

Differential T Cell Function and Fate in Lymph Node and Nonlymphoid Tissues

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Abstract

The functions and fate of antigen-experienced T cells isolated from lymph node or nonlymphoid tissues were analyzed in a system involving adoptive transfer of in vitro-activated T cells into mice. Activated T cells present in the lymph nodes could be stimulated by antigen to divide, produce effector cytokines, and migrate to peripheral tissues. By contrast, activated T cells that had migrated into nonlymphoid tissues (lung and airway) produced substantial effector cytokines upon antigen challenge, but were completely unable to divide or migrate back to the lymph nodes. Therefore, activated T cells can undergo clonal expansion in the lymph node, but are recruited and retained as nondividing cells in nonlymphoid tissues. These distinct regulatory events in lymph node and nonlymphoid tissues reveal simple key mechanisms for both inducing and limiting T cell immunity.

Key words: T cell • lung • cell migration • cell division • cytokines

Introduction

The development of an effector T cell immune response involves a defined sequence of events. The first event involves the delivery of Ag from an exposed tissue site to the draining lymph node. This initiates the activation of naive T cells that constantly recirculate from blood to lymph node via specialized high-walled endothelium (1–3), and then back to the blood via lymphatic drainage. The final event that occurs is the differentiation and expansion of effector and memory T cells. These effector T cells express new cell surface molecules that allow them to recirculate back to nonlymphoid tissue sites (3). Here effector T cells influence with other leukocytes such as eosinophils, mast cells, neutrophils, and macrophages, to cause inflammation and to clear Ag.

It is clear that some effector T cells must be retained in the lymphoid organs to contact B cells and orchestrate humoral responses. However, what has not been delineated is the response of effector T cells at nonlymphoid tissue sites when they reencounter Ag. Do they proliferate again, return to the draining lymph node to proliferate, or simply carry out effector functions such as cytokine production? In this study we attempt to determine whether effector T cells present in nonlymphoid organs display similar functions to

those effector T cells resident in lymph node, after contact with Ag. We also follow the fate of those cells that enter nonlymphoid tissues.

We generated populations of in vitro-activated T cells that expressed CD62L^{med-lo} and CD62L^{hi} phenotypes, which, after adoptive transfer, localize to nonlymphoid tissue and lymph nodes, respectively. When adoptively transferred mice were challenged with Ag, the activated T cells in both the nonlymphoid tissues and lymph node produced effector cytokines within 2 h. Activated T cells in the lymph node divided extensively after Ag challenge. By contrast, activated T cells in nonlymphoid tissues, such as the lung and airway, could not be induced to divide in vivo under any circumstance, despite being able to divide to the same antigenic stimulus in vitro. Those activated T cells resident in the airway remained fully competent with respect to effector cytokine production, but were apparently unable to return to the draining lymph nodes nor to the circulating pool of lymphocytes.

Materials and Methods

Mice. All mice were bred and maintained at the Animal Facility of the Wellington School of Medicine (Wellington, New Zealand). B10.A mice were originally obtained from The Jackson Laboratory and maintained by brother x sister mating. The –I line 5C.C7 transgenic mice (4, 5) were backcrossed to B10.A

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mice and maintained by breeding transgenic males to B10.A females. The TCR used to generate the 5C.C7 transgenic strain was derived from the cytochrome c-specific T cell clone 5C.C7. All animal experimental procedures used in this study were approved by the Wellington School of Medicine Animal Ethics Committee, and performed in accordance with the guidelines set by the University of Otago (Dunedin, New Zealand).

T Cell Culture, Carboxyl Fluorescein Succinimidyl Ester Labeling, and Adoptive Transfer. Naive T cells from 5C.C7 mice were purified from the lymph nodes by gradient centrifugation over 60 and 70% layered Percoll® (Amersham Pharmacia Biotech), followed by depletion of CD8⁺ T cells and B cells using Dynabeads® (Dyna). CD4⁺ T cells were incubated on anti-CD3 mAb (2C11, 10 µg/ml) coated six-well plates (Falcon; Becton Dickinson) in complete IMDM plus 20 U/ml recombinant human IL-2 (IL2L6 supernatant), 1,000 U/ml recombinant mouse IL-4 supernatant, 10 ng/ml recombinant human IL-6 and anti-CD28 mAb (1:50 dilution of 37.51 supernatant). Complete IMDM consisted of IMDM (Sigma-Aldrich) with 5% FCS (GIBCO BRL), 2 mM glutamine (Sigma-Aldrich), 1% penicillin-streptomycin (Sigma-Aldrich) and 5 × 10⁻⁵ M 2-ME (Sigma-Aldrich). Cells were cultured for 5–6 d with media. Floating cells were removed every 2–3 d and replaced with fresh cIMDM + cytokines. At the end of the anti-CD3 culture, plate-bound cells were removed, washed extensively, and then cultured for another 3–4 d in cIMDM + 20 U/ml IL-2. After this rest period, the percentage of Vα11⁺Vβ3⁺ T cells was determined by FACS® analysis. Cells were labeled with carboxyl fluorescein succinimidyl ester (CFSE)* by incubation with 5 µM CFSE (Molecular Probes) for 8 min at room temperature in PBS, followed by quenching of the unlabeled CFSE with excess FCS and extensive washing. Between 5 × 10⁶ and 2 × 10⁷ CFSE⁺Vα11⁺Vβ3⁺ T cells were injected into the tail vein of sex-matched B10.A mice in a total volume of 500 µl IMDM, as indicated for each experiment. Alternatively 10⁶ CFSE⁺Vα11⁺Vβ3⁺ T cells were administered intranasally to naive B10.A mice in a total volume of 30 µl IMDM.

Peptide and Cytokine Challenge. At the stated times after adoptive transfer, recipient mice were anesthetized by injection of a mixture of Ketamine and Xylazine (Phoenix), and then challenged intranasally with 100 µg moth cytochrome c (MCC)_{88–103} in 50 µl PBS, or with PBS alone. Alternatively, 100 µg MCC_{88–103} in 200 µl alum adjuvant (SERVA) was injected into the peritoneum (i.p. challenge). For intranasal administration of IL-2, mice were given an inoculation of 1,000 U recombinant human IL-2 in 50 µl cIMDM, or 50 µl cIMDM alone.

Bone Marrow Dendritic Cells (BMDC) Culture and Administration. Bone marrow cells from B10.A mice were cultured in six-well plates (Falcon) at 4 × 10⁵ cells/ml in cIMDM containing 20 ng/ml IL-4 and 20 ng/ml GM-CSF, as described previously (6). Cultures were provided with fresh cIMDM + cytokines every 2 d and incubated at 37°C for a total of 7 d. BMDC (10⁶ cells/ml) were loaded with MCC_{88–103} by incubation for 2 h at 37°C in cIMDM + 16 µg/ml MCC_{88–103}. Cells were washed three times with IMDM to remove excess Ag, and then 10⁶ BMDC ± MCC_{88–103} administered intranasally to anesthetized mice in a total volume of 30 µl IMDM.

