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Cutting Edge

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Cutting Edge: Intravascular Staining Redefines Lung CD8 T Cell Responses

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Nonlymphoid T cell populations control local infections and contribute to inflammatory diseases, thus driving efforts to understand the regulation of their migration, differentiation, and maintenance. Numerous observations indicate that T cell trafficking and differentiation within the lung are starkly different from what has been described in most nonlymphoid tissues, including intestine and skin. After systemic infection, we found that >95% of memory CD8 T cells isolated from mouse lung via standard methods were actually confined to the pulmonary vasculature, despite perfusion. A respiratory route of challenge increased virus-specific T cell localization within lung tissue, although only transiently. Removing blood-borne cells from analysis by the simple technique of intravascular staining revealed distinct phenotypic signatures and chemokine-dependent trafficking restricted to Ag-experienced T cells. These results precipitate a revised model for pulmonary T cell trafficking and differentiation and a re-evaluation of studies examining the contributions of pulmonary T cells to protection and disease. *The Journal of Immunology*, **2012, 189: 2702–2706.**

A dense network of pulmonary capillaries underlying the alveoli forms the structural basis of respiration. Gas exchange is most efficient at the thinnest portions of the air–blood barrier, where narrow capillaries share a fused basal lamina with alveolar epithelium. This intimate association between the capillary bed, a thin permeable membrane, and the outside world—coupled with the fact that inflammation can disrupt the delicate architecture necessary for gas exchange—creates vulnerabilities. Indeed, lower respiratory infections account for the single greatest cause of death from infectious disease, and the incidence of chronic T cell-dependent inflammatory diseases such as asthma is increasing (1, 2).

T cell differentiation is coupled with anatomic distribution. Naive and central memory T cells (T_{CM}) recirculate through secondary lymphoid organs, blood, and lymphatic vessels. This restricted homing pattern optimizes interaction with professional APCs and subsequent proliferation in response to cognate Ag recognition. Effector memory T cells (T_{EM}) patrol nonlymphoid tissues, where they are positioned for more immediate interception of pathogens at the most common points of exposure (3). Indeed, resident T_{EM} within skin contribute most rapidly to control of local reinfection (4, 5). Resident T_{EM} populations have been defined in many nonlymphoid tissues, and are characterized by unique phenotypic signatures not represented in blood, including CD69 and CD103/β7 integrin (4–8).

Regardless of route, infection or immunization gives rise to extraordinarily large effector and memory T cell populations that can be isolated from perfused mouse lung (9, 10). However, lung T cell migration and differentiation are less clear than in tissues such as the intestinal mucosa, skin, brain, or lymph nodes (LNs). In contrast to the stereotypic three-step model of lymphocyte extravasation (11), some evidence demonstrates that T cell homing to lung is chemokine independent (12). Expression of chemokine receptors by T cells, including CCR5 and CXCR3, however, is required for normal distribution and differentiation of lung T cells following local infection (13). In some infection models, the lung contains a large fraction of T_{CM} (14). In fact, even naive lymphocytes can be isolated from the perfused lung (15–17). These observations contrast with those in most other nonlymphoid compartments, which do not contain T_{CM}, exclude naive T cells, and require chemokine signaling for entry. This study sheds light on these issues by refining our understanding of the anatomic compartmentalization of CD8 T cells within the lung.

Materials and Methods

Mice and infections

P14 chimeric immune mice were generated as described (6). Mice were either infected i.p. with 2×10^5 PFU lymphocytic choriomeningitis virus (LCMV) or intratracheally (i.t.) with 1×10^5 PFU LCMV (18). The University of Minnesota Institutional Animal Care and Use Committee approved all experiments.

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Abbreviations used in this article: ILN, inguinal lymph node; i.t., intratracheal(ly); LCMV, lymphocytic choriomeningitis virus; LN, lymph node; PTx, pertussis toxin; T_{CM}, central memory T cell; T_{EM}, effector memory T cell.

