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Immunopathology and Infectious Diseases

Distinct Compartmentalization of CD4$^+$ T-Cell Effector Function Versus Proliferative Capacity during Pulmonary Cryptococcosis

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The activation and expansion of T cells and their acquisition of effector function are key steps in the development of the adaptive immune response. Most infections are predominantly outside of the lymphoid tissues, and it is unclear at what point developmentally and anatomically T cells acquire effector function *in vivo*. In these studies, we compared the activation and polarization of T cells during murine pulmonary *Cryptococcus neoformans* infection in the secondary lymphoid tissues and at the site of primary infection. Few CD4$^+$ and CD8$^+$ T cells expressed an activated phenotype (CD44^{hi}, CD25$^+$, CD69$^+$, CD62L^{lo}, CD45RB^{lo}) at the sites of clonal expansion (lymph nodes, spleen, and blood). In contrast, a high percentage of T cells expressed activation markers at the site of primary infection, the lungs. Additionally, the polarization of CD4$^+$ T cells to interferon-γ-producing effector cells occurred at the site of infection, the lungs. CD4$^+$ and CD8$^+$ T cells from secondary lymphoid organs responded to TCR restimulation by proliferating, whereas T cells from the lungs proliferated poorly. This report demonstrates for the first time that T-cell activation and effector function in secondary lymphoid tissues during fungal infection is characteristically different from that at the site of primary infection. *(Am J Pathol 2006, 168:847–855; DOI: 10.2353/ajpath.2006.050522)*

The activation and expansion of T cells represent key steps in the development of the adaptive immune response. Naïve T cells, and central memory T cells, continuously recirculate through secondary lymphoid tissues.[1] If naïve T cells encounter stimulated antigen-presenting cells bearing their cognate antigen in secondary lymphoid tissues, the naïve T cells ultimately become activated, proliferate, and acquire effector function. Recent evidence suggests that much of this proliferation and differentiation pathway of T cells may be established on initial encounter with antigen.[2] Effector T cells perform their functions directly through cytolytic function and indirectly through the production of cytokines and other mediators.

Most infections are predominantly outside of the lymphoid tissues, and it is unclear at what point developmentally and anatomically T cells acquire effector function *in vivo*. It would be beneficial to the host for T cells to undergo clonal expansion in the lymph nodes but subsequently to acquire effector function after trafficking to the site of infection. This process would allow effector activity to be focused at the site of infection. This stepwise, compartmentally distinct process would minimize T-cell-mediated damage to secondary lymphoid organs and uninfected tissues. Although this model makes teleological sense, experimental evidence for this model is lacking.

In these studies, we wanted to compare the activation, proliferative capacity, and polarization of T cells during pulmonary *Cryptococcus neoformans* infection in the secondary lymphoid tissues and at the site of primary infection. *C. neoformans* is an encapsulated fungal pathogen that survives both extracellularly and intracellularly.[3] In humans, cryptococcal pneumonia and disseminated cryptococcosis primarily affect immunocompromised hosts. Among these populations, CD4$^+$ T-cell deficiency results in markedly enhanced morbidity and mortality,

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highlighting the importance of $CD4^+$ T cells. Data from our laboratory and others using a murine model of pulmonary *C. neoformans* infection have reinforced the importance of $CD4^+$ and $CD8^+$ T cells in clearance of the pulmonary infection,[4–8] as well as prevention of central nervous system dissemination.[9] Both $CD4^+$ and $CD8^+$ T-cell responses are generated during pulmonary murine *C. neoformans* infection, no doubt because of the combined extra- and intracellular nature of the pathogen. In this study, we wanted to assess the function of $CD4^+$ and $CD8^+$ T cells at the primary site of infection (lungs) and in secondary lymphoid tissue [spleen and lung-associated lymph nodes (LALNs)[3]]. Our objective was to determine the sites *in vivo* where T cells proliferate, are activated (CD44, CD25, CD69, CD62L, and CD45RB expression), and acquire effector function (cytokine production) during a pulmonary infection by a pathogen (*C. neoformans*) that grows both intracellularly and extracellularly.

Materials and Methods

Mice

Female CBA/J mice (weight, 23 ± 5 g) were obtained from the Jackson Laboratories (Bar Harbor, ME). Mice were housed under pathogen-free conditions in enclosed filter-topped cages. Clean food and water were given *ad libitum*. The mice were handled and maintained using microisolator techniques, with daily veterinarian monitoring. Bedding from the mice was transferred weekly to cages of uninfected sentinel mice that were subsequently bled at weekly intervals and found to be negative for antibodies to mouse hepatitis virus, Sendai virus, and *Mycoplasma pulmonis.* All studies involving mice were approved by the University Committee on Use and Care of Animals at the University of Michigan.

Cryptococcus neoformans

Cryptococcus neoformans strain 52D was obtained from the American Type Culture Collection, Rockville, MD (ATCC no. 24067). For infection, yeasts were grown to stationary phase (48 to 72 hours) at 35°C in Sabouraud dextrose broth (1% neopeptone and 2% dextrose; Difco, Detroit, MI) on a shaker. The cultures were then washed in nonpyrogenic saline, counted on a hemocytometer and diluted to 3.3×10^5 CFU/ml in sterile nonpyrogenic saline. The precise number of organisms delivered was determined by a CFU count of inoculum plated on Sabaraud dextrose agar (Difco).

C. neoformans *Lysate*

C. neoformans lysate was prepared from stationary phase organisms as follows. One 30-ml culture of yeast was centrifuged at $1500 \times g$ for 20 minutes, washed three times with phosphate-buffered saline (PBS), and resuspended in a minimal volume of PBS. Next, 0.5-mm glass beads were added to cover the yeast (Biospec Products, Bartlesville, OK). Yeasts were lysed via 25 cycles each consisting of 30 seconds of vortexing followed by 30 seconds in an ice bath. The yeast lysate was diluted with 10 ml of PBS, separated from cellular debris by low-speed centrifugation ($750 \times g$), and the supernatant filtered through a 0.22-μm bottle top filter. Sterile lysate was frozen at −70°C until use.