Lymphocyte Isolation. Cell suspensions were made from lymph node or spleen, and RBC lysed by incubation for 5 min at

37°C in 3 ml ammonium-chloride-Tris buffer. Mice used for the preparation of lung lymphocytes were killed and the lungs perfused via the right ventricle of the heart with ~5 ml PBS to remove circulating blood. Minced lung tissue was incubated for 1 h in cIMDM containing 2.4 µg/ml collagenase type II (GIBCO BRL) and 0.1% DNase 1 (Sigma-Aldrich), and mononuclear cells purified by gradient centrifugation over 30% Percoll®. When lung and airway cells were used for in vitro culture, macrophages were depleted by culturing cells on plastic dishes in cIMDM for 1–2 h at 37°C. Alternatively, lung or liver tissue was minced for 3 min in a Medimachine (Becton Dickinson) in IMDM containing 0.1% DNase 1, and mononuclear cells purified by gradient centrifugation over 30% Percoll®. Lymphocytes were isolated from peripheral blood by collecting 100 µl of tail blood into 1 ml Alsever's solution, followed by a 5–10-min incubation in 3 ml ammonium-chloride-Tris buffer to lyse RBC. Mononuclear cells were purified from airway by broncho-alveolar lavage (BAL). BAL was performed by flushing 3 × 1 ml PBS a total of five times each into the airway. Analysis of cellular infiltration into the airway was performed by BAL and differential cell counting as described previously (7). Intraperitoneal wash mononuclear cells were isolated by flushing 8 ml PBS three times into the peritoneal cavity.

Flow Cytometric Analysis. FACS® analysis of lymphocytes was performed by staining as described (7). Anti-CD4 (GK1.5) was purified from hybridoma culture supernatant, and conjugated to biotin. Anti-Vα11-PE or Biotin, anti-Vβ3-Biotin, anti-CD44-PE, anti-3G-11-Biotin, anti-CD62L-PE, and Streptavidin-PerCP or -APC were all obtained from PharMingen.

Intracellular Cytokine Detection. Lymphocytes were cultured for 2 h on uncoated, or anti-CD3 mAb (2C11, 10 µg/ml) coated, 24-well plates (Falcon) in cIMDM and anti-CD28 mAb (1/50 37.51 supernatant) at a concentration of 10⁶ cells/well. Monensin (Calbiochem-Novabiochem) was added to the cultures at a final concentration of 2 µM and the incubation continued for 4 h. Alternatively, lymphocytes were collected directly into cIMDM containing 2 µM monensin, and then incubated at 37°C for 20 min without stimulation. At the end of the culture, cells were harvested, washed once in PBS buffer, and fixed in 4% paraformaldehyde (Sigma-Aldrich) for 15 min at room temperature. Fixed cells were washed and resuspended in FACS® buffer containing anti-Fc receptor mAb (24.G2). Indicated cells were then surface stained with Vα11-Biotin mAb followed by SA-PerCP (PharMingen). After washing to remove unbound antibody, cells were subjected to intracellular cytokine staining in saponin buffer (PBS, 10 mM HEPES, 0.1% saponin, 0.1% BSA, 0.02% Na azide) for 30 min at room temperature. Anti-IL-4-APC, -IL-5-PE, -IFN-γ-APC, and -IL-2-PE were all obtained from PharMingen and used at a 1:100 dilution.

Results

Lymph Node and Nonlymphoid Tissues Select Distinct T Cell Populations after Adoptive Transfer of In Vitro Activated Vα11⁺Vβ3⁺ T Cells. Activated T cells were prepared for adoptive transfer to B10.A recipients according to the following protocol. Naive CD4⁺ T cells from 5C.C7 mice were purified by gradient centrifugation over Percoll® and negative depletion using Dynabeads®. Between 50 to 70% of CD4⁺ T cells from 5C.C7 mice expressed both transgenic Vα11 and Vβ3 TCR chains specific for either pigeon cytochrome c fragment 81–104 and I-E^k, or MCC (8,

*Abbreviations used in this paper: BAL, broncho-alveolar lavage; BMDC, bone marrow dendritic cells; CFSE, carboxyl fluorescein succinimidyl ester; DC, dendritic cells; MCC_{88–103}, moth cytochrome c fragment 88–103; med LN, mediastinal lymph node.

9). The remaining CD4⁺ T cells expressed the transgenic V β 3 TCR chain along with an endogenously rearranged TCR α chain and did not respond to the MCC peptide (unpublished data).

The naive 5C.C7 CD4⁺ T cells were activated with plate-bound anti-CD3 and anti-CD28 mAb in the presence of IL-2, IL-6, and IL-4. Cells were cultured for 5–6 d, with floating cells removed every 2 d and replaced with fresh media plus additives. Cells were cultured for an additional 2–4 d in IL-2 alone. At the end of this period the large majority of harvested T cells were viable and expressed elevated levels of CD44 (Pgp-1), and medium to low levels of CD62L (L-selectin, Mel-14) (Fig. 1 A). These cells completely lacked expression of 3G-11 (Fig. 1 A), a marker that is lost after the activation of naive cells (10). If restimulated with anti-CD3, these T cells were found to be predominantly IL-4 producers (68%), whereas a smaller number produced IL-2 (33%), IL-5 (18%), and IFN- γ (19%) (unpublished data).

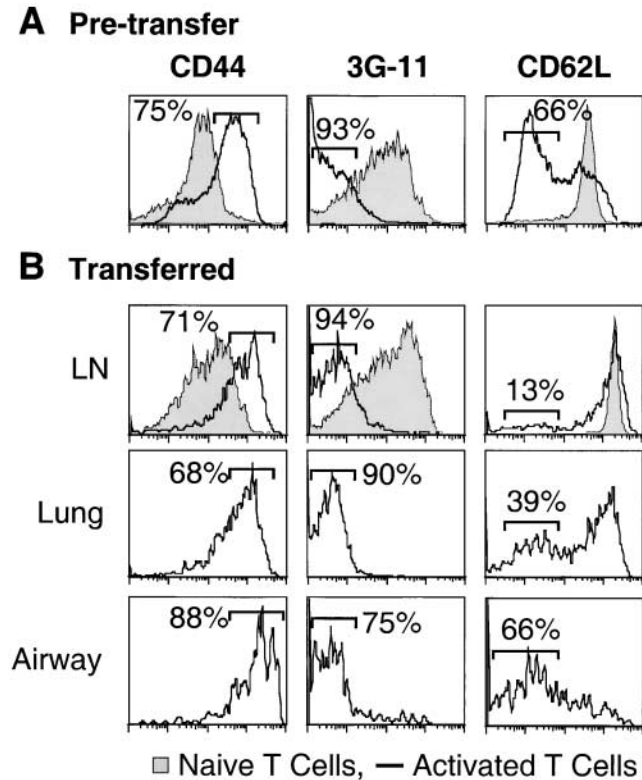


Figure 1. Activation marker expression by pre- and postadoptively transferred V α 11⁺V β 3⁺ T cells. (A) Activated T cells were generated in vitro, and then examined for expression of CD44, 3G-11 and CD62L. Profiles shown are for live V α 11⁺V β 3⁺ T cells. Activation marker expression on naive peripheral blood V α 11⁺V β 3⁺ T cells from 5C.C7 mice is shown as a control. (B) In vitro-activated T cells were CFSE labeled, and 2×10^7 CFSE⁺V α 11⁺V β 3⁺ T cells injected into the tail vein of naive syngenic B10.A hosts. 2–5 d later, lymphocytes were purified from the peripheral lymph nodes, lung, and airway, and their expression of CD44, 3G-11, and CD62L examined. Profiles shown are for live CFSE⁺V α 11⁺V β 3⁺ T cells. Numbers reflect the percentage of cells that fall within the shown boundaries. The data shown are from one representative experiment using six to eight mice per group. Similar results were obtained from three separate experiments.

