Preferential migration of effector CD8+ T cells into the interstitium of the normal lung

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The respiratory tract is a primary site of infection and exposure to environmental antigens and an important site of memory T cell localization. We analyzed the migration and retention of naive and activated CD8+ T cells within the noninflamed lungs and quantitated the partitioning of adoptively transferred T cells between the pulmonary vascular and interstitial compartments. Activated but not naive T cells were retained within the lungs for a prolonged period. Effector CD8+ T cells preferentially egressed from the pulmonary vascular compartment into the noninflamed pulmonary interstitium. T cell retention within the lung vasculature was leukocyte function antigen-1 dependent, while the egress of effector T cells from the vascular to the interstitium functions through a pertussis toxin–sensitive (PTX-sensitive) mechanism driven in part by constitutive CC chemokine ligand 5 expression in the lungs. These results document a novel mechanism of adhesion receptor– and pulmonary chemokine–dependent regulation of the migration of activated CD8+ T cells into an important nonlymphoid peripheral site (i.e., the normal/noninflamed lung).

Introduction

Naive and central memory T cells continuously circulate from the blood to secondary lymphoid tissues (1, 2) whereas effector and memory effector T cells preferentially home to peripheral tissues in search of antigen (3, 4). The entry of lymphocytes into lymphoid tissues is a well-orchestrated, nonrandom process that involves the interaction of the circulating lymphocytes with high endothelial venules and is regulated by both adhesion and chemoattractant receptor/ligand interactions (5). Recently, extensive studies were performed to dissect the mechanism of T cell homing to LNs (6), the skin, and the gut (7), but much less is known about normal/homeostatic T cell migration to nonlymphoid tissues such as lungs.

Unlike most tissues, the lungs are perfused by a dual circulation: the bronchial circulation, which supplies blood to the larger conducting airways, and the pulmonary circulation, which perfuses the bulk of the pulmonary interstitium and branches into alveolar capillaries. The nature and type of T cell interaction with the alveolar capillary endothelium and the contribution of this interaction to T cell transmigration into the lung interstitium are largely unknown. The potential importance of T cell migration to the lungs for the host response to infection is suggested by the recent finding that after systemic viral infection, the lungs are a major site of localization of effector/memory CD8+ T cells following virus clearance (8).

Early studies on the fate of activated cloned CD8+ T cells after adoptive transfer into normal recipient mice indicated that these differentiated effector T cells are retained in the noninflamed lungs for a prolonged period — possibly due to physical trapping of these large blast cells within the pulmonary circulation. The homing and retention of adoptively transferred T cells in the lungs after systemic or pulmonary infection/inflammation (9–11), migration of memory CD8+ T cells during respiratory virus infection, and CD8+-dependent allergen-induced airway inflammation to the lung airways have been well documented (8, 12, 13). Adhesion molecules such as selectins (14), ICAM-1 and -2 (15), and leukocyte function antigen-1 (LFA-1) (16) are believed to be involved in T cell retention within the lungs. In these studies, the scope of the kinetic analysis of T cell retention in the lungs was limited by the necessity of sacrificing recipient mice, and the localization of T cells within intravascular versus extravascular compartments was not fully determined.

We have previously shown that adoptive transfer of influenza HA–specific effector CD8+ T cells into HA-expressing recipients results in the development of lethal pulmonary injury over 3–5 days after cell transfer (17). Since in this model, the target antigen (HA) is expressed selectively on alveolar type 2 cells, it has been hypothesized that the transferred T cells must migrate from the vascular compartment of the noninflamed lungs into the interstitium and induce the injury process.

In this report, we examine the migration, the kinetics of retention in the normal lungs, and the fate of adoptively transferred naive and activated CD8+ T cells by whole-body dual modality imaging and then examine the mechanism of T cell retention within the normal pulmonary vasculature and interstitium. To analyze the localization process, we developed a new flow cytometry-based method to quantitate cell partitioning between lung compartments. We demonstrate that effector CD8+ T cells selectively localize and retain within the pulmonary vasculature and subsequently actively transmigrate into the pulmonary interstitium. T cell retention within the vascular compartment is dependent on LFA-1 expression by T cells while pulmonary RANTES (CC chemokine ligand 5 [CCL5]) expression contributes to the active T cell transmigration into the interstitium through a pertussis toxin–sensitive (PTX-sensitive) signaling mechanism.