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 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 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, Spleen, and Blood Leukocyte Isolation

Lungs from each mouse were excised, washed in PBS, minced, and digested enzymatically for 30 minutes in 15 ml/lung of digestion buffer [RPMI, 5% fetal calf serum, 1 mg/ml collagenase (Boehringer Mannheim Biochemical, Chicago, IL), and 30 μg/ml DNase (Sigma Chemical Co., St. Louis, MO)]. After erythrocyte lysis using NH_4Cl buffer, cells were washed, resuspended in complete media, and centrifuged for 30 minutes at $2000 \times g$ in presence of 20% Percoll (Sigma) to separate leukocytes from cell debris and epithelial cells. Total lung leukocyte numbers were assessed in the presence of trypan blue using a hemocytometer; viability was >85%. LALNs and spleens were excised and cells dispersed with the plunger of a 3-ml syringe. Erythrocytes were lysed using NH_4Cl buffer, and cells were resuspended in complete media (RPMI, 5% fetal calf serum, 2 mmol/L L-glutamine, 50 μmol/L 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate). Peripheral blood was obtained from mice by cardiac puncture and drawn into heparinized 1-ml syringes through a 21-gauge needle.

Flow Cytometry

For surface staining alone, leukocytes were washed and resuspended at a concentration of 10^7 cells/ml in FA buffer (Difco) + 0.1% NaN_3, Fc receptors were blocked by the addition of unlabeled anti-CD16/32 (Fc block; BD Pharmingen, San Diego, CA). After Fc receptor blocking, 10^6 cells were stained in a final volume of 120 μl in 12 × 75-mm² polystyrene tubes (Becton Dickinson, Franklin Lakes, NJ) for 20 minutes at 4°C. All stains [CD4 (RM4-4 and H129.19), CD8 (5H10-1), αβTCR (H57-597), CD25 (7D4), CD44 (IM7), CD69 (H1.2F3)] were obtained from BD Pharmingen and used per the manufacturer's instruc-

tions. Cells were washed twice with FA buffer, resuspended in 100 μl, and an equal volume of 4% formalin was added to fix the cells. A minimum of 20,000 events were acquired on a FACScaliber flow cytometer (BD Pharmingen) using Cell-Quest software (BD Pharmingen). For activation markers (ie, CD25, CD44, CD69), gates were set based on positive (splenocytes cultured with high-dose PMA/ionomycin, 50 ng/500 ng per ml) and negative (isotype) controls.

Intracellular Flow Cytometry

Leukocytes were cultured for 12 hours at 2×10^6 cells/ml in 12-well plates in the presence of 0.1 μg/ml of soluble anti-CD3 with or without 0.1 μg/ml anti-CD28. Brefeldin A or monensin (in the form of Golgi-stop or Golgi-block) were added for the last 4 hours of culture as per the manufacturer's instructions (BD Pharmingen). Nonadherent cells were harvested, washed twice with FA buffer, and stained for cell-surface molecules as described above.

In some experiments, T cells were enriched from the lungs via fluorescence-activated cell sorting (FACS) and from lymph nodes and spleens via magnetic-activated cell sorting (MACS). For FACS, lung leukocytes were stained using anti-CD4 (RM4-4) and anti-CD8 (5H10-1). FACS analysis was performed on a FACSVantage SE Cell Sorter (BD Immunocytometry systems, San Jose, CA). The purity of the sorted population was >99% as determined by postsort analysis. For MACS, cell suspensions from secondary lymphoid tissues were stained using a panel of biotinylated antibodies: anti-CD19 (1D3), anti-CD49b (DX5), anti-Gr-1 (RB6-8C5), anti-erythroid cells (TER-119) (all from BD Pharmingen), and anti-mouse F4/80 (CI:A3-1; Caltag Laboratories, Burlingame, CA). T cells were enriched via negative selection using anti-biotin microbeads on a SuperMACS separator (Miltenyi Biotec, Auburn, CA). Enriched T cells (10^6) were co-cultured with 10^6 adherent lung cells from uninfected mice and stimulated with either anti-CD3 and anti-CD28 or *C. neoformans* lysate.

For intracellular staining, cells were washed of excess surface stains, fixed, and permeabilized using Cytofix/Cytoperm (BD Pharmingen), and stained using anti-interferon (IFN)-γ (XMG1.2) and/or interleukin-4 (BVD4-1B11, 11D11) (BD Pharmingen) in permeabilization buffer (FA buffer + 0.1% saponin; Sigma) at 4°C for 30 minutes. Flow cytometry was performed as for surface staining above, except that >50,000 events per sample were collected. The specificity of IFN-γ staining by XMG1.2 was tested by comparing staining of experimental samples to a minimum of two of three negative controls: 1) isotype control, 2) excess unlabeled antibody, and/or 3) preincubation of antibody with recombinant cytokine.

Proliferation

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 μmol/L 5-(and 6-) carboxyfluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) in PBS and 5% fetal calf serum for 7 minutes at room temperature. Cells were washed several times to remove 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 on a FACScaliber flow cytometer (BD Pharmingen) using Cell-Quest software (BD Pharmingen).

Results

$CD4^+$ and $CD8^+$ T-Cell Expansion in Response to Pulmonary C. neoformans *Infection*

To determine the kinetics of the $CD4^+$ and $CD8^+$ T-cell responses to pulmonary *C. neoformans* infection, T cells from the lungs and LALNs of *C. neoformans*-infected mice were isolated and quantified at various time points during infection. After intratracheal challenge, *C. neoformans* grew rapidly in the lungs of mice during the first week of infection (Figure 1A). Clearance of the yeast began between weeks 1 and 2 of infection and correlated with a significant recruitment of lymphocytes to the lungs (Figure 1B). The recruitment/expansion of $CD4^+$ T cells in the lungs was more vigorous than that of $CD8^+$ T cells. During the first week of infection, few $CD4^+$ T cells were recruited to the lungs. Between weeks 1 and 2 after infection, the number of $CD4^+$ T cells in the lungs increased greater than 10-fold ($10.7 \pm 1.8 \times 10^6$), with similar numbers of $CD4^+$ T cells in the lungs at week 4 after infection. In contrast, $CD8^+$ T cells accumulated in the lungs less efficiently than $CD4^+$ T cells ($2.77 \pm 0.5 \times 10^6$), with only a fourfold increase in lung $CD8^+$ T-cell number between weeks 1 and 2 after infection (Figure 1B).