Before adoptive transfer, the activated T cells were labeled with CFSE (11) and injected intravenously into naive syngenic B10.A hosts. 5 d later, cell suspensions were prepared from lymph nodes, lung, and airway tissue of adoptive hosts. Airway cells were recovered by performing a broncho-alveolar lavage. Lung cells included all cells remaining in the airways after lavage, as well as cells resident in the lung tissue itself. Reisolated CFSE⁺V α 11⁺V β 3⁺ T cells from each tissue were identified by fluorescent staining and examined for the expression of CD44, CD62L, and 3G-11 (Fig. 1 B). CFSE⁺V α 11⁺V β 3⁺ T cells from the lymph nodes, lung, and airway of adoptive hosts all displayed an activated phenotype (CD44^{hi}, 3G-11^{lo}). Cells recovered from the lung contained two populations that were either CD62L^{med} or CD62L^{lo}, whereas the majority of cells recovered from the airway were CD62L^{lo}. In contrast, CFSE⁺V α 11⁺V β 3⁺ T cells recovered from the lymph nodes were almost all CD62L^{hi}.

Thus, adoptively transferred activated T cells appear to separate into distinct subpopulations that migrate to either lymph nodes or nonlymphoid tissues according to their expression of high or low levels of CD62L, respectively.

In Vivo Ag Challenge Induces Clonal Expansion of Adoptively Transferred V α 11⁺V β 3⁺ T Cells Migrating to the Lymph Node, but Not Those Migrating to Nonlymphoid Tissue. We compared the ability of previously activated V α 11⁺V β 3⁺ T cells to divide after contact with Ag in a lymph node, versus contact in a nonlymphoid tissue environment. Mice were injected intravenously with in vitro-activated CFSE⁺V α 11⁺V β 3⁺ T cells and then challenged with 100 μ g MCC_{88–103} via the intranasal route. Intranasal challenge is a reliable means of delivering either soluble Ag, infectious agents, or cells to the lower airways, and results in a specific T cell response in the airways, lung, and draining mediastinal lymph node (med LN) (12, 13). Cell division of the adoptively transferred CFSE⁺V α 11⁺V β 3⁺ T cells at various host tissue sites was followed by FACS[®] analysis. Although in vitro-activated T cells do not label with CFSE in a sufficiently narrow peak to enable accurate quantitation of cell division, we were able to demonstrate that V α 11⁺V β 3⁺ T cells in the draining med LN divided vigorously in response to MCC_{88–103} intranasal challenge (Fig. 2 A). Strikingly, V α 11⁺V β 3⁺ T cells isolated from the airway had not divided, even by 90 h after intranasal Ag challenge (Fig. 2 A). Similarly, no division of V α 11⁺V β 3⁺ T cells from lung tissue was detected (unpublished data). It is important to note that a small proportion (~0.8%) of T cells in naive B10.A hosts express the V α 11 and V β 3 TCR chains. These cells are not specific for MCC and can be distinguished from the adoptively transferred cells because they are CFSE negative in the FACS[®] plots shown (Fig. 2 A).

The Ag-induced accumulation of V α 11⁺V β 3⁺ T cells in the draining med LN and airway was also determined at various time points after intranasal challenge. A marked accumulation of V α 11⁺V β 3⁺ T cells was observed in both the med LN and airway of MCC_{88–103} intranasally challenged, but not PBS challenged, mice (Fig. 2 B). These

data indicate that the MCC₈₈₋₁₀₃-induced V α 11⁺V β 3⁺ T cell accumulation in the airway occurs solely as a result of cellular recruitment from the blood and lymph nodes, since T cells resident in the airway did not divide (Fig. 2 A). Analysis of the inflammatory exudate induced by intranasal MCC₈₈₋₁₀₃ challenge also revealed that eosinophils had been recruited to the airway (Fig. 2 C), an event dependent on local IL-5 production by CD4⁺ T cells (7, 14, 15). This result indicated that airway V α 11⁺V β 3⁺ T cells had recognized Ag and were producing IL-5. No airway eosinophilia or V α 11⁺V β 3⁺ T cell accumulation was observed in B10.A mice that had been intranasally challenged with MCC₈₈₋₁₀₃, and had not received in vitro-activated V α 11⁺V β 3⁺ T cells (unpublished data).

To further compare the function of T cells encountering Ag in either the lymph nodes or lung and airway tissue, we analyzed in vivo cytokine production by adop-

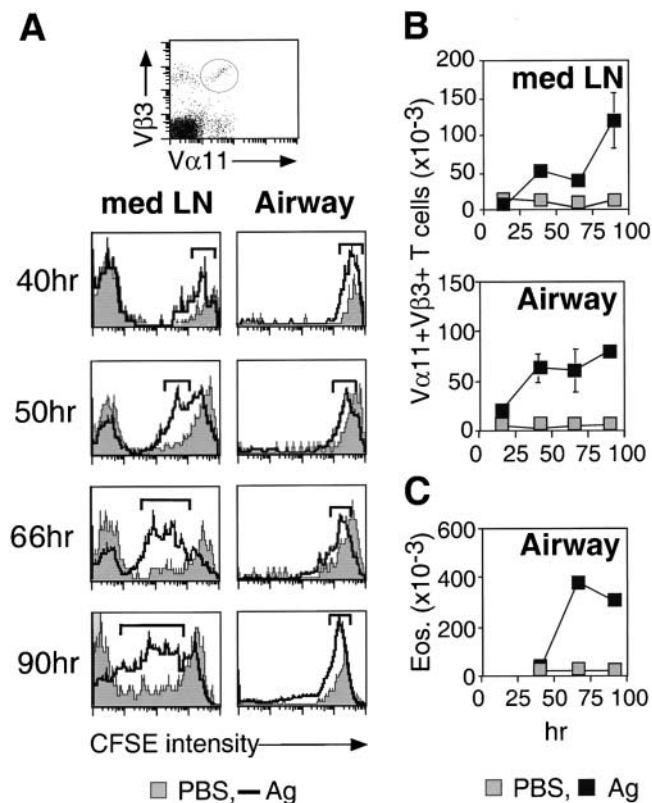


Figure 2. In vivo accumulation and division of activated V α 11⁺V β 3⁺ T cells in the med LN and airway of mice challenged intranasally with MCC₈₈₋₁₀₃. Activated V α 11⁺V β 3⁺ T cells were generated in vitro, CFSE labeled, and 5×10^6 CFSE⁺V α 11⁺V β 3⁺ T cells injected into the tail vein of naive syngenic B10.A hosts. On that day mice were challenged intranasally with PBS or MCC₈₈₋₁₀₃. Responses were measured at indicated time points after intranasal challenge. (A) Cell division was examined by FACS[®] analysis of CFSE intensity. Profiles shown are for live V α 11⁺V β 3⁺ T cells. (B) Accumulation of live V α 11⁺V β 3⁺ T cells in the med LN and airway of intranasally challenged mice was examined by FACS[®]. (C) Eosinophil infiltration into the airway of intranasally challenged mice was examined by BAL and differential cell counting of Giemsa-stained cytopins. The data shown represents mean \pm SE of two to three mice per group from one representative experiment. Similar results were obtained from three separate experiments.

