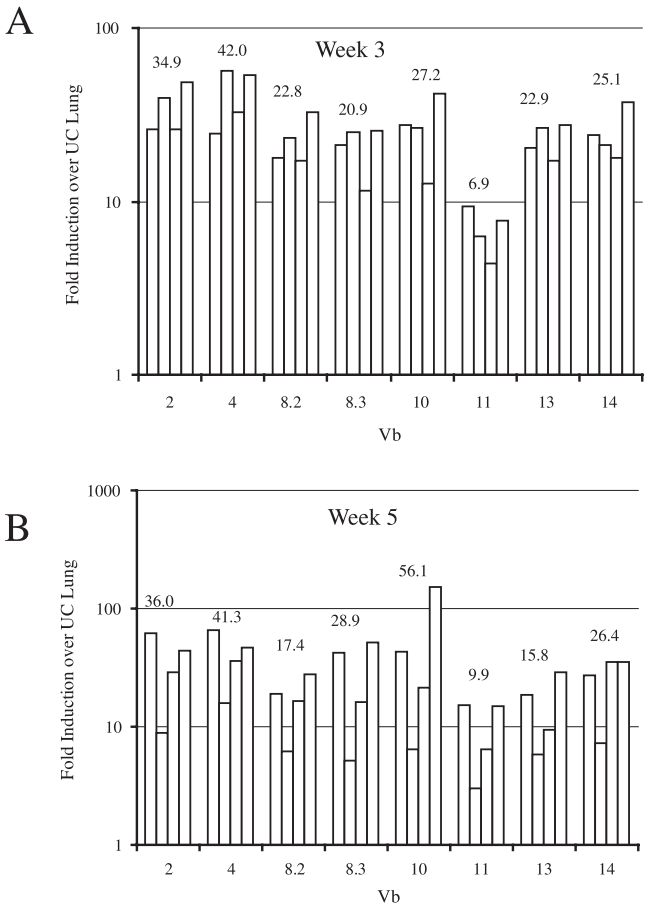

FIG. 3. Effect of $CD4^+$ T-cell deficiency on the $CD8^+$ Vβ repertoire during pulmonary *C. neoformans* infection. The increases in the numbers of lung CD8 T cells at weeks 3 (A) and 5 (B) postinfection relative to the numbers in $CD4^+$ T-cell-sufficient, uninfected control (UC) lungs are shown. CBA/J mice were made $CD4^+$ T cell deficient throughout the infection by using anti-CD4 depleting monoclonal antibodies, as described in Materials and Methods. The TCR Vβ repertoire of lung $CD8^+$ T cells in CBA/J mice was assayed as described above. Each bar represents a single animal, and the numbers indicate the mean for each group.

TCR Vβ repertoire during the secondary immune response to pulmonary *C. neoformans* infection. To determine whether mice maintained a diverse T-cell Vβ repertoire during secondary infection, mice were infected with 10^3 CFU of *C. neoformans* and then housed in specific-pathogen-free conditions for 16 weeks to allow resolution of this primary infection. The mice were then given a secondary challenge consisting of 10^4 CFU, and the Vβ repertoire was characterized 10 days after the secondary challenge. In the $CD8^+$ T-cell compartment, very little expansion of $CD8^+$ T cells was observed, and four of