T-cell numbers from the LALNs of uninfected mice housed in specific pathogen-free conditions are typically $<10^5$ cells. (Figure 1C). However, by week 1 after infection, $2.2 \pm 0.3 \times 10^6$ $CD4^+$ T cells were present in the LALNs. The number of $CD4^+$ T cells in the LALNs continued to rise at week 2 to a peak of $4.6 \pm 0.2 \times 10^6$ cells. $CD8^+$ T-cell numbers in the LALNs were more modest in quantity but followed a similar kinetic trend: $1.5 \pm 0.2 \times 10^6$ $CD8^+$ T cells were present in the LALNs at 1 week after infection. LALN $CD8^+$ T-cell number continued to rise through week 2, to a peak of $2.8 \pm 0.2 \times 10^6$, with a similar number at week 4 after infection. Thus, during *C. neoformans* infection, both $CD4^+$ and $CD8^+$ T cells expand in the LALNs, but $CD4^+$ T cells are significantly elevated in the lungs.

Expression of Activation Markers in the Lungs and Secondary Lymphoid Organs of C. neoformans*-Infected Mice*

To determine the activation profiles of T cells in the spleens, LALNs, and lungs of *C. neoformans*-infected mice, we determined the expression of the activation markers CD44, CD25, CD69, CD62L, and CD45RB on T

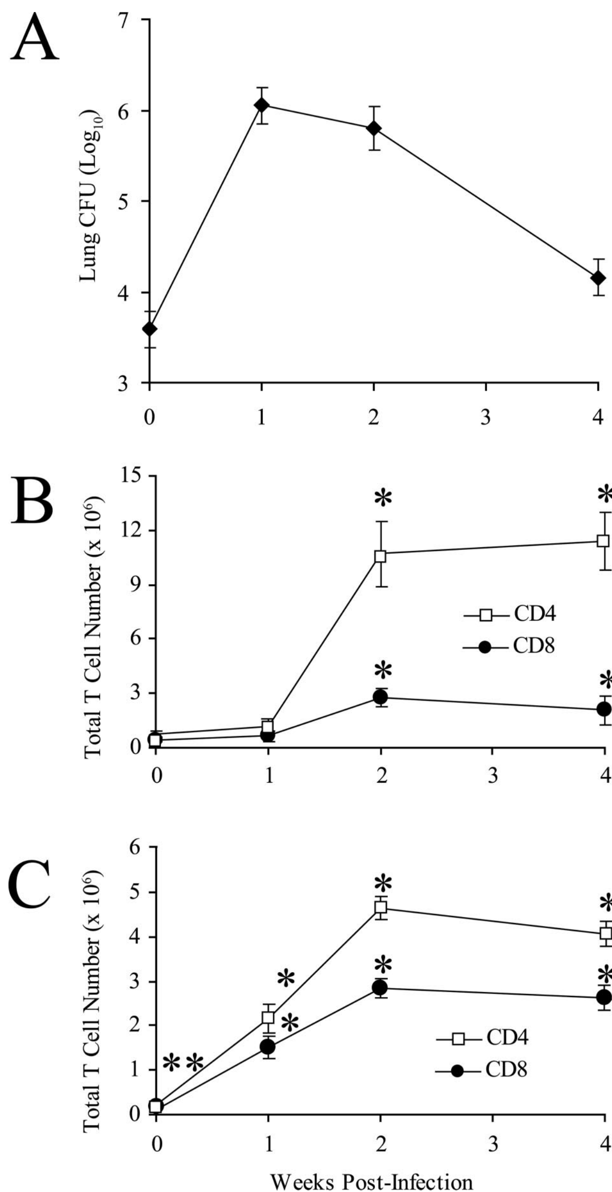

Figure 1. Kinetics of the host response to pulmonary *C. neoformans* infection at the time of infection and weeks 1, 2, and 4 after infection. Pulmonary fungal burden (**A**), lung T-cell (**B**), and LALN T-cell (**C**) numbers were assessed as described in the Materials and Methods. Each time point represents the mean ± SEM of six animals. Data are from two independent experiments. Note that the *y* axis in **A** is in $\log_{10}$ scale. **In **C**, T-cell numbers in the LALNs of uninfected, specific pathogen-free mice are less than 10^5 cells. $^*P < 0.05$, compared to $CD4^+$ or $CD8^+$ T-cell numbers at week 0 (uninfected controls).