tively transferred V α 11⁺V β 3⁺ T cells after intranasal MCC₈₈₋₁₀₃ challenge. Mice were given an intranasal challenge with 100 μ g MCC₈₈₋₁₀₃ 2 d after receiving an adoptive transfer of in vitro-activated CFSE⁺V α 11⁺V β 3⁺ T cells. 2 h after the intranasal challenge, lymphocytes were isolated from med LN, lung, and airway tissue. Once isolated, these cells were incubated for 20 min in monensin-containing medium (without stimulation) to inhibit the loss of any cytokines that were being synthesized in vivo at the time of tissue removal. At the end of the incubation period, cells were fixed and cytokine production analyzed by intracellular cytokine staining.

The data in Fig. 3 shows the proportion of CFSE⁺ T cells that could produce IL-2, IFN- γ , or IL-4 after the intranasal Ag challenge. CFSE⁺ T cells from either the med LN, or the lung and airway of MCC₈₈₋₁₀₃ challenged mice, were able to produce IL-2, IFN- γ , and IL-4. Slightly more CFSE⁺ T cells from the lung and airways produced IL-2 as compared to CFSE⁺ T cells from the med LN after intranasal challenge with Ag (Fig. 3). In the experiment shown (Fig. 3), a greater proportion of CFSE⁺ T cells in the med LN appeared to be producing IL-4, as compared with lung and airway T cells. However, in other experiments this situation was reversed (unpublished data), indicating there is some background variation.

The percentage of CFSE⁺ T cells found to produce effector cytokines in these experiments was very low. We believe a much greater number of CFSE⁺ T cells are capa-

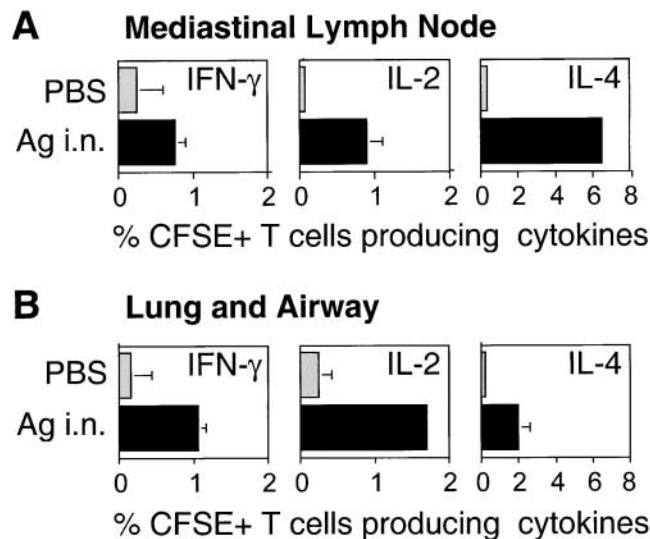


Figure 3. In vivo cytokine production by activated V α 11⁺V β 3⁺ T cells in the med LN or lung and airway, after intranasal challenge with MCC₈₈₋₁₀₃. Activated V α 11⁺V β 3⁺ T cells were generated in vitro, CFSE labeled, and 7.5×10^6 CFSE⁺V α 11⁺V β 3⁺ T cells injected into the tail vein of naive syngenic B10.A hosts. The next day mice were challenged intranasally with MCC₈₈₋₁₀₃ or PBS. 2 h later mice were killed and lymphocytes from the med LN, or lung and airway, were incubated for 20 min in cIMDM containing 2 μ M monensin. T cells were then fixed and examined for expression of IL-4, IL-2, or IFN- γ using intracellular cytokine staining. Profiles shown are for CFSE⁺ T cells. The data shown represents mean \pm SE of three mice per group from one representative experiment. Similar results were obtained from three separate experiments.

ble of producing effector cytokines in the lung, airway, and med LN, but that these are not detected by the intracellular cytokine staining protocol for ex vivo T cells. Indeed we found no IL-5 production in any of the experiments performed. This likely reflects a poor sensitivity of the intracellular cytokine staining protocol used, as the Ag-dependent eosinophilia indicated that significant IL-5 production was occurring in the airways. Our results contrast with those of Lee Reinhardt et al. (16) who reported that production of the effector cytokine IFN- γ is largely confined to the nonlymphoid tissues. However, these authors were investigating memory T cells, and the experimental protocol used involved the coadministration of LPS, which could have significant effects on T cell activation and recirculation patterns. Furthermore, our analysis of IL-4 expression supports our finding that effector cytokines can be produced in lymph node.

Taken together, our data demonstrates that previously activated T cell populations resident in nonlymphoid tissues, such as the lung and airway, do not divide and do not clonally expand when activated by Ag (Fig. 2 A). However, these T cells are not anergic as they are able to produce cytokines (Fig. 3) and initiate profound inflammatory cell exudates in the airway (Fig. 2 C). By contrast, previously activated T cells resident in the draining lymph node undergo rapid cell division after Ag challenge (Fig. 2 A). These lymph node-homing T cells also produce effector cytokines (Fig. 3).

Reisolated Lung and Airway $V\alpha 11^+V\beta 3^+$ T Cells Can Divide In Vitro. We questioned whether the lack of detectable division by Ag-challenged T cells in the airway was an intrinsic property of those T cells. In vitro-activated CFSE $^+$ $V\alpha 11^+V\beta 3^+$ T cells were adoptively transferred into naive hosts, and then recovered from the peripheral lymph nodes, lung, and airway, without additional in vivo Ag challenge. These cells were then cultured with T cell-depleted splenocytes from naive B10.A mice \pm 10 μ g/ml MCC $_{88-103}$. Cell division was detected by recording the changes in CFSE intensity. Reisolated CFSE $^+$ $V\alpha 11^+V\beta 3^+$ T cells from all tissues could divide in response to MCC $_{88-103}$ stimulation in vitro (Fig. 4 A). In each of the experiments, previously activated T cells reisolated from the lung and airway were found to divide at a slightly faster rate than those reisolated from peripheral lymph nodes (Fig. 4 A and unpublished data). All the T cells from the lung and airways appeared to divide in vitro.