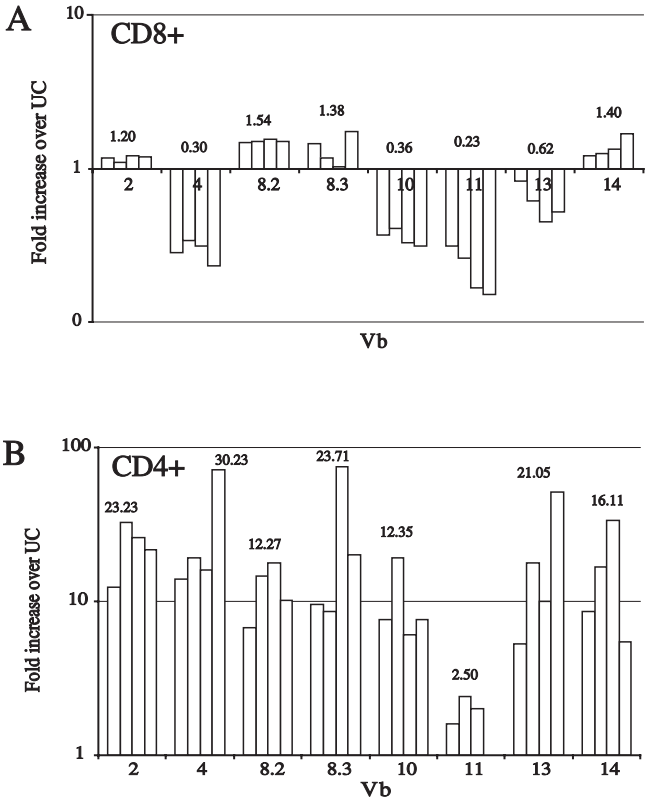

FIG. 4. Relative expansion of Vβ TCR subsets in lung $CD8^+$ (A) and $CD4^+$ (B) T cells during the secondary response to *C. neoformans*. Mice were infected with 10^3 CFU of *C. neoformans* and rested to allow resolution of the primary infection, as described in Materials and Methods. The mice were given a secondary infection consisting of 10^4 CFU *C. neoformans*, and the Vβ TCR usage of $CD4^+$ and $CD8^+$ T cells in the lungs was assayed by flow cytometry at day 10 after the secondary infection. Each bar represents a single animal, and the numbers indicate the mean for each group. UC, uninfected control.

eight Vβ subsets were contracted compared to uninfected controls (Fig. 4A). Whether increased numbers in the other four Vβ subsets represented skewing of the $CD8^+$ Vβ repertoire in the secondary response is unclear, because the total number of $CD8^+$ T cells at day 10 after the secondary challenge was similar to the number of cells in uninfected controls (data not shown). In the $CD4^+$ T-cell compartment, preferential expansion of some Vβ subsets was observed in individuals (Fig. 4B). For example, mouse 4 exhibited skewing of the $CD4^+$ repertoire to Vβ 4 and Vβ 13, whereas mouse 3 preferentially expanded Vβ 8.3 and Vβ 14 (Fig. 4B). However, preferential usage of Vβ subsets by $CD4^+$ T cells was not consistent between individuals. Thus, during the secondary T-cell response to pulmonary *C. neoformans* infection, individuals exhibited skewing of the $CD4^+$ T-cell repertoire, but consistent, preferential expansion of any Vβ subset was not observed across the group.

Proliferative responses of T cells from immunized mice in response to *C. neoformans* antigens. The results described above demonstrated that a diverse TCR Vβ repertoire was maintained in the T-cell response to pulmonary *C. neoformans* infection. Additionally, these results suggested that tracking any Vβ subset would likely be a poor surrogate for antigen specificity in the T-cell response to pulmonary *C. neoformans* infection. Therefore, we shifted the focus of our investigation to determining whether we could identify a method for more specifically assaying the T-cell responses to *C. neoformans*.

To do this, we studied the proliferative responses of $CD4^+$ and $CD8^+$ T cells from mice immunized with *C. neoformans*. CBA/J mice were infected with 10^3 CFU of *C. neoformans* and allowed to recover from the infection. Previous studies demonstrated that there were enhanced T-cell responses to secondary *C. neoformans* infection in such mice (D. Lindell, M. Ballinger, R. McDonald, G. Towes, and G. Huffnagle, submitted for publication). At 16 weeks postinfection, single-cell suspensions were prepared from pooled lymph nodes and spleens of immunized mice. To assay for *C. neoformans*-specific responses, we generated a variety of antigen preparations from *C. neoformans*, as described in Materials and Methods. *C. neoformans* can persist in the lungs of resistant mice long after a primary infection (Lindell et al., submitted). The levels of cells recovered from the secondary lymphoid organs of mice immune to *C. neoformans* mice were below the detectable levels of viable yeast cells (<50 CFU in the pooled sample for five mice).

The proliferation of $CD4^+$ and $CD8^+$ T cells from mice at week 16 postinfection was assessed using a flow cytometry-based proliferation assay, as described in Materials and Methods. This assay has an advantage over [^{3}H]thymidine incorporation assays because the proliferation of multiple cell populations can be determined independently in a single treatment well. Following 2 days in culture, there was minimal proliferation (as indicated by a decrease in CFSE staining) of either $CD4^+$ or $CD8^+$ T cells from immunized mice in the absence of stimulation (Fig. 5). In response to anti-CD3/anti-CD28 restimulation, a majority of $CD4^+$ and $CD8^+$ T cells proliferated (Fig. 5). A slight decrease in CFSE intensity was observed in both $CD4^+$ and $CD8^+$ T cells stimulated with *C. neoformans* lysate (Fig. 5). In contrast, after 4 days in culture, significant proportions of both $CD4^+$ and $CD8^+$ T cells from immunized mice had proliferated in response to *C. neoformans* lysate (Fig. 5).