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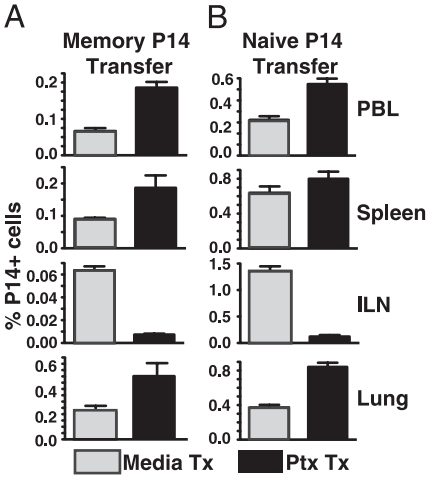

FIGURE 1. PTx treatment of transferred T cells yields increased recovery from lung. Naive or memory P14 splenocytes were treated with PTx or media control and transferred i.v. into naive recipients. Tissues were harvested 1 (naive) or 3 (memory) d post transfer. Frequency of memory (**A**) or naive (**B**) P14 cells in PBL, spleen, ILN, or lung. Data are representative of three independent experiments totaling >12 mice per condition. Error bars indicate SEM.

Intravascular staining and cell isolations

A total of 3 μg anti–CD8α-APC or anti–CD8α-PE (clone 53-6.7 from eBioscience) or purified rabbit anti-mouse collagen IV (Novus Biologicals) was injected i.v. At 3 min after injection, the animals were sacrificed, lavaged to remove cells in the airway, bled, and perfused with 10 ml cold PBS. The spleen, LNs, lung, liver, and small intestine were harvested within 12 min, and lymphocytes were isolated as described (19). Immunofluorescence staining was performed as noted (6).

Pertussis toxin treatment

Purified splenocytes from P14 immune chimeric mice or naive P14 transgenic mice were incubated in RPMI 1640 containing 10% FBS ± 25 ng/ml pertussis toxin (PTx) (R&D Systems) at a concentration of 1.5×10^7 cells/ml for 1 h at 37°C, as described (8). Following incubation, $1.5–3.5 \times 10^7$ cells were injected i.v. into C57BL/6 recipient mice.

Results and Discussion

PTx treatment of T cells yields increased recovery from lung

We wished to confirm the PTx sensitivity of memory CD8 T cell homing to lung and other tissues, as well as to address this issue for naive CD8 T cells. Gp33-specific P14 memory CD8 T cells were generated in vivo in response to i.p. LCMV infection (referred to hereafter as P14 immune chimeras; see *Materials and Methods*). Eight weeks later, splenocytes containing memory P14 CD8 T cells were treated with PTx or control media, then transferred i.v. into naive recipients. At 3 d after transfer, various tissues were harvested to assess T cell migration. Consistent with the known requirement for CCR7, migration to inguinal LNs (ILNs) was blocked by PTx treatment, and a reciprocal increase of donor cells among spleen and PBLs was noted. As reported previously, PTx treatment did not inhibit migration to lung and, like blood, actually led to an increase in recovered cells (Fig. 1A). This experiment was repeated with naive P14 CD8 T cells, with similar results (Fig. 1B). These data suggest that even naive CD8 T cells home to lung in a chemokine-independent process, particularly when LN homing was inhibited.

In vivo staining distinguishes between anatomic compartments

We wished to define the lung compartment to which naive T cells migrated. We injected anti-CD8α Ab i.v. into naive mice that had received untreated naive P14 CD8 T cells. At 3 min after injection, blood was isolated; mice were immediately sacrificed and perfused with PBS; and tissues were quickly dissected, minced, and rinsed of free Ab. Lymphocytes were isolated via standard methods, then stained with anti-CD8β surface Ab and other markers of interest. The permissiveness of donor naive T cells to i.v. staining varied among distinct tissues (Fig. 2A): 100% of donor cells within blood, a subset of cells within spleen, and virtually no cells within LN were labeled. Like blood, 100% of naive donor cells isolated from lung were labeled with injected anti-CD8α