The ability of reisolated CFSE $^+$ $V\alpha 11^+V\beta 3^+$ T cells to produce cytokines after reactivation in vitro was also examined. Lymphocytes were isolated from lymph nodes, lung, and airway, and stimulated for 6 h on plate-bound anti-CD3 plus anti-CD28 mAb. Monensin was added for the last 4 h of culture, after which cells were fixed in 4% paraformaldehyde. The production of IL-4, IL-5, IL-2, or IFN- γ by the reisolated CFSE $^+$ $V\alpha 11^+V\beta 3^+$ T cells was examined by intracellular cytokine staining. The data presented in Fig. 4 B demonstrate that CFSE $^+$ $V\alpha 11^+V\beta 3^+$ T cells from either the lymph nodes, or lung and airway, can produce IL-4, IFN- γ , and IL-2. For each of these cyto-

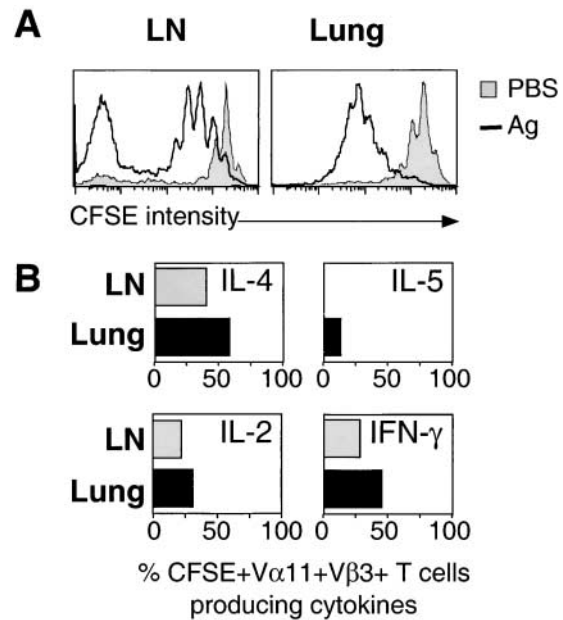


Figure 4. In vitro division and cytokine production by activated $V\alpha 11^+V\beta 3^+$ T cells reisolated from the lymph nodes, or lung and airway. Activated $V\alpha 11^+V\beta 3^+$ T cells were generated in vitro, CFSE labeled, and 2×10^7 CFSE $^+$ $V\alpha 11^+V\beta 3^+$ T cells injected into the tail vein of naive syngenic B10.A hosts. 2–5 d later lymphocytes were purified from the peripheral lymph nodes, and lung and airway. (A) 5×10^5 lymph node, or lung and airway, cells were cultured with 1.5×10^6 T cell-depleted splenocytes from naive B10.A mice \pm 10 μ g/ml MCC $_{88-103}$. After 48 h of culture, T cell division was examined by FACS $^{\text{®}}$ analysis of CFSE intensity. Profiles shown are for live $V\alpha 11^+V\beta 3^+$ T cells. (B) Peripheral lymph node, or lung and airway, T cells were restimulated with anti-CD3 and anti-CD28 in the presence of 2 μ M monensin for 6 h. T cells were then examined for expression of IL-4, IL-5, IL-2, or IFN- γ using intracellular cytokine staining. Profiles shown are for CFSE $^+$ $V\alpha 11^+V\beta 3^+$ T cells. In both A and B, lung represents lung and airway cells. The data shown are from representative experiments, using six to eight mice per group. Each experiment was repeated two to three times with similar results.

kines a slightly larger proportion of CFSE $^+$ $V\alpha 11^+V\beta 3^+$ T cells from the lung and airway were cytokine positive, as compared with those from the lymph nodes (Fig. 4 B). Using this technique, IL-5 producing CFSE $^+$ $V\alpha 11^+V\beta 3^+$ T cells could only be detected in small numbers, and only in the lung (Fig. 4 B).

In summary, our data indicate that activated T cells present in the lung and airway are more sensitive to Ag stimulation in vitro (as measured by division and cytokine production) than those from the lymph nodes. Our findings also indicate that it is the microenvironment of the lung and airway that prevents T cell division in vivo (Fig. 2 A), since the inability to divide is not an intrinsic property of the T cells present at these sites.

Immunization With Peptide-loaded BMDC Cannot Induce Division of Airway Resident $V\alpha 11^+V\beta 3^+$ T Cells. We considered it likely that the environmental factor limiting T cell division in the lung and airway was APC function. Although dendritic cells (DC) are considered to be the most potent lung APC (17, 18), numerous studies (19, 20) have shown that lung DC exhibit an immature phenotype

with a low level of expression of MHC class II and costimulatory molecules. In our laboratory we routinely generate *in vitro*-cultured bone marrow DC (BMDC) that exhibit a mature phenotype, and express high levels of MHC class II and B7-1 (unpublished data). Lambrecht et al. and Havenith et al. have shown that DC delivered via the intratracheal route can activate naive T cells present in the med LN (13, 21). In addition, we found that CFSE-labeled BMDC delivered intranasally could later be isolated from the lung and med LN tissues, indicating their ability to establish in the airway and to further migrate to local lymph nodes (unpublished data).

To test whether the lack of T cell division in the lung and airway was due to poor APC function by airway resident DC, we challenged mice with *in vitro*-generated BMDC presenting MCC₈₈₋₁₀₃. As shown in Fig. 5 A, Ag-loaded BMDC induced vigorous division of CFSE⁺V α 11⁺V β 3⁺ T cells in the med LN. However, no division of airway CFSE⁺V α 11⁺V β 3⁺ T cells was observed at 50

or 66 h after intranasal challenge. It is important to note that at the 90-h time point we observed the appearance of CFSE^{dull} V α 11⁺V β 3⁺ T cells in the airway (Fig. 5 A). The level of CFSE staining in the 90-h airway population was comparable to that seen in the med LN at 66 h (Fig. 5 A). This suggested that the CFSE^{dull} T cells present in the airway at 90 h had been recently recruited from the med LN, and were not resident airway T cells that had divided. Interestingly, the adoptively transferred CFSE⁺V α 11⁺V β 3⁺ T cells had undergone several rounds of division in the med LN of the PBS-challenged mice at 66 h. Although this phenomenon did not occur consistently throughout the experiments, it suggests that *in vitro*-activated T cells may be capable of dividing *in vivo* in the absence of Ag. This may reflect the activity of homeostatic mechanisms operating to maintain memory T cells. Taken together, these data indicate that the lack of division observed for airway V α 11⁺V β 3⁺ T cells in Fig. 2 A, cannot be overcome by Ag presentation on BMDC. This suggests that lack of cell division in the airway is not simply a result of reduced airway APC function.