Proliferative responses by both $CD4^+$ and $CD8^+$ T cells were also observed in response to a number of other *C. neoformans*-derived antigen preparations, including heat-killed organisms, culture filtrate antigen (CneF), and purified cryptococcal mannoprotein (Fig. 6A). These results demonstrated that a portion of $CD4^+$ and $CD8^+$ T cells in the secondary lymphoid tissues of immune mice proliferate in response to a number of *C. neoformans* antigen preparations.

Effector cytokine production by T cells from immunized mice in response to *C. neoformans* antigens. Our next objective was to determine whether the preparations of *C. neoformans* antigens (heat-killed organisms, lysate, CneF, or mannoprotein) could be used to assay effector cytokine production. To do this, lymphocyte suspensions from immune mice were cultured with uninfected adherent splenocytes in the presence of each stimulus, and the production of IFN-γ and TNF-α was assayed by intracellular flow cytometry, as described in Materials and Methods. In response to each of the antigen preparations, $CD4^+$ T cells produced both IFN-γ and TNF-α (Fig. 6B). In contrast, $CD8^+$ T cells produced only IFN-γ in response to *C. neoformans* antigens (Fig. 6C). These results dem-

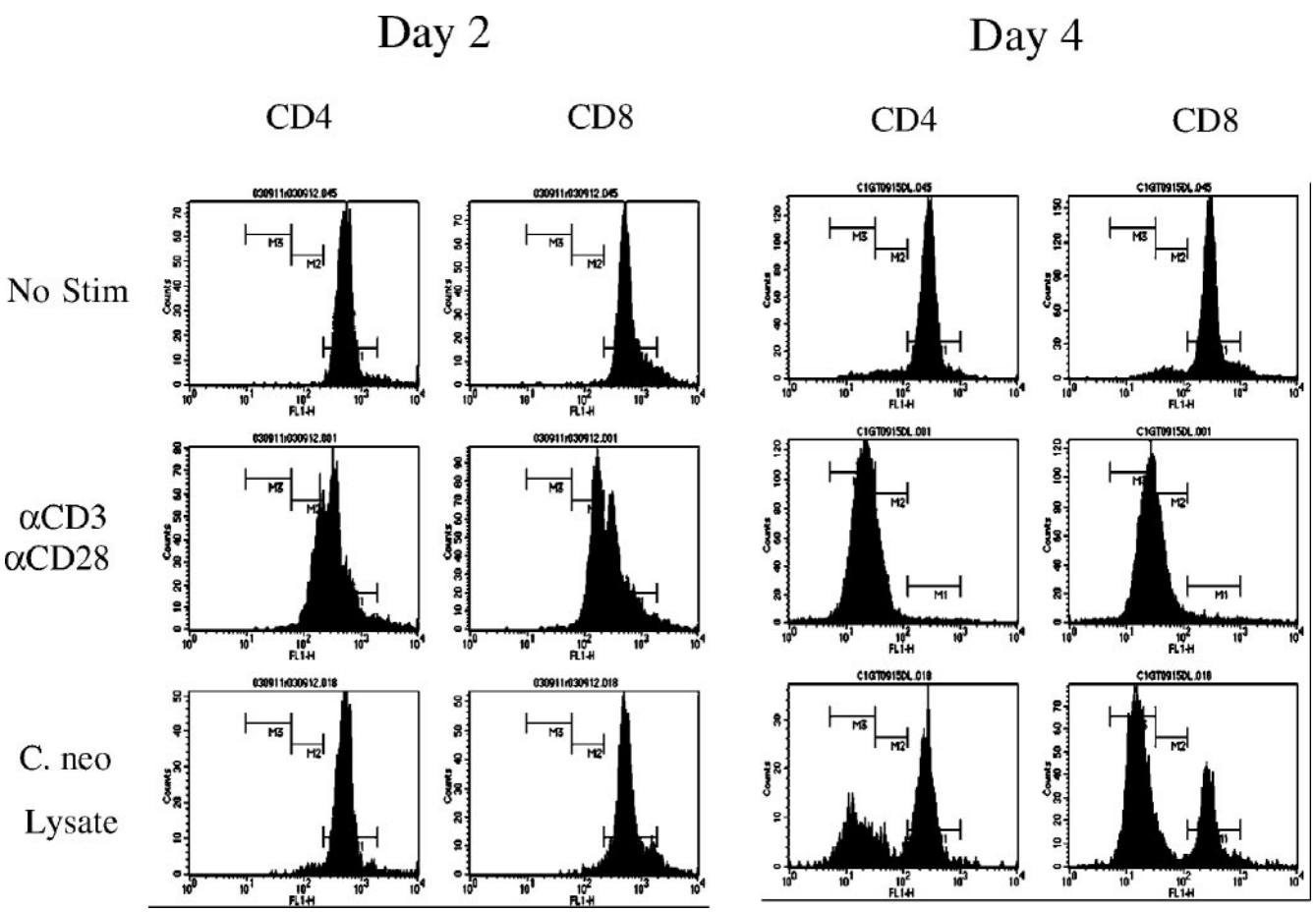

FIG. 5. Proliferation of T cells from secondary lymphoid tissues of immune mice. Cells from LALN and spleens of immunized mice were stained with CFSE and cultured with (i) no restimulation (No Stim), (ii) anti-CD3/anti-CD28 (αCD3 αCD28), or (iii) *C. neoformans* lysate (C. neo Lysate). The histograms show the results for $CD4^+$ or $CD8^+$ gated populations after 2 and 4 days in culture.

onstrate that T cells from the secondary lymphoid tissues of immune mice produced effector cytokines in response to nonviable *C. neoformans* antigen preparations.

Proliferation of $CD4^+$ and $CD8^+$ T cells from LALN of mice with pulmonary cryptococcal infections. Our next objective was to determine whether T cells from the LALN of mice during primary pulmonary infection proliferate in response to *C. neoformans* antigens. To do this, we assayed proliferation of LALN $CD4^+$ and $CD8^+$ T cells in response to *C. neoformans* lysate. The results obtained with immune mice suggested