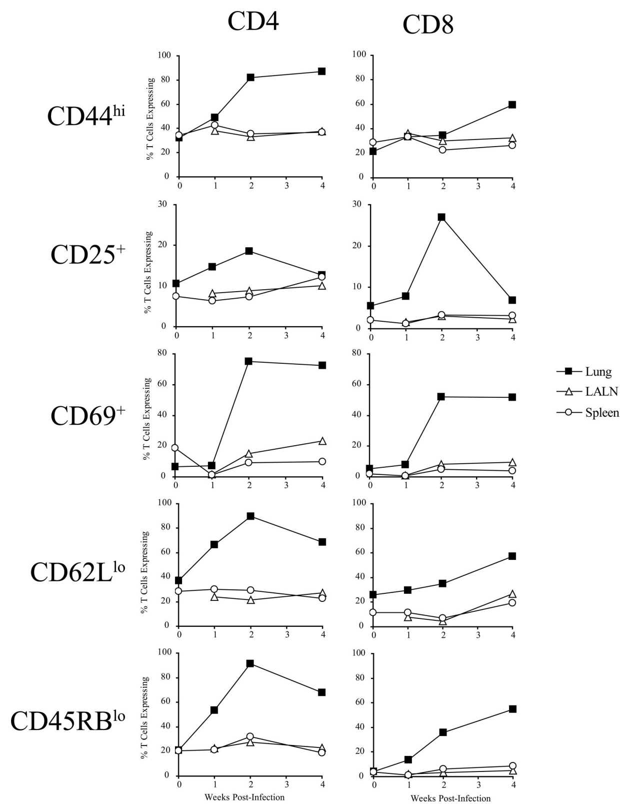

Figure 2. Activation profiles of T cells isolated from the spleens, LALNs, and lungs of uninfected mice and mice 1, 2, and 4 weeks after infection. The expressions of CD44, CD25, CD69, CD62L, and CD45RB were determined by flow cytometry on freshly isolated cells, as described in the Materials and Methods. Data points are the means of pooled samples from three mice per time point. Similar results were obtained in three independent experiments.

cells freshly isolated from the various organs. CD44 is a hyaluronic acid-binding glycoprotein that is up-regulated on activated T cells and persists at high levels on memory T cells.[10,11] CD25, the α-chain of the interleukin-2 receptor, and CD69 (very early activation antigen) a C-type lectin, are rapidly up-regulated on activated T cells.[12,13] Conversely, CD62L (L-selectin) and CD45RB are down-regulated on T-cell activation and differentiation.[14]

Our first objective was to examine activation marker expression by $CD4^+$ and $CD8^+$ T cells in the secondary lymphoid tissues. Compared to other time points, a modest decline in the percentage of $CD69^+$ $CD4^+$ T cells was observed in the spleen at week 1 after infection (Figure 2). The percentage of splenic $CD4^+$ T cells expressing each of the other activation markers assessed (CD44, CD25, CD62L, and CD45RB) was relatively constant throughout the infection (Figure 2). Similarly, the percentage of LALN $CD4^+$ T cells expressing activation markers was unchanged from weeks 1 to 4 after infection (Figure 2). Consistent with the results observed for $CD4^+$ T cells, with the exception of CD62L at week 4 after infection, the percentage of $CD8^+$ T cells expressing activation markers remained unchanged throughout the course of infection. However, the number of $CD4^+$ and $CD8^+$ T cells in the LALNs increased greater than 20-fold during the first week of infection and continued to increase through the second week (Figure 1C). Thus, the percentage of activated T cells in the LALNs remained constant throughout the course of infection, but there was a dramatic increase in the absolute number of activated T cells in the LALNs in response to pulmonary *C. neoformans* infection.

The percentage of $CD4^+$ T cells in the lungs expressing an activated phenotype ($CD44^{hi}$, $CD25^+$, $CD62L^{lo}$, $CD45RB^{lo}$) increased as early as week 1 after infection (Figure 2) and peaked at week 2 after infection (Figure 2). Between weeks 2 and 4 after infection, modest declines in the percentages of lung $CD4^+$ T cells expressing $CD25^+$, $CD69^+$, $CD62L^{lo}$, and $CD45RB^{lo}$ were observed (Figure 2). The percentages of $CD8^+$ T cells in the lungs

expressing each of the activation markers followed similar kinetics as those observed for CD4$^+$ T cells. The percentages of lung CD8$^+$ T cells expressing CD44^{hi}, CD25$^+$, CD69$^+$, CD62L^{lo}, and CD45RB^{lo} increased in the first week of infection, with dramatic up-regulation observed between weeks 1 and 2 (Figure 2). In contrast to CD4$^+$ T cells, however, the percentages of CD8$^+$ T cells expressing CD62L^{lo} and CD45RB^{lo} continued to rise between weeks 2 and 4 after infection (Figure 2). The decline in CD25 expression by both CD4$^+$ and CD8$^+$ T cells in the lungs at week 4 after infection correlates with decreased pulmonary fungal load, with 80-fold fewer yeasts present in the lungs at week 4 compared to week 2 (Figure 1A). The striking increase in the percentage of activated CD4$^+$ and CD8$^+$ T cells between weeks 1 and 2 after infection coincides with an influx of T cells into the lungs during this time period (approximately fivefold). Thus, activated T cells are significantly enriched in the lungs during the cell-mediated immune response to pulmonary *C. neoformans* infection.

Effector Function of T Cells from the Lungs and LALNs

To assess the effector function of T cells from the LALNs and lungs at various time points after infection, leukocytes were isolated from each organ, cultured for 16 hours in the presence of anti-CD3 and anti-CD28 antibodies (0.2 μg/ml each), and assayed for IFN-γ production by intracellular flow cytometry. After restimulation, LALN CD8$^+$ T cells produced IFN-γ to a high degree, with a maximum of ~30% IFN-γ^+ at week 4 after infection (Figure 3, A and B). In contrast, the percentage of IFN-γ-producing CD4$^+$ T cells from the LALNs was much lower (~2%). The lack of IFN-γ production by LALN CD4$^+$ T cells was consistent at all time points measured (Figure 3B). Consequently, T-cell-derived IFN-γ in the LALNs, as measured by the absolute number of IFN-γ-positive T cells (Figure 3B, bottom left), was almost exclusively from CD8$^+$ T cells.

In contrast to LALN CD4$^+$ T cells, a high percentage of lung CD4$^+$ T cells produced IFN-γ, with ~26% of lung CD4$^+$ T cells producing IFN-γ at week 4 after infection (Figure 3). At weeks 2 and 4 after infection, the percentage of IFN-γ^+ CD4$^+$ T cells was substantially higher than IFN-γ^+ CD8$^+$ T cells (Figure 3B, top right). Furthermore, because more CD4$^+$ T cells are present in the lungs at later time points, T-cell-derived IFN-γ in the lungs, as measured by the absolute number of IFN-γ-positive T cells (Figure 3B, bottom right), is almost exclusively from CD4$^+$ T cells. Thus, a high percentage of IFN-γ-producing effector CD4$^+$ T cells were present at the site of primary infection, but at a much lower number in secondary lymphoid tissues.