To evaluate whether donor DC transferred Ag to host DC, a separate group of mice were challenged with ethanol-killed BMDC + MCC₈₈₋₁₀₃. Ethanol-fixed BMDC expressed levels of MHC class II that were equivalent to that seen for live BMDC. The conditions used to fix the BMDC were adjusted to avoid the loss of peptide from MHC class II molecules. Intranasal delivery of live BMDC + MCC₈₈₋₁₀₃ induced the accumulation of V α 11⁺V β 3⁺ T cells in both the med LN and the airway (Fig. 5 B). It also led to the development of a profound airway eosinophilia (Fig. 5 C). These responses were not observed after intranasal administration of ethanol-killed BMDC + MCC₈₈₋₁₀₃ (Fig. 5, B and C), indicating that MCC₈₈₋₁₀₃ presentation occurs exclusively by the donor BMDCs.

Exogenous IL-2 Cannot Restore Division of Airway Resident V α 11⁺V β 3⁺ T Cells. The lack of division of T cells in the airway did not appear to be due to poor airway APC function. We speculated that it could instead be caused by limited local IL-2 production. We have shown in Fig. 3 that CFSE⁺V α 11⁺V β 3⁺ T cells in the lymph node, lung, and airway, all produced similar levels of IL-2 after intranasal Ag challenge. Nevertheless, it was possible that bystander IL-2 production was confined to the lymph node environment. Therefore, to address the possibility of IL-2 deficiency in the lung environment, we delivered large doses of exogenous IL-2 to the airway of mice. The same mice were also given CFSE⁺V α 11⁺V β 3⁺ T cells \pm MCC₈₈₋₁₀₃ via the intranasal route.

Intratracheal adoptive transfer of lung memory CD4⁺ T cells has already been shown by Hogan et al. (22) to result in a functional population of cells that remain in the airway for several weeks after transfer. In our experiments, intranasal adoptive transfer of *in vitro*-activated CFSE⁺V α 11⁺V β 3⁺ T cells resulted in a population of airway-resident T cells that were viable and could respond to intranasal-delivered Ag by producing IL-5 and generating an airway eosinophilia (Fig. 6 B). At day 4 after intranasal

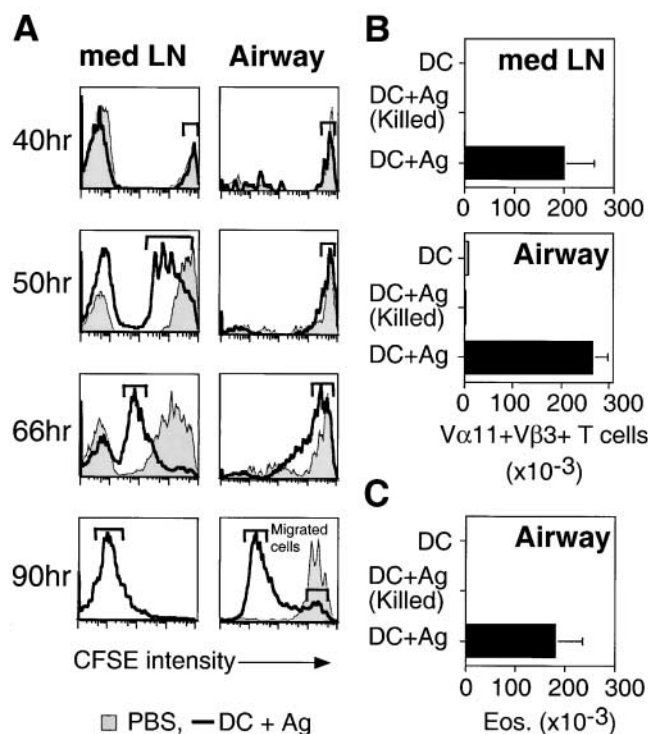


Figure 5. Effect of intranasal challenge with BMDC + MCC₈₈₋₁₀₃ on division of adoptively transferred V α 11⁺V β 3⁺ T cells. Activated V α 11⁺V β 3⁺ T cells were generated *in vitro*, CFSE labeled, and 5×10^6 CFSE⁺V α 11⁺V β 3⁺ T cells injected into the tail vein of naive syngeneic B10.A hosts. On that day mice were challenged intranasally with 10^6 ethanol-killed or live BMDC \pm MCC₈₈₋₁₀₃. Responses were measured at indicated time points after intranasal challenge. (A) The extent of cell division was examined by FACS[®] analysis of CFSE intensity. Profiles shown are for live V α 11⁺V β 3⁺ T cells (B) Accumulation of live V α 11⁺V β 3⁺ T cells in the med LN or airway of intranasally challenged mice was examined by FACS[®]. (C) Eosinophil infiltration into the airway of intranasally challenged mice was enumerated by BAL and differential cell counting of Giemsa-stained cytopins. The data shown represent mean \pm SE of three to four mice per group from one representative experiment. Similar results were obtained in two to four separate experiments.

adoptive transfer, ~20% of input CFSE⁺ T cells could be recovered from the airway by broncho-alveolar lavage (BAL). In addition, a significant population of viable CFSE⁺ T cells that were not isolated by BAL, could be identified in lung and airway tissue that had been subjected to collagenase digestion (Fig. 7 A).

Despite being able to generate an airway eosinophilia after MCC₈₈₋₁₀₃ stimulation, CFSE⁺V α 11⁺V β 3⁺ T cells administered to the airways remained undivided even 4 d after Ag challenge (Fig. 6 A). The addition of exogenous IL-2 to the airway was not found sufficient to restore cell division (Fig. 6 A). However, IL-2 resulted in a large increase in the number of airway eosinophils seen after MCC₈₈₋₁₀₃ stimulation (Fig. 6 B). This indicates that the adoptively transferred T cells were capable of responding to IL-2 by producing increased amounts of IL-5. These data verify our earlier observations that previously activated T cells present

in the airway do not divide, and indicate that this is not due to a lack of IL-2 production.

Airway Resident V α 11⁺V β 3⁺ T Cells Do Not Return To the Circulation. One of the surprising findings of this study was that while undivided, CFSE^{bright} V α 11⁺V β 3⁺ T cells were abundant in the airway at all time points, undivided cells were lost from the med LN by 50 h and did not return (Fig. 5 A). We hypothesized that T cells in the airway were unable to recirculate back to the draining lymph node, even when it was inflamed.