that maximal proliferation and effector cytokine production could be assayed using lysate, relative to the other antigen preparations (Fig. 6). Adherent lung cells from uninfected mice were cultured with LALN cells from mice 2 weeks after a primary infection, as described in Materials and Methods. Minimal proliferation was observed when LALN T cells were not stimulated; however, LALN T cells from infected mice proliferated in response to *C. neoformans* lysate (Fig. 7). We also investigated whether *C. neoformans* lysate induced proliferation from naive T cells. Although no such activity has been demonstrated for murine T cells, components of the *C. neoformans* cell wall have been shown to have mitogenic activity with human T cells (21, 25). After 4 days in culture, there was no difference in CFSE staining between unstimulated T cells from uninfected spleens and T cells stimulated with *C. neoformans* lysate (Fig. 7). T cells from uninfected spleens were capable of proliferation, however, and divided extensively in response to anti-CD3/anti-CD28 stimulation (Fig. 7). Both $CD4^+$ and $CD8^+$ T cells obtained from the LALN of mice at week 2 postinfection proliferated in response to *C. neoformans* lysate (Fig. 7). These results demonstrated that *C. neoformans* lysate did not induce mitogenic proliferation in naive murine T cells and that both $CD4^+$ and $CD8^+$ T cells recovered from the LALN proliferated in response to *C. neoformans* lysate. These data support the conclusion that a portion of the T cells in the LALN during pulmonary *C. neoformans* infection are antigen specific.

Effector cytokine production by $CD4^+$ and $CD8^+$ T cells from *C. neoformans*-infected mice. Our next objective was to determine the frequencies of $CD4^+$ and $CD8^+$ T cells producing the effector cytokines IFN-γ and TNF-α in the lungs and secondary lymphoid tissues of *C. neoformans*-infected mice. To do this, T cells from infected lungs, LALN, spleens, uninfected lungs, and uninfected spleens were enriched by MACS or FACS, as described in Materials and Methods. T cells were cultured with adherent cells from uninfected control lungs in the presence of no stimulus or *C. neoformans* lysate. The results were expressed as the increase in the number of