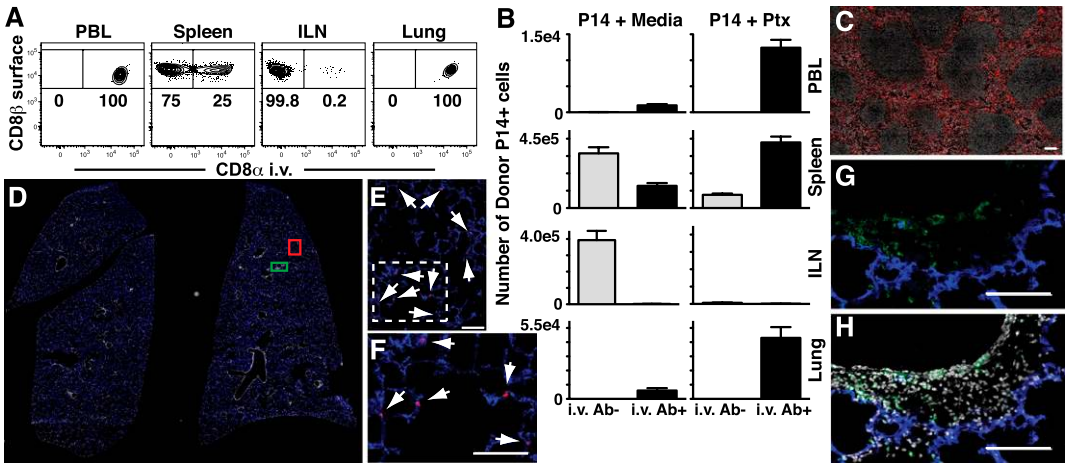

FIGURE 2. In vivo staining distinguishes between anatomic compartments. (**A**) Naive Thy1.1$^+$ P14 splenocytes were transferred i.v. into C57BL/6J mice. Anti-CD8α was injected i.v. Three minutes later, tissues were harvested. Cells were isolated and then stained ex vivo with anti-CD8β. Plots were gated on Thy1.1$^+$ lymphocytes. (**B**) Number of i.v. Ab$^+$ and i.v. Ab$^-$ naive P14 cells, ± PTx treatment, isolated from tissues after transfer. (**C**) Spleen after anti–CD8α-PE i.v. injection. (**D–H**) Whole-lung sections of P14 immune chimeras were imaged. (E) and (F) or (G) and (H) represent enlarged images defined by red or green boxes, respectively, in (D). CD8 T cells that stain with i.v. injected Ab (red) colocalize with blood vessels [CD31, blue; see (E) and enlarged *inset* (F)], whereas those protected from i.v. Ab (green surface stain) are spatially distinct [(G) and (H), ± DAPI]. Arrows designate red i.v. Ab$^+$ cells. (A) and (B) are representative of three independent experiments totaling ≥12 mice per condition. Error bars indicate SEM. Images are representative of two independent experiments totaling six mice. Scale bars [(C), (E)–(H)], 100 μm.

Ab. PTx treatment increased the number of labeled donor cells recovered from blood, spleen, and lung, but reduced the number of unlabeled cells recovered from spleen and ILN (Fig. 2B).

To assess whether intravascular staining was associated with distinct anatomic compartments, we injected anti–CD8α-PE Ab into P14 immune chimeras, sacrificed and perfused recipients, and then immediately froze spleen and lung for immunofluorescence. Injected Ab labeled CD8 T cells in red pulp but not those in white pulp of spleen (Fig. 2C). Lung tissue sections were also surface stained for CD8α-AF488, CD31-AF647 (which labels endothelium), and DAPI (which labels cell nuclei). Analysis of the lung revealed that the vast majority of CD8 T cells were labeled only with injected Ab (Fig. 2D). Closer inspection revealed that these cells (red) appeared to be closely associated with pulmonary capillaries (blue; Fig. 2E, 2F). These data indicate that most cells were exposed to injected Ab and that in vivo staining blocked ex vivo surface staining on tissue sections. However, a small fraction of $CD8\alpha^+$ cells, typically surrounding airways and large blood vessels, were labeled only by ex vivo staining (green) of tissue sections, suggesting that they were protected from i.v. injected Ab (Fig. 2G, 2H).