Nonstandard abbreviations used: APC, allophycocyanin; CCL5, CC chemokine ligand 5; CCR6, CC chemokine receptor 6; CFSE, carboxyfluorescein diacetate, succinimidyl ester; CMTMR, CellTracker Orange (5-(and-6)-(((4-chloromethyl)benzoyl) amino)tetramethylrhodamine); CXCR2, CXC chemokine receptor 2; ^{111}In, ^{111}Indium; LFA-1, leukocyte function antigen-1; MCP-1, monocyte chemoattractant protein-1; PTX, pertussis toxin; ROI, region of interest; VLA-4, very late antigen-4.

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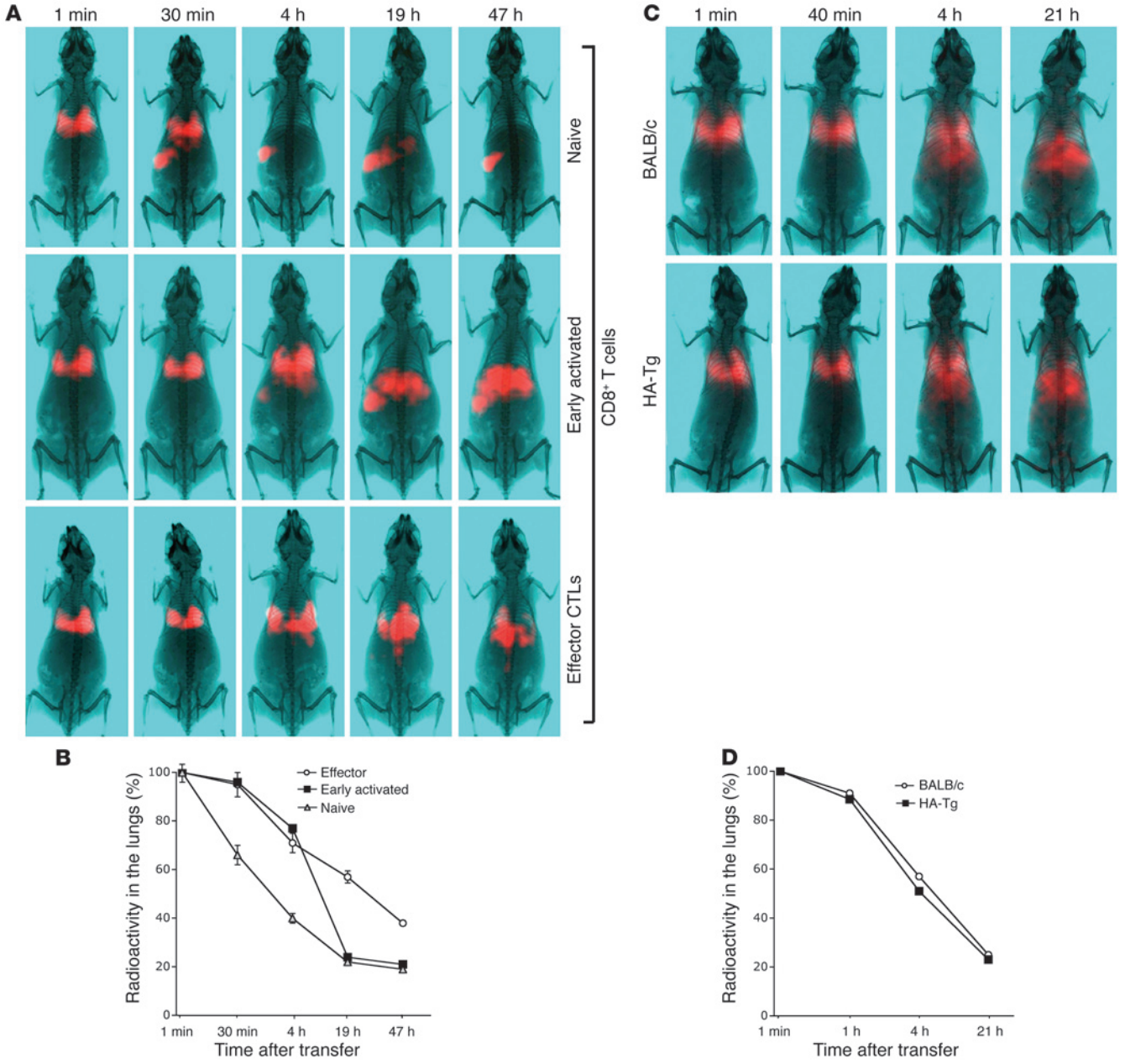

Figure 1
Activated CD8+ T cells but not naive T cells are retained within lungs after adoptive transfer in an antigen-independent manner. (**A**) Dual modality image (merged image combining γ-ray and x-ray images) following adoptive transfer of ^{111}In-labeled naive, early-activated, or effector CD8+ T cells into WT (non-Tg) recipient mice (top view, prone mouse). In the merged RGB images shown, the red channel indicates γ-counts. (**B**) Distribution of ^{111}In-labeled CD8+ T cells at the indicated time points after transfer. Data is represented as percentage of radioactivity in ROI defined for the lungs, with the fraction of transferred cells/radioactivity localized to the lungs (~80%) at 1 minute defined as 100%. Results are representative of 6 independent experiments. (**C**) Dual modality image and (**D**) quantitation following adoptive transfer of ^{111}In-labeled effector CTLs into HA-transgenic (HA-Tg) and WT mice.