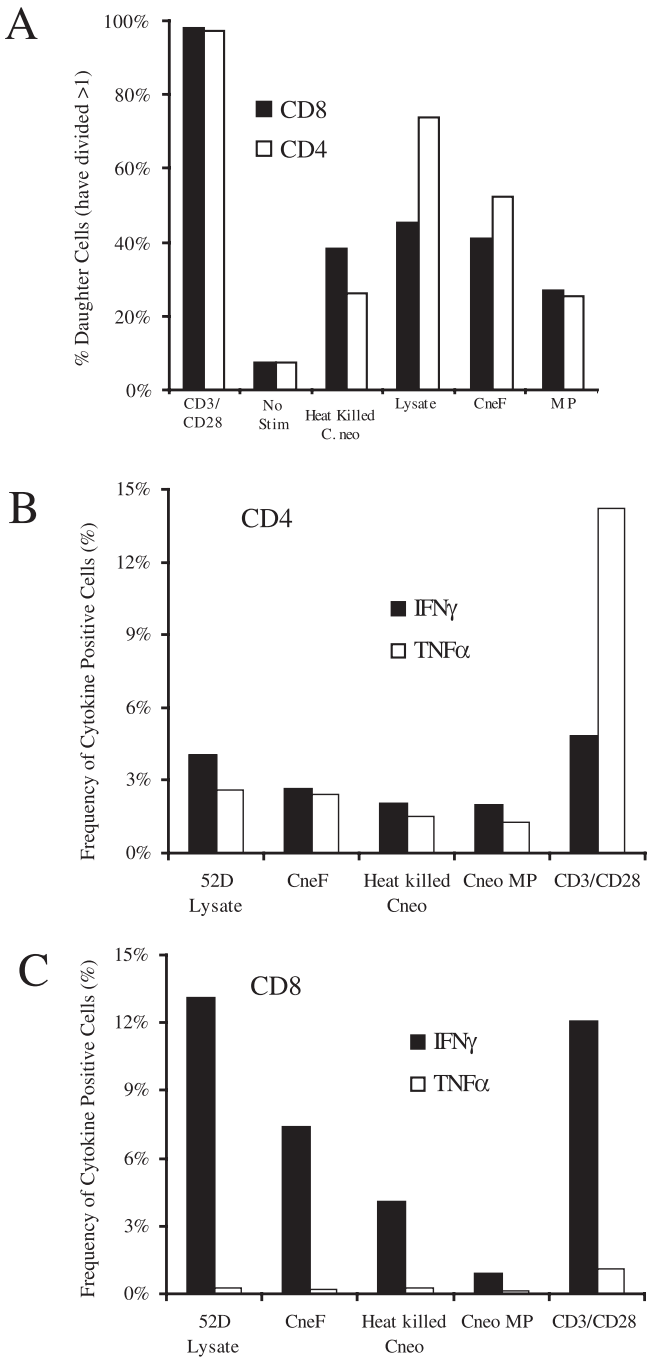

FIG. 6. Proliferation of (A) and effector cytokine production by (B and C) T cells from immunized mice in response to a variety of *C. neoformans* antigens. (A) Pooled LALN cells and splenocytes from immunized mice were cultured with the following stimuli, as described in Materials and Methods: anti-CD3/anti-CD28, no stimulus (No Stim), heat-killed *C. neoformans* (Cneo), *C. neoformans* lysate, *C. neoformans* culture filtrate antigen (CneF), and purified *C. neoformans* mannoprotein (MP). The data are the data from one of two experiments in which similar results were obtained. (B and C) Frequency of production of IFN-γ and TNF-α by $CD4^+$ (B) and $CD8^+$ (C) T cells from immunized mice in response to *C. neoformans* antigens. Pooled LALN cells and splenocytes from immunized mice were cultured with the various stimuli, as described in Materials and Methods. The frequency is the percentage of cytokine-positive T cells in the stimulated wells minus the percentage in unstimulated wells. The data are the data from one of two experiments in which similar results were obtained.

cytokine-positive cells compared with the number in unstimulated samples.

$CD4^+$ T cells from infected lungs, but not $CD4^+$ T cells from secondary lymphoid tissues, produced IFN-γ at a high frequency in response to CD3/CD28 restimulation (Fig. 8). $CD4^+$ T cells in infected lungs produced TNF-α in response to CD3/CD28 restimulation, but the frequency of TNF-α^+ $CD4^+$ T cells in uninfected tissues was equal to or higher than the frequency of TNF-α^+ $CD4^+$ T cells in infected lungs (Fig. 8). These results demonstrated that in response to CD3/CD28 restimulation, lung $CD4^+$ T cells from infected mice produced IFN-γ. $CD4^+$ T cells regulated the production of TNF-α less stringently than they regulated the production of IFN-γ, however, as CD3/CD28 stimulation resulted in production of TNF-α by $CD4^+$ T cells from uninfected mice. $CD8^+$ T cells from lungs and secondary lymphoid tissues produced IFN-γ in response to CD3/CD28 restimulation (Fig. 8). However, similar frequencies of $CD8^+$ T cells from uninfected tissues produced IFN-γ in response to CD3/CD28 (Fig. 8). Thus, $CD8^+$ T cells can produce TNF-α and IFN-γ in response to low-dose CD3/CD28 restimulation in the absence of infection.