Our next objective was to determine whether this paradigm would hold true using a *C. neoformans*-derived stimulus in a more defined culture environment. To this end, T cells from infected lungs, LALNs, spleens, uninfected lungs, and uninfected spleens were enriched by MACS or FACS, as described in Materials and Methods.

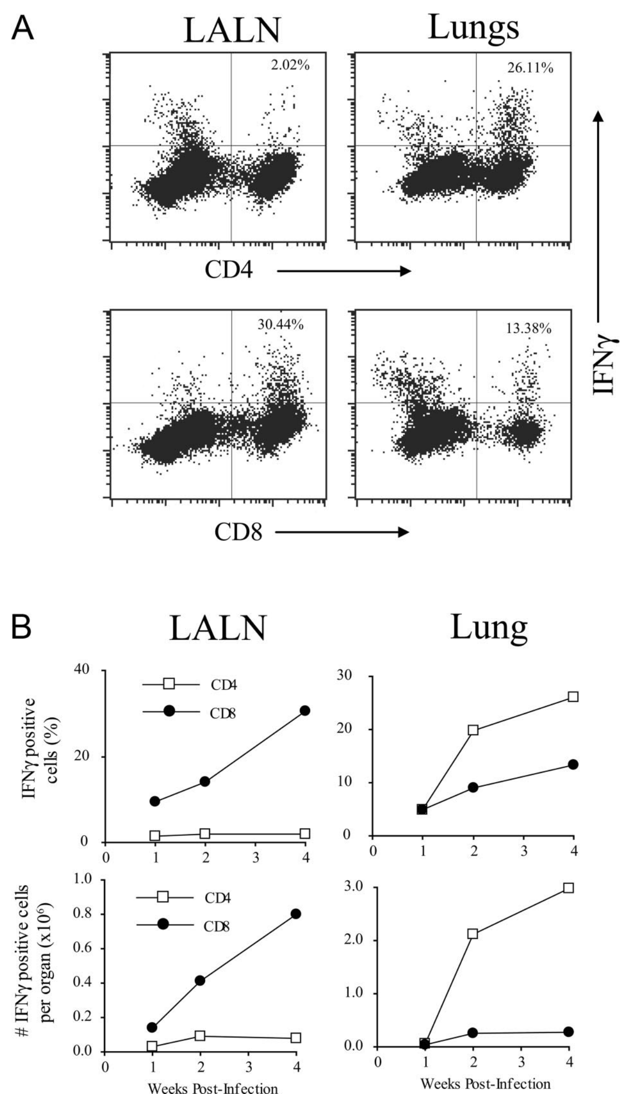

Figure 3. Production of IFN-γ in stimulated T cells from the lymph nodes and lungs of *C. neoformans*-infected mice. Leukocytes were cultured overnight in the presence or absence of anti-CD3 cross-linking antibodies, as described in the Materials and Methods, stained for the presence of IFN-γ and analyzed by intracellular flow cytometry. In **A**, representative dot plots from week 4 after infection are shown. In **B**, the percentage of IFN-γ-positive CD4$^+$ and CD8$^+$ T cells in lungs or lymph nodes after restimulation with anti-CD3 and anti-CD28 at weeks 1, 2, and 4 after infection. The percentage of IFN-γ-positive cells was multiplied by the absolute number of each T-cell population to determine the absolute number of IFN-γ-positive T cells per LALN and per lung. Data points represent pooled samples of three mice per time point. Similar results were obtained in three independent experiments.

T cells were cultured with adherent cells from uninfected control lungs in the presence of the following: no stimulus, *C. neoformans* lysate, or anti-CD3/anti-CD28 antibodies. Results are reported as an increase in cytokine-positive cells over unstimulated samples.

Similar to the results found using cultures of whole infected lungs, the percentage of IFN-γ-producing CD4$^+$ T cells from infected lungs was much higher (fourfold to fivefold) than for CD4$^+$ T cells from secondary lymphoid tissues after CD3/CD28 restimulation (Figure 4). In response to restimulation with *C. neoformans* lysate, the percentage of CD4$^+$ T cells from infected lungs that produced IFN-γ was much higher (approximately seven-

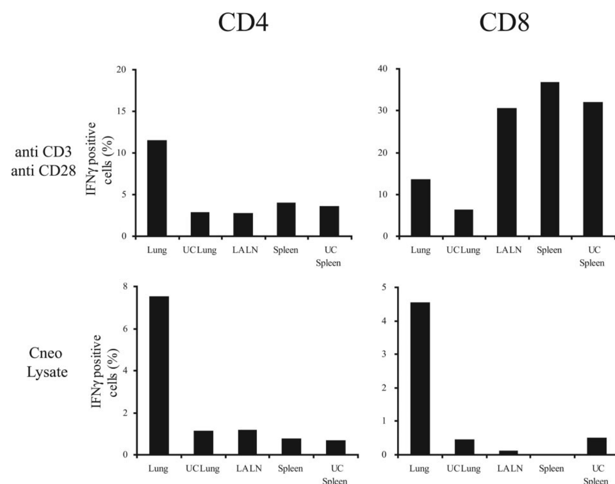

Figure 4. IFN-γ production by T cells from the lungs and secondary lymphoid tissues. T cells from the lungs, LALNs, and spleens of infected mice and the lungs and spleens of uninfected (UC) mice were enriched via MACS or FACS and co-cultured with adherent lung cells from uninfected mice, as described in Materials and Methods. Cultures received no additional stimulus, *C. neoformans* lysate, or anti-CD3/anti-CD28. Bars represent the percentage of IFN-γ^+ T cells in the stimulated wells minus IFN-γ^+ T cells in unstimulated wells. Data points represent pooled samples of three mice per time point and are from one of two experiments with similar results.