Results

Analysis of transferred CD8+ T cell retention in lung and subsequent migration by dual modality imaging. We employed dual modality whole-body γ-camera/x-ray imaging (18) to analyze in real time the retention time in the lungs and the subsequent migration of ^{111}Indium-labeled (^{111}In-labeled) CD8+ T cells after i.v. adoptive transfer into normal recipients. In this analysis, the dominant sites of radioactivity localization over time are displayed pictorially by false color red, which represents the γ-camera data acquisition, with pixel count (red intensity) superimposed on the x-ray image. As described below, at 1 minute after cell transfer, approximately 80% of the radioactivity is localized to the lungs, with the remaining radioactivity dispersed throughout the circulation. As Figure 1A demonstrates, naive splenic CD8+ T cells are initially

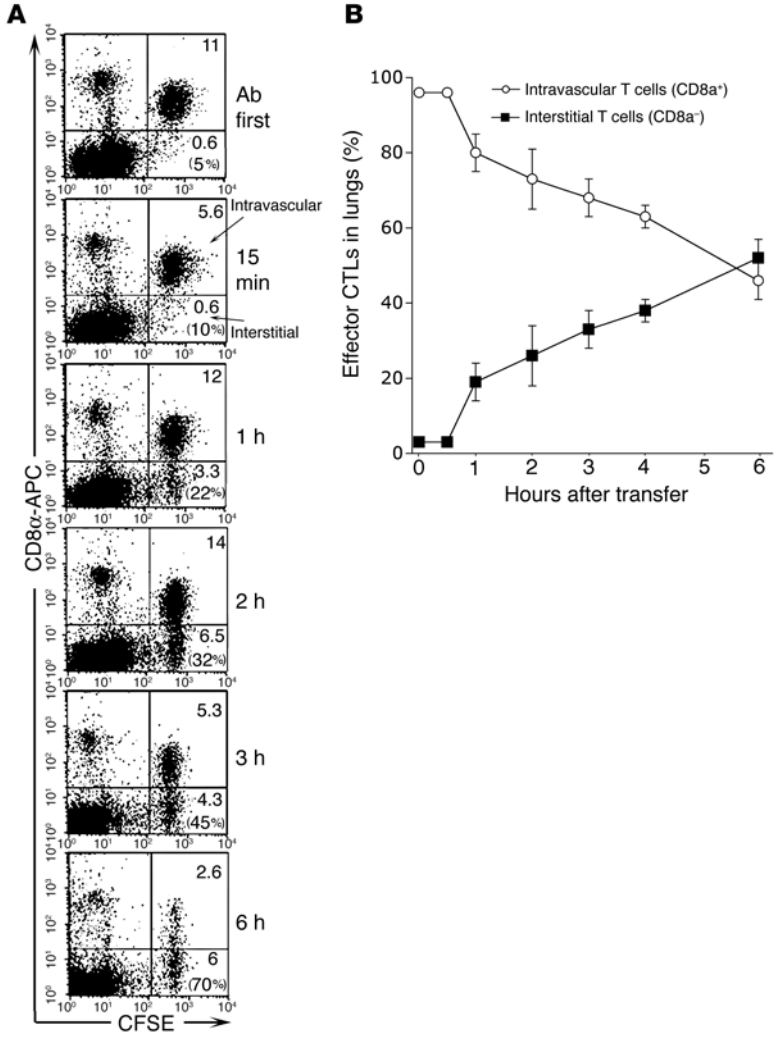

Figure 2

Effector CD8+ T cells migrate into the interstitium of noninflamed lungs. (**A**) Kinetic analysis of intravascular versus extravascular compartmentalization of effector CD8+ T cells in lungs. We injected 4 × 10^6 CFSE-labeled CD8+ T cells into recipient mice. Anti–CD8α-APC mAbs were injected at 5 minutes or 1, 2, 3, or 6 hours after transfer; then lungs were harvested 10 minutes later. The percentage of CFSE-labeled cells is shown; the percentage of CFSE-labeled