Migration to uninflamed lung tissue is chemokine dependent

On the basis of these data, it was possible that intravascular labeling of cells in the lung was restricted to cells contained within pulmonary capillaries or other blood vessels that were refractory to removal by perfusion. Alternatively, injected Ab may have leaked or been exported out of the pulmonary capillary bed, thereby staining perivascular cells. A collagen IV-containing basement membrane underlies the lung vascular endothelium (as revealed by surface staining; Fig. 3A, *upper right panel*). We injected anti-collagen IV Ab and found that the basement membrane remained unlabeled, suggesting that the capillary bed did not allow rapid perivascular leakage of Abs (Fig. 3A, *lower right panel*). For controls, we examined surface and injected collagen IV staining in the spleen (where only red pulp was exposed to injected Ab) and liver (which contains fenestrated endothelium within sinusoids, permitting intravascular staining of basement membrane; Fig. 3A).

To further test the interpretation that intravascular staining identifies cells contained within the capillary bed of the lungs of perfused mice, we revisited the issue of PTx-insensitive lung trafficking. As in Fig. 1A, PTx-treated or control memory CD8 T cells were transferred i.v., and recipient tissues were harvested 3 d later. The vast majority of untreated donor cells harvested from lung became labeled with injected Ab. However, a small fraction (~6%) was within a compartment of the lung protected from injected Ab. Importantly, appearance of this subset was PTx sensitive (Fig. 3B, 3C), suggesting that it represented a chemokine-dependent trafficking event.

Most lung memory CD8 T cells are capillary associated post infection

Our results thus far indicate that the vast majority of transferred CD8 T cells recovered from perfused lung were actually present within the narrow capillary network associated with alveoli. We next sought to determine what proportion of memory CD8 T cells recovered from the lungs of infected mice were within blood. To this end, we generated P14 immune chimeras, using an i.p. route of infection. We found that