In response to *C. neoformans* lysate, $CD4^+$ T cells from secondary lymphoid tissues did not produce IFN-γ or TNF-α. Markedly higher frequencies of IFN-γ and TNF-α production were observed with $CD4^+$ T cells from infected lungs (Fig. 8). Although anti-CD3/anti-CD28 restimulation resulted in significant IFN-γ production by $CD8^+$ T cells from the secondary lymphoid tissues (Fig. 8), $CD8^+$ T cells from secondary lymphoid tissues restimulated with lysate were poor effector cytokine producers (Fig. 8). Similar to the results obtained with $CD4^+$ T cells, only lung $CD8^+$ T cells from infected lungs produced IFN-γ and TNF-α in response to *C. neoformans* lysate (Fig. 8). These data show that *C. neoformans* lysate induces minimal effector cytokine production by T cells in uninfected mice. Furthermore, these results demonstrate that lung T cells from *C. neoformans*-infected mice produce effector cytokines in response to in vitro restimulation with *C. neoformans* lysate and that effector cytokine production occurs at a higher frequency than it occurs in T cells from secondary lymphoid tissues.

DISCUSSION

In this paper we report that in immunocompetent mice, a diverse TCR Vβ repertoire is maintained throughout the primary response to pulmonary *C. neoformans* infection. While Vβ 13 $CD4^+$ T cells were preferentially expanded at week 3 postinfection, they comprised a minority of the overall lung $CD4^+$ T-cell population. Furthermore, this preferential expansion did not persist until week 5 postinfection. $CD4^+$ T-cell deficiency resulted in relative expansion of all $CD8^+$ T-cell subsets. During the secondary immune response, preferential usage of $CD4^+$ T-cell Vβ subsets occurred in individuals, but not overall. Both $CD4^+$ and $CD8^+$ T cells from the secondary lymphoid tissues of immunized mice proliferated in response to a variety of *C. neoformans* antigens, including heat-killed whole *C. neoformans*, culture filtrate antigen (CneF), *C. neoformans* lysate, and purified cryptococcal mannoprotein. $CD4^+$ and $CD8^+$ T cells from the secondary lymphoid tissues of immune mice produced IFN-γ in response to these stimuli, and