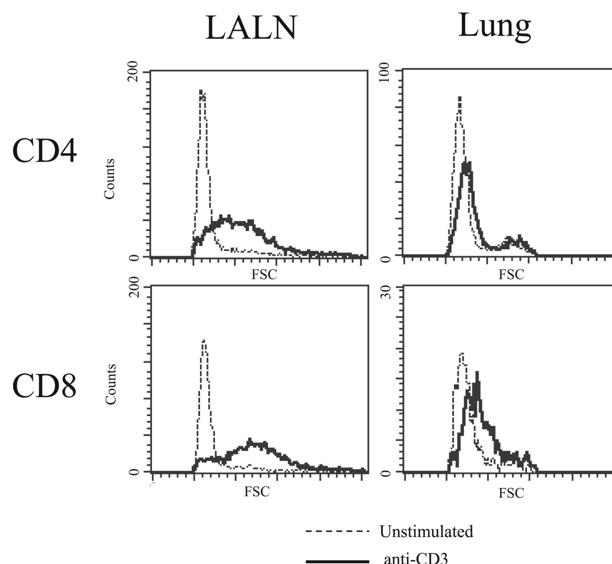

Figure 5. Effect of anti-CD3 stimulation on lymphoblast formation by T cells obtained from the LALNs and lungs of mice 2 weeks after *C. neoformans* infection. T cells were cultured overnight with or without anti-CD3 antibodies and forward scatter was assessed by flow cytometry. Data are representative of three independent experiments consisting of three mice per time point.

fold) than for CD4$^+$ T cells from the secondary lymphoid tissues of infected mice (Figure 4). IFN-γ production by CD4$^+$ T cells from the LALN and spleen was similar to CD4$^+$ T cells from uninfected control tissues (Figure 4). Thus, similar enrichment of IFN-γ-producing CD4$^+$ T-cell effectors was observed using antigen-independent stimulus (anti-CD3/anti-CD28), as well as *C. neoformans*-derived stimulus (cryptococcal lysate).

In response to anti-CD3/anti-CD28 restimulation, a smaller proportion of CD8$^+$ T cells from the lungs of infected mice produced IFN-γ than from secondary lymphoid tissues of these mice (Figure 4). An even higher percentage of CD8$^+$ T cells from the spleens of uninfected mice produced IFN-γ in response to anti-CD3/anti-CD28 than did CD8$^+$ T cells from infected lungs (Figure 4). In response to restimulation with *C. neoformans* lysate, however, CD8$^+$ T cells from secondary lymphoid tissues were poor IFN-γ producers (Figure 4). Similar to the results obtained with CD4$^+$ T cells, only lung CD8$^+$ T cells from infected lungs produced IFN-γ in response to *C. neoformans* lysate (Figure 4). These results demonstrate that compared T cells from secondary lymphoid tissues, a larger percentage of lung CD4$^+$ and CD8$^+$ T cells from *C. neoformans*-infected mice restimulated *in vitro* with *C. neoformans* lysate produced the effector cytokine IFN-γ.

Proliferative Responses of T Cells from the Lungs and LALNs

Leukocytes from each site were cultured overnight in the presence of anti-CD3 antibodies, to determine the ability of T cells from the lungs and LALNs to proliferate on TCR restimulation. Lymph node T cells were initially uniformly low in forward scatter (Figure 5). In response to stimulation via the TCR, lymph node T cells had a dramatic increase in forward scatter, reflecting a larger blast-like morphology indicative of proliferation or activation (Figure 5). In contrast to T cells from the LALN, freshly isolated lung T cells displayed a more heterogeneous forward scatter profile, which did not change on anti-CD3 stimulation. After CD3 restimulation, lung CD4$^+$ T cells did not dramatically increase in forward scatter. Lung CD8$^+$ T cells displayed a low-level increase in forward scatter on restimulation, but not to the extent reached by lymph node T cells (Figure 5). To determine whether the blast-like morphology observed after short-term restimulation correlated with proliferative capacity, the proliferation of T cells isolated from the lungs, LALNs, and spleens at week 2 after infection were assessed using an *in vitro* CFSE-based proliferation assay. In this assay, each cell division results in a decrease in CFSE staining intensity. In response to CD3 cross-linking, splenic and LALN CD4$^+$ and CD8$^+$ T cells divided extensively, whereas lung CD4$^+$ and CD8$^+$ T cells did not (Figure 6 and Table 1). Thus, T cells from secondary lymphoid organs responded to TCR restimulation by proliferating whereas T cells from the lungs (the primary site of infection) proliferated poorly.

Discussion

In this report, we present evidence that although CD4$^+$ T cells proliferated in secondary lymphoid tissues, they did not acquire effector function until reaching the site of fungal infection. As assessed by the expression of CD44, CD25, CD69, CD62L, and CD45RB, activated CD4$^+$ T cells were present in the LALN at low numbers beginning at week 1 after infection. At the site of primary infection (the lungs), a high percentage of activated CD4$^+$ T cells

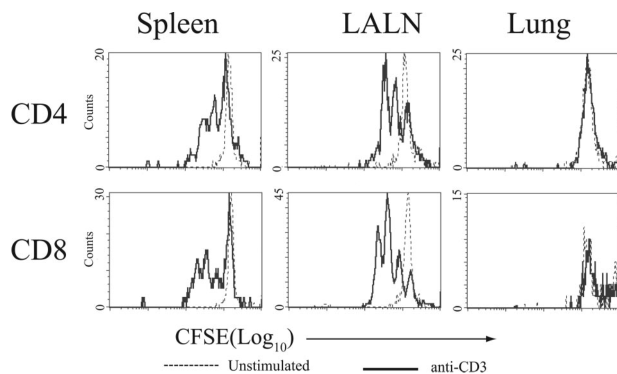

Figure 6. Effect of anti-CD3 stimulation on proliferation of T cells obtained from the spleen, LALNs, and lungs of mice after *C. neoformans* infection. Cells from each of the organs were labeled with CFSE and cultured for 3 days in the presence or absence of anti-CD3 antibodies. The intensity of CFSE staining on $CD4^+$ and $CD8^+$ T cells was assessed by flow cytometry. Data are representative of two independent experiments consisting of three mice per time point.