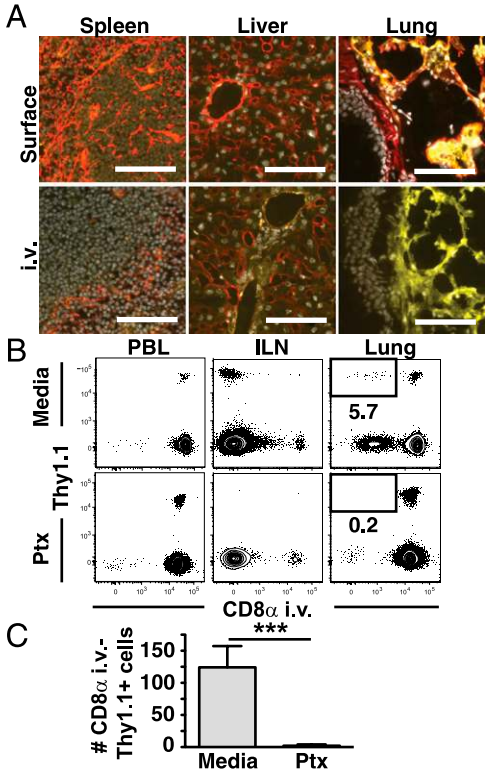

FIGURE 3. Migration of memory CD8 T cells to uninflamed lung tissue is chemokine dependent. (**A**) Anti-collagen IV Ab (red) was injected i.v. prior to tissue harvest, or tissue sections were surface stained ex vivo along with anti-CD31 (yellow) and DAPI (gray). (**B** and **C**) $Thy1.1^+$ memory P14 splenocytes were treated with PTx or media and transferred i.v. into naive C57BL/6J mice. Intravascular anti-CD8α staining and tissue harvest occurred 3 d later. (B) Representative flow cytometric analysis of the indicated recipient tissues. Plots gated on $CD8\beta^+$ lymphocytes. (C) Number of donor memory P14 cells recovered from lung that were protected from intravascular staining (intravascular anti-$CD8\alpha^-$). Images are representative of two independent experiments totaling four mice. Scale bars represent 100 μm. Plots in (B) and (C) are representative of three independent experiments totaling ≥12 mice per condition. $^{***}p = 0.006$, unpaired Student *t* test. Error bars represent SEM.

>96% of LCMV-specific CD8 T cells isolated from the lung were labeled with intravascular Ab (Fig. 4A). To determine whether this vascular-biased localization was unique to an i.p. infection, mice were instead infected via the i.t. route. Although local infection established a significantly greater CD8 T cell response in the lung stroma and inducible BALT, the majority of cells isolated from lung were still capillary derived (Fig. 4A).

Post infection, mice inoculated i.p. maintained a constant proportion of P14 CD8 T cells in the lung tissue versus capillaries. In contrast, i.t. infected mice experienced a dynamic elevation and contraction in the proportion of P14 CD8 T cells within lung tissue relative to capillaries (Fig. 4B), correlating with the transient presence of inducible BALT (data not shown). In spleen, P14 CD8 T cells initially predominated in red pulp, then gradually shifted to white pulp (Fig. 4B), correlating with T_{CM} differentiation (20).

Differential phenotype of lung tissue and vascular CD8 T cells

We then examined the phenotype of P14 CD8 T cells 15 d after i.t. LCMV infection. P14 cells present in peripheral blood bore a striking resemblance to those cells isolated