To test this hypothesis, mice were intranasally administered with in vitro-activated CFSE⁺V α 11⁺V β 3⁺ T cells. This population contains T cells that are capable of migrating to the lymph node and nonlymphoid tissues after intravenous injection (Fig. 1). 4 d after intranasal transfer of CFSE⁺V α 11⁺V β 3⁺ T cells, relevant tissues were examined for the presence of V α 11⁺V β 3⁺ T cells, and the number of these cells that were CFSE⁺ determined. As expected from the previous data, all the V α 11⁺V β 3⁺ T cells recovered by lavage from the airway of nonimmunized

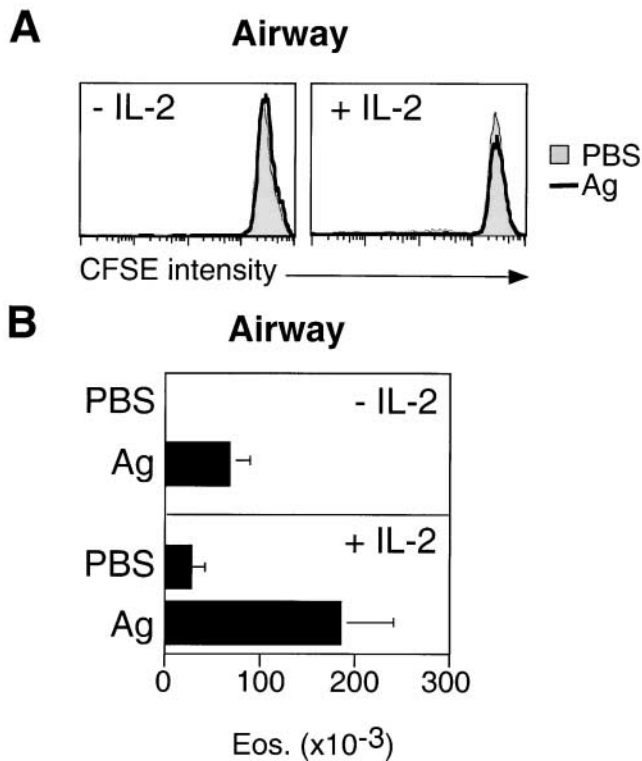


Figure 6. In vivo division of intranasally administered, activated V α 11⁺V β 3⁺ T cells, after intranasal challenge with MCC₈₈₋₁₀₃ ± IL-2. Activated V α 11⁺V β 3⁺ T cells were generated in vitro, CFSE labeled, and 10⁶ CFSE⁺V α 11⁺V β 3⁺ T cells administered intranasally to naive syngenic B10.A hosts. On that day mice were challenged intranasally with PBS or MCC₈₈₋₁₀₃. At days 0 and 2 after challenge, mice were additionally treated with an intranasal inoculation of 500 U rhIL-2 in cIMDM, or cIMDM alone. (A) On day 4 after MCC₈₈₋₁₀₃ intranasal challenge, cell division was examined by FACS[®] analysis of CFSE intensity. Profiles shown are for live V α 11⁺V β 3⁺ T cells. (B) Accumulation of eosinophils in the airway 4 d after MCC₈₈₋₁₀₃ intranasal challenge was determined by BAL and differential cell counting of Giemsa-stained cytopspins. The data shown represents the mean ± SE of three to four mice from one representative experiment. Similar results were obtained from two to four separate experiments.

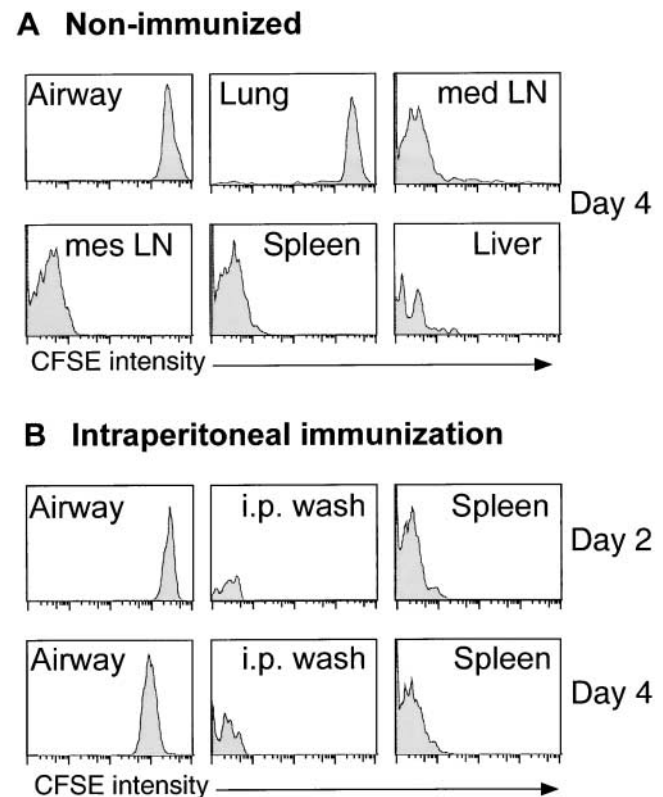


Figure 7. Migration of intranasally administered, activated V α 11⁺V β 3⁺ T cells. Activated V α 11⁺V β 3⁺ T cells were generated in vitro, CFSE labeled, and 10⁶ CFSE⁺V α 11⁺V β 3⁺ T cells administered intranasally to naive syngenic B10.A hosts. (A) 4 d later lymphocytes were isolated from the airway, lung, med LN, mes LN, liver, and spleen. (B) Alternatively, recipient mice were immunized intraperitoneally with 100 μ g MCC₈₈₋₁₀₃ in 200 μ l alum adjuvant, and lymphocytes isolated from the airway, spleen, and peritoneal cavity at days two and four after immunization. Profiles shown are for live V α 11⁺V β 3⁺ T cells. The data shown are from representative experiments, using three mice per group. Each experiment was repeated two to four times with similar results.

mice were CFSE⁺ (Fig. 7 A). The majority of V α 11⁺V β 3⁺ T cells found in the lung were also CFSE⁺. In contrast, no V α 11⁺V β 3⁺ T cells recovered from the draining med LN, mesenteric LN, or spleen, were CFSE⁺ (Fig. 7 A). Nor were any CFSE⁺V α 11⁺V β 3⁺ T cells found in the liver, an organ that has been suggested to act as a “graveyard” for activated T cells (23).

We were concerned that CFSE⁺V α 11⁺V β 3⁺ T cells that left the airway might have been distributed to systemic tissues in numbers below our threshold of detection. To address this possibility we challenged mice with MCC₈₈₋₁₀₃ in alum adjuvant via the intraperitoneal route. Intraperitoneal immunization of naive or primed mice results in Ag drainage to the spleen (24), and an accumulation of Ag-specific T cells in both the peritoneum and spleen (unpublished data). Therefore, any CFSE⁺V α 11⁺V β 3⁺ T cells present in the circulation after intranasal inoculation should be recruited to the peritoneum and spleen after intraperitoneal MCC₈₈₋₁₀₃-alum challenge. At days 2 and 4 after intraperitoneal Ag challenge, we examined the spleen and peritoneal cavity of mice that had received an intranasal transfer of CFSE⁺V α 11⁺V β 3⁺ T cells. No CFSE⁺V α 11⁺V β 3⁺ T cells were detected in either the spleen or the peritoneum at day 2, before any cell division would be expected to occur, or day 4, after intraperitoneal challenge with MCC₈₈₋₁₀₃ in alum (Fig. 7 B).

In summary, these data indicate that adoptively transferred CFSE⁺V α 11⁺V β 3⁺ T cells immigrating to the lung and airway are unable to recirculate to other tissue sites, even after specific Ag challenge. Together these data support our hypothesis that previously activated T cells resident in (or recruited to) the lung and airway are unable to return to the lymph node or recirculating pool.