were observed by week 2 after infection. As assessed by the production of IFN-γ, effector $CD4^+$ T cells were highly enriched in the lungs. $CD4^+$ T cells from secondary lymphoid organs responded to TCR restimulation by proliferating, whereas those from the effector site proliferated poorly. Similar to $CD4^+$ T cells, $CD8^+$ T cells in the LALN proliferated extensively on TCR restimulation. In contrast to LALN $CD4^+$ T cells, LALN $CD8^+$ T cells could elaborate the effector cytokine IFN-γ in response to anti-CD3/anti-CD28 restimulation. In response to restimulation with *C. neoformans* lysate, however, neither $CD4^+$ nor $CD8^+$ T cells from the draining lymph nodes produced IFN-γ. Cumulatively, these results demonstrate that, during fungal infection, $CD4^+$ T cells in secondary lymphoid tissues are specialized for proliferation, but do not acquire effector function until they subsequently encounter antigen in infected tissues. $CD8^+$ T cells follow a similar progression except that $CD8^+$ T cells in the LALN can produce IFN-γ, and the elaboration of IFN-γ by $CD8^+$ T cells from the LALN is dependent on the nature of restimulation.

Our studies found that the percentage of $CD4^+$ T cells in the secondary lymphoid tissues (LALN and spleen) expressing an activated phenotype ($CD44^{hi,}$ $CD25^+$, $CD69^+$, $CD62L^{lo}$, $CD45RB^{lo}$) did not increase substantially at any point during infection (Figure 2). Similarly, the percentage of $CD8^+$ T cells from secondary lymphoid tissues expressing activation markers remained similar throughout the course of infection. Only slight increases in the percentages of $CD8^+CD62L^{lo}$ cells in the spleen and LALN were observed at week 4 after infection. In response to pulmonary infection with *C. neoformans*, the number of T cells in the LALN increased dramatically in size during the first week of infection (Figure 1C). Thus, although the percentage of activated T cells remained unchanged, the absolute number of activated T cells in the LALN increased dramatically during the first week of infection. In the lungs, a dramatic increase in the percentage of activated T cells was observed during the second week of infection (Figure 2). In addition to cognate antigen presentation, cytokine or other bystander activation may contribute to T-cell activation in the lungs. These results suggest that a steady-state percentage of T-cell activation is present in the LALNs. *C. neoformans*-specific T cells in the LALNs encounter APCs bearing cognate antigen and respond by proliferating. These expanded, activated T cells then rapidly traffic to the lungs where they accumulate to high numbers.

In response to anti-CD3/anti-CD28 restimulation, $CD8^+$ T cells from LALNs of *C. neoformans*-infected mice produced IFN-γ whereas $CD4^+$ T cells from LALNs did not, demonstrating that $CD4^+$ and $CD8^+$ T cells regulate IFN-γ differently during fungal infection. The polarization of naive $CD4^+$ T cells to IFN-γ-producing Th1 effectors has been extensively studied and results from a combination of factors including T-cell receptor stimulation, co-stimulation, and coordinated integration of intracellular signal involving T-bet, Stat-4, and others.[15] The differentiation of $CD8^+$ T cells to IFN-γ-producing effectors is less well understood and has a number of differences from the $CD4^+$ T-cell polarization paradigm. 1) $CD8^+$ T cells may be less dependent on co-stimulation because LCMV-specific $CD4^+$ T-cell responses are impaired in CD40L, CD28, and OX-40 knockout mice, but $CD8^+$ T

Table 1. Proliferation of $CD4^+$ and $CD8^+$ T Cells from the Spleens, Lung-Associated Lymph Nodes (LALNs), and Lungs of Mice Infected with *C. neoformans*

	CD4		CD8	
	≥1 Div (%)†	≥2 Div (%)	≥1 Div (%)	≥2 Div (%)
Week 1*				
Spleen	49.93	40.91	69.37	64.04
LALNs	83.30	36.60	91.23	82.19
Lungs	1.75	0.23	1.46	0.15
UC spleen‡	69.43	62.11	81.91	76.15
Week 2				
Spleen	49.43	28.65	55.61	43.41
LALNs	65.26	40.32	76.32	60.94
Lungs	3.97	2.41	1.10	0.74
UC spleen	83.57	65.61	79.96	68.59

*Cells were stained with CFSE, as described in Materials and Methods, and cultured in the presence of anti-CD3 antibodies.
†The percentage of $CD4^+$ or $CD8^+$ T cells from the various organs that had undergone ≥1 and ≥2 cell divisions was determined by a decrease in the intensity of CSFE staining by flow cytometry.
‡UC, uninfected control. Data points represent pooled samples of three mice per time point. Similar results were obtained in two independent experiments.

responses remained intact.[16] 2) Although impaired under more stringent conditions, IFN-γ-producing CD8$^+$ T cells can be generated in T-bet-null mice.[17] 3) CD8$^+$ T cells have both Stat 4-dependent and -independent (TCR-mediated) pathways of IFN-γ induction.[18] 4) The differentiation of CD8$^+$ T cells to functional effectors involves another T-box transcription factor not normally expressed in CD4$^+$ T cells, eomesodermin (Eomes), which effects both IFN-γ production and cytolytic function.[19]

The elaboration of IFN-γ by CD4$^+$ T cells has been linked to progression through the cell cycle, in part due to the remodeling of inaccessible chromatin.[20–22] Differences in the accessibility of the IFN-γ locus in CD4$^+$ and CD8$^+$ T cells may account for the differences in IFN-γ regulation observed in this study. Although these studies did not directly address the mechanism responsible for this observation, our results demonstrate that CD8$^+$ T cells have less stringent activation requirements for IFN-γ production than CD4$^+$ T cells during fungal infection.