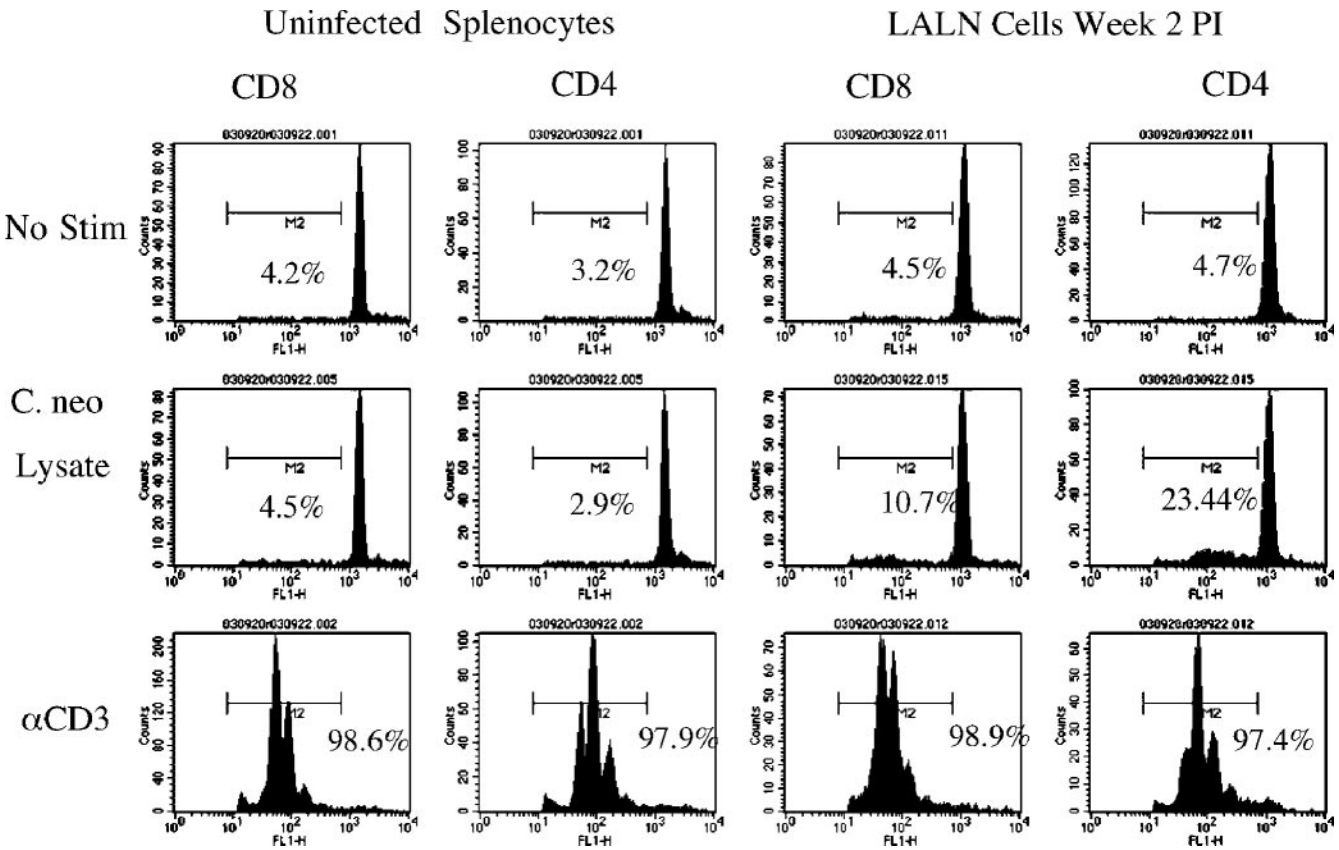

FIG. 7. Proliferative responses of T cells from the LALN of mice infected with *C. neoformans*. Splenocytes from uninfected mice were used as a control, because there were insufficient numbers of T cells from the LALN of uninfected specific-pathogen-free mice for analysis. T cells were enriched by MACS, stained with CSFE, and cultured with adherent splenocytes from uninfected controls for 4 days in the presence of (i) no additional stimulus (No Stim), (ii) *C. neoformans* lysate (C. neo Lysate), or (iii) anti-CD3 antibodies (αCD3). The histograms show data for viable cells gated on $CD4^+$ or $CD8^+$ cells, and the percentages indicate the percentages of daughter cells (cells which had undergone ≥1 cell division). The data are the data from one of two experiments in which similar results were obtained.

$CD4^+$ T cells also produced TNF-α. Both $CD4^+$ and $CD8^+$ T cells from the secondary lymphoid tissues of mice exhibiting a primary response to *C. neoformans* proliferated in response to *C. neoformans* lysate. In response to stimulation with *C. neoformans* lysate, lung $CD4^+$ and $CD8^+$ T cells produced the effector cytokines TNF-α and IFN-γ.

Our studies demonstrated that a diverse TCR Vβ repertoire is maintained throughout the primary response, but focusing of the TCR Vβ repertoire occurred in $CD4^+$ T-cell populations of individuals during the secondary immune response. Pulmonary infection of mice with *H. capsulatum* leads to significant skewing of the TCR Vβ repertoire, as does protective vaccination with *H. capsulatum* heat shock protein 60 (9, 10, 23). Depletion of the preferentially expanded population in either case adversely affects clearance (9, 10, 23). Evidence obtained with other experimental systems, however, suggests that Vβ preferences during primary infection are the exception rather than the rule. During primary *Listeria monocytogenes* infection, epitope-specific T cells have a diverse TCR Vβ repertoire (4). In a secondary response, however, focusing of the Vβ repertoire occurs in the responding antigen-specific T-cell populations (4). Similar to the results reported here, variability between genetically identical individuals was also observed (4). A T-cell response in which a common TCR Vβ repertoire is found in all individuals has been termed a "public" repertoire, whereas a "private" repertoire is specific to an individual (6). In a number of recent studies workers have sought to determine the relative roles of private and public repertoires, chiefly in models of T-cell responses to viruses (14, 26). The $CD8^+$ T-cell response to influenza in C57BL/6 mice is directed primarily against the immunodominant epitopes NP_{366} and PA_{224}. The response to NP_{366} is characteristically public, whereas the response to PA_{224} is largely private (14, 15, 26). Although similar numbers of NP_{366}- and PA_{224}-specific T cells are generated in the primary response, the secondary response is dominated by the public NP_{366} response, suggesting that public specificity may play a more prominent role in secondary T-cell responses (2). However, private specificity may play a more important role in regulating differential responses to heterologous infections in genetically identical mice (15). Although in our study we did not utilize techniques such as spectratyping, the differences in Vβ profiles between individual mice suggest that the $CD4^+$ T-cell Vβ repertoire during secondary *C. neoformans* infection could be characterized as largely private.