Discussion

Adoptively transferred CFSE-labeled *in vitro*-activated T cells were found to separate into distinct subpopulations based on their migration to lymph node, lung, or airway tissue. Although we have no understanding of what *in vitro* factors determine the commitment of naive T cells into each of the activated T cell subpopulations, their migration into lymph nodes correlated with higher levels of expression of CD62L. CD62L is known to be important for lymphocyte entry into lymph nodes (25), and acts as a ligand for MAdCAM-1 that is expressed on high-walled endothelium of the lymph node (26). Our subpopulations of activated T cells closely resemble the proposed T_{EM} and T_{CM} subpopulations of memory T cells proposed by Sallusto et al. (27), with respect to their expression of CD62L. This resemblance in terms of CD62L expression may be explained by the finding that T_{EM} and T_{CM} subpopulations of memory T cells also reside in nonlymphoid or lymphoid tissues, respectively (16, 28). CCR7, a chemokine receptor expressed by naive and central memory (T_{CM}) T cells (27), also controls homing to secondary lymphoid tissues and may be expressed by T cells found to enter lymph node in our experiments.

We compared the functions of the adoptively transferred CFSE⁺ T cells that migrate to the lymph node, with CSFE⁺ T cells that migrate to nonlymphoid tissues such as the lung and airway. CFSE⁺ T cells present in either the lymph nodes, or the lung and airway, could all produce similar levels of cytokines after encountering MCC₈₈₋₁₀₃ *in vivo* (Fig. 3). CFSE⁺ T cells in the med LN also divided vigorously in response to MCC₈₈₋₁₀₃ (Fig. 2 A). In striking contrast, CFSE⁺ T cells in the lung and airway could not be induced to divide upon exposure to MCC₈₈₋₁₀₃ (Fig. 2 A and unpublished data). However, CFSE⁺ T cells reisolated from either the lymph nodes, or the lung and airway, could divide extensively when restimulated with Ag during *in vitro* culture (Fig. 4 A). Therefore, the inability to divide is not an intrinsic property of T cells that go to the lung, but is likely to be due to the microenvironment of the lung and airway not being supportive for T cell division.

We endeavored to determine the reason for the lack of *in vivo* division observed for T cells in the lung and airway. To our surprise, MCC₈₈₋₁₀₃ presentation by donor-mature BMDC could not induce CFSE⁺ T cells in the airway to divide (Fig. 5 A), although an IL-5-dependent airway eosinophilia was observed (Fig. 5 C). These data strongly indicate that it is not the poor T cell costimulatory function, nor inadequate Ag processing and presentation, of lung and airway APC that is responsible for the absence of T cell division and clonal expansion. Nor was this lack of division found to be the result of limited IL-2 production in the lung environment, since the addition of large amounts of IL-2 could not induce intranasally delivered CFSE⁺ T cells to divide (Fig. 6 A).

There are several reports that indicate that alveolar macrophages are able to suppress T cell division *in vitro* (for review see 29). These findings were also extended to lung immune responses (30). In two separate studies, it was demonstrated that alveolar macrophages could mediate this suppressive effect on proliferation through modification of the signaling properties of the IL-2 receptor (31, 32). This effect was found to be mediated by nitric oxide (33, 34). Our results extend these *in vitro* findings by reporting the key observation that the airway environment does not affect the production of effector cytokines. Although we have not resolved whether the absence of T cell division in the airway is mediated through nitric oxide, there are several other candidate mediators and cytokines, such as IL-10 and TGF- β , which could exert this effect. Alternatively, agents that induce production of pro-inflammatory cytokines, or that have mitogenic properties, may be required for the proliferation of lung and airway T cells. Lastly, factors (other than IL-2) that normally support T cell division in the lymph nodes may be absent from the lung and airway. We are currently investigating all these possibilities in an attempt to further define the mechanism by which the suppression of T cell proliferation in the lung and airway occurs. The question of the physiological significance of the inability of T cells to divide in the lung and airway raises some intriguing possibilities. An important consideration is that suppression of T cell division in peripheral tis-

sues may serve to prevent potentially pathological T cell activation in response to harmless environmental Ag. Additionally, the development of cross-reactive autoimmunity by pathogen-activated T cells in response to sequestered tissue Ag would be avoided in tissue environments where division is prevented.

Our data indicates that activated T cells cannot divide in the lung or airway, although cells that have divided can enter these tissues after exiting the lymph node. We posed the question as to whether these airway-homing T cells can migrate from the airway back to the lymph node or general circulation. The data presented in Fig. 7 indicates that CFSE⁺ T cells delivered to the airway do not return to the recirculating pool. This failure to migrate out of the airway and lung tissue is likely to be due to an inability of these cells to move across epithelial barriers or into draining-afferent lymphatics. It is not due to the inability of the intranasally transferred cells to survive in the airway microenvironment, since these cells were shown to respond to Ag and induce an airway eosinophilia (Fig. 6 B). It is interesting to note that although intranasally administered, activated T cells cannot leave the airway, BMDC administered intranasally are capable of migrating to the draining med LN and inducing a response here (unpublished data and 13). Our data appears to contrast that of Mackay et al. who reported the presence of CD4⁺ T cells (35) exhibiting a memory phenotype (3) in popliteal-afferent lymph. We do not rule out the possibility that activated T cells leave other nonlymphoid tissue sites to enter afferent lymphatics and return to the lymph node. However, it would appear that this is not the fate of the large majority of activated T cells resident in the lung and airway.

In the context of devising vaccine strategies or immunotherapeutic interventions, one key implication of our work is that the extent and degree of T cell accumulation at an inflammatory site is tightly regulated by two distinct events. The first is the amplification of T cell number by expansion in the lymph node. The second involves recruitment of T cells to the site of Ag exposure. There is no apparent on-site amplification of the T cell response. In addition, we found that the cells that migrate to the airway are lost to this site, and fail to return to the recirculating pool. Thus, previously activated T cells that have entered the airway appear to be trapped, and must either remain in the airway as long-lived cells or be lost to the mucosal surface. This finding raises the possibility that resting memory T cells may suffer the same fate. The question as to whether memory T cells can recirculate, or whether they form two populations destined solely for lymphoid or nonlymphoid tissues, has important implications for our understanding of secondary immune responses and requires further investigation.

Our studies have focused on the lung, airway and draining mediastinal lymph node, and on an acute immune response. It remains to be determined whether the observations we have made are relevant to other nonlymphoid tissues. It would also be of considerable interest to investigate whether chronic infections and/or autoimmune dis-

eases are subject to the same control mechanisms. Experimental evidence suggests that chronic inflammation involves ectopic formation of de novo lymphoid structures (for reviews see 36, 37) that may allow T cell division to occur. Formation of de novo lymphoid structures occurs in infectious diseases such as *Helicobacter pylori*-induced gastritis (38) and in autoimmune disorders including Hashimoto's thyroiditis (39), rheumatoid arthritis (40, 41), Sjogren's syndrome (42), and experimental murine diabetes (43). It is also not clear what physiological controls exist that prevent activated T cells from dividing in the lung and airway tissues. Elucidation of such mechanisms would have clear potential for the development of new therapeutics.

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