Our results demonstrate that CD4$^+$ T cells from the LALNs of *C. neoformans*-infected mice proliferated but did not produce IFN-γ. CD4$^+$ T cells that proliferate but remain in a nonpolarized state have been termed Thpp, or proliferating precursors.[23] Thpp are generated in response to keyhole limpet hemocyanin immunization with or without adjuvant, suggesting that they arise during both Th1 and Th2 polarizing conditions.[24] Other studies have shown that the duration of TCR stimulation can affect T-cell polarization.[25] Antigen-primed T cells that receive only a short TCR stimulation *in vitro* remain in a nonpolarized state and home to lymph nodes on adoptive transfer.[26] In the current studies, we demonstrated that CD4$^+$ T cells isolated from secondary lymphoid tissues proliferated extensively on anti-CD3 restimulation but did not produce the effector cytokine IFN-γ. Thus, during *C. neoformans* infection, CD4$^+$ T cells from the LALNs are a proliferating but nonpolarized population.

Our results demonstrate that although CD4$^+$ T cells from the LALNs made little IFN-γ, lung CD4$^+$ T cells were potent IFN-γ producers. The difference in effector function observed between CD4$^+$ T cells from the LALNs and the lungs suggests that organ-specific signals from the local environment control the polarization of CD4$^+$ T cells. In other studies, using a CD8$^+$ T-cell adoptive transfer model for graft-versus-host disease, CD8$^+$ T cells with identical antigen specificities simultaneously gave rise to a type 1 response in the testis but a type 2 response in the spleen.[27] In a model of oral *Listeria monocytogenes* infection, the anti-LM CD8$^+$ T-cell response after oral infection was dependent on CD40/CD40L in the lamina propria but was primarily independent of CD40/CD40L in the spleen.[28] Tissue-specific requirements for CD4$^+$ T-cell responses were found as well.[29] Cumulatively, these results highlight the role organ-specific factors may play in the magnitude and character of responding T-cell populations.

It is tempting to speculate that CD8$^+$ T cells may be producing IFN-γ in response to intracellular infection of macrophages or dendritic cells that have trafficked to the LALNs. Viable *C. neoformans* can be cultured from the LALNs after pulmonary *C. neoformans* inoculation. However, IFN-γ production by LALN CD8$^+$ T cells in response to *C. neoformans* lysate was minimal (Figure 4), whereas CD8$^+$ T cells from naïve spleens produced IFN-γ in response to anti-CD3 and anti-CD28 (Figure 4). These results suggest that IFN-γ production by LALN CD8$^+$ T cells may not be antigen-specific. It should also be noted that clearance of the fungus began before the peak of IFN-γ-producing T cells in the lungs. Other studies in our laboratory have shown that, although pulmonary infection is controlled, low-level persistent *C. neoformans* infection (~100 CFU) is maintained in the lungs of CBA/J mice long after infection (>14 weeks). In these mice, most of the T cells present at week 4 after infection are gone, but a smaller population ($\sim 2 \times 10^6$) of CD4$^+$ T cells are maintained in the lungs, where they can produce IFN-γ at high frequency (D.M. Lindell et al, unpublished data). A number of published studies have sought to define the role of CD8$^+$ T cells during pulmonary *C. neoformans* infection in mice. Although macrophage activation via IFN-γ production has been a consistent theme of these studies, cytolytic function has not been observed but may be important much later in the infection.

Our findings suggest that the acquisition of CD4$^+$ T-cell effector function during fungal infection is anatomically compartmentalized; proliferation occurs in secondary lymphoid tissues, but polarization occurs at the site of infection. We acknowledge that many of the data presented here are also consistent with a model in which CD4$^+$ T cells acquire activation markers, gain the ability to produce IFN-γ, and lose proliferative capacity concomitantly with trafficking to the lungs. However, the majority of CD4$^+$ T cells recruited to the lungs did not express activation markers or produce IFN-γ. Thus, differentiation did not appear to be required for trafficking. Additionally, the percentage of T cells expressing activation markers in the peripheral blood was extremely low, and consistently lower than the percentage of activated T cells in the LALNs (data not shown). Evidence from other experimental systems supports this view: using *in vitro* activation and adoptive transfer of transgenic CD4$^+$ T cells, T cells that migrated to nonlymphoid tissues were CD62L$^{lo\text{-}med}$, whereas those recovered from lymph nodes were CD62L^{hi}.[30] This compartmentalization correlated with specialized function as well. Consistent with our findings, CD4$^+$ T cells from the lungs and airways produced effector cytokines on *in vivo* antigen challenge but did not proliferate, whereas T cells from the lymph nodes proliferated extensively on antigenic rechallenge. T cells that differ in their functional capacities have also been reported in another infection system: murine pulmonary influenza infection.[31] In this model, T cells from the draining lymph node proliferated well, provided B-cell help, but had poor cytolytic function whereas T cells from the effector site proliferated poorly, were poor at providing B-cell help, but produced IFN-γ. Thus, data from another infection system, as well as a more controlled, clonotypic T-cell-cognate antigen model, support the concept of compartmentalized acquisition of CD4$^+$ T-cell effector function.

A common theme from all of these studies[23,26,28–31] is evidence for a compartmentalized model of effector T-

cell development in which T cells receive initial stimulation in secondary lymphoid tissues. On receiving primary stimulation, these primed T cells proliferate and then migrate to peripheral sites. In peripheral tissues, primed T cells re-encounter antigen and give rise to effector cells. This report demonstrates for the first time that T-cell activation and effector function in secondary lymphoid tissues is characteristically different from that at the site of fungal infection. Whereas T cells from the secondary lymphoid tissues are specialized for proliferation, they are poor effectors. In contrast, T cells from the primary site of infection (nonlymphoid) produce effector cytokines much more readily, but at the expense of proliferative capacity. These findings support the possibility that pathological T cells in other diseases, including allergy and asthma, may not yet be committed to a polarized phenotype, and manipulation of signals from the local environment may lessen pathology.

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