Our results indicated that during $CD4^+$ T-cell deficiency, *C. neoformans* infection led to enhanced expansion of all $CD8^+$ Vβ subsets compared to the expansion observed with $CD4^+$

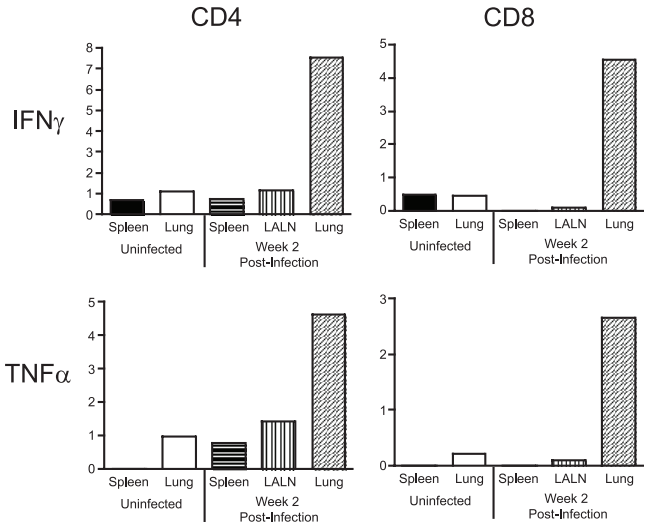

FIG. 8. Frequency of production of effector cytokines by T cells from the lungs and secondary lymphoid tissues. T cells from the lungs, LALN, and spleens of infected mice and from the lungs and spleens of uninfected mice were enriched by MACS or FACS and cocultured with adherent lung cells from uninfected mice, as described in Materials and Methods. Cultures received no additional stimulus or *C. neoformans* lysate. The bars indicate the percentage of cytokine-positive T cells in the stimulated wells minus the percentage of cytokine-positive cells in unstimulated wells. The data are the data from one of two experiments in which similar results were obtained.

T-cell sufficiency. Previous studies demonstrated that hyperexpansion of $CD8^+$ T cells occurs in response to *C. neoformans* during $CD4^+$ T-cell deficiency (18). The IFN-γ-producing $CD8^+$ T-cell effectors mediate a level of protection in a $CD4^+$ T-cell-deficient host (18). Together, these results demonstrate that while the absence of $CD4^+$ T cells increases the magnitude of the $CD8^+$ T-cell response to *C. neoformans* infection, the $CD8^+$ response to *C. neoformans* in a $CD4^+$ T-cell-deficient host is also characterized by diversity.

If *C. neoformans* has superantigen activity in vivo in mice, we would have expected specific Vβ subsets to be expanded in response to *C. neoformans* infection. Superantigens are microbially derived proteins which stimulate the proliferation of T cells in a non-antigen-specific manner via direct binding of the Vβ region of the T-cell receptor (17). Superantigens may benefit a pathogen by inducing a high frequency of nonspecific T cells, which can interfere with the antigen-specific response (12). *C. neoformans* produces a mitogen (CnM) for human T cells, whose activity is localized to proteins found in the cell wall and membrane and can stimulate naive T-cell proliferation (20, 21). The mitogenic effect is similar to that of staphyloccocal enterotoxin B, a known superantigen, and requires phagocytosis and protein processing (24). In our study, we observed no proliferation by $CD4^+$ or $CD8^+$ T cells in response to *C. neoformans* lysate, even after 5 days in culture. Thus, our results suggest that *C. neoformans* mitogen is not active in mice.

Our data demonstrated that while T cells from the lymph nodes or spleens of mice at 2 weeks postinfection produced little TNF-α and IFN-γ in response to stimulation with *C. neoformans* lysate, a significant number of T cells in the lungs produced each of these effector cytokines (Fig. 8). These results show that there is compartmentalization of T-cell effector function during pulmonary *C. neoformans* infection. T cells encounter antigen-presenting cells in the LALN bearing cognate antigen, where they respond by proliferating. Concomitant with trafficking to the lungs, both $CD4^+$ and $CD8^+$ T cells acquire an antigen-dependent effector cytokine-producing phenotype. Throughout the immune response to pulmonary *C. neoformans* infection, both $CD4^+$ and $CD8^+$ T cells maintain a diverse TCR Vβ repertoire, and the response is driven by multiple *C. neoformans* antigens rather than by one or two immunodominant antigens.

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