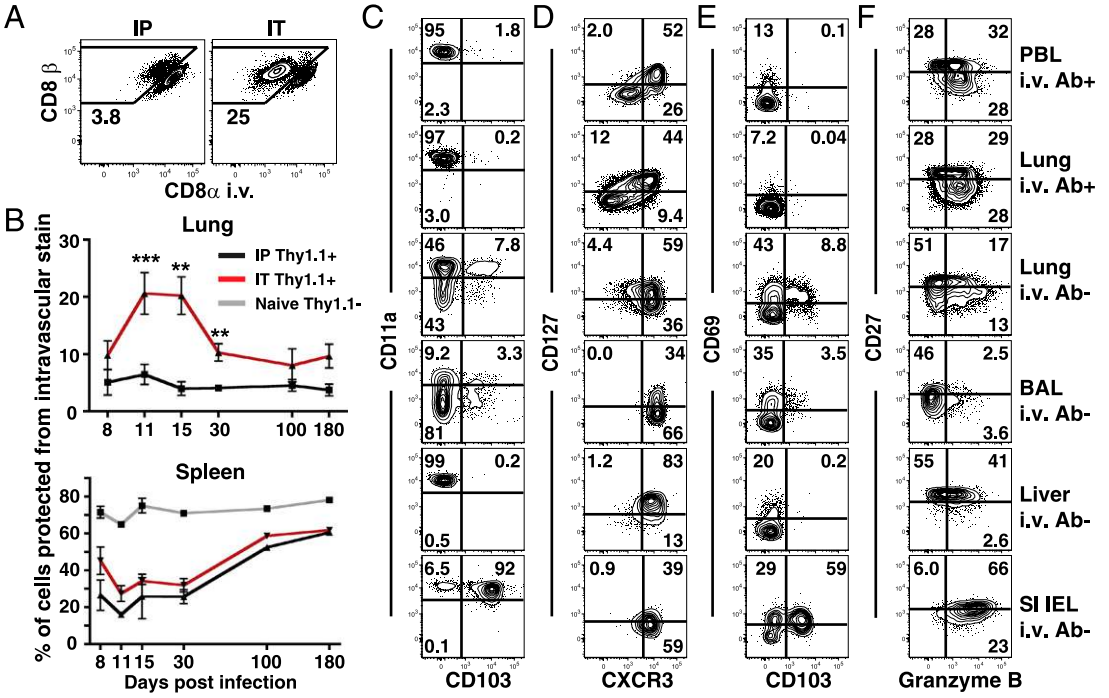

FIGURE 4. Most lung memory CD8 T cells are capillary associated post infection. (**A**) P14 immune chimeras were infected with LCMV via the i.p. or i.t. route. At 15 d after infection, mice were subjected to intravascular staining (as in Fig. 2), and lymphocytes were isolated from perfused lung. Plots are gated on Thy1.1$^+$ P14 cells. (**B**) Frequency of P14 cells that were protected from intravascular staining isolated from lung or spleen after i.p (black) or i.t. (red) infection. Proportion of naive (CD44^{lo}) CD8 T cells (gray) that were protected from intravascular staining was also determined in spleen. (**C–F**) The phenotype of P14 cells was evaluated in the indicated tissues 15 d after i.t. LCMV infection. Data are representative of two or three independent experiments per time point, totaling ≥8 mice per condition. Error bars represent SEM. $^{***}p = 0.0003$, $^{**}p < 0.006$, unpaired Student t test.

from the lung that stained with injected Ab (Fig. 4C–F, *rows 1* and *2*). In contrast, cells protected from i.v. staining were distinct, contained subsets of CD103$^+$ and CD69$^+$ cells, and were uniformly CXCR3$^+$ (Fig 4C–F, *rows 2* and *3*). Airway T cells (removed prior to lung digestion) exhibited a distinct phenotype, including pronounced downregulation of CD11a, as previously described (Fig. 4C–F, *rows 3* and *4*) (21). P14 CD8 T cells isolated from the liver or small intestine epithelium that were protected from i.v. staining (~5% or >99% of total P14s isolated from each tissue, respectively; data not shown) were also uniformly CXCR3$^+$ but were distinct from lung tissue cells in many respects (Fig. 4C–F, *rows 5* and *6*).

A previous report quite elegantly demonstrated that CD8 T cells stimulated in vitro under varying conditions, and then transferred i.v., were differentially labeled by intravascular staining when recovered from lung, and exhibited distinct homing requirements (22). Our study validates that intravascular staining discriminates CD8 T cells present within the lung tissue from those trapped in the vasculature. We used this approach to re-evaluate the anatomic distribution of CD8 T cells isolated from lung after in vivo infection. This analysis revealed the major finding that, in some contexts, up to 96% of effector or memory T cells isolated from lung represent cells in the capillary vessels rather than lung tissue. Unique phenotypic signatures were expressed among cells protected from labeling, including CD69 and CD103/β7 integrin expression, which has been associated with resident T_{EM} populations (4–7). Moreover, only a small fraction of memory CD8 T cells transferred into naive recipients that were recovered from lung actually migrated into the tissue. This process was chemokine dependent, and naive T cells were excluded. These results precipitate a re-evaluation of previous studies that examined the distribution, phenotype, and homing requirements of pulmonary T cells, and also have ramifications for T cell-dependent protection studies that may overestimate localization of transferred or established T cell populations within lung tissue (20, 23). Vaccine modalities that promote true T cell homing to lung tissue, as evaluated by intravascular staining, may be developed to enhance protection against respiratory infections (24–26).

In summary, our results demonstrate that intravascular staining is a useful tool to define vascular and tissue pulmonary lymphocytes, to indicate that the majority of T cells are often within the vasculature of perfused lung, and to support a revised model of the regulation of cellular immunity within the respiratory mucosa.

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Disclosures

The authors have no financial conflicts of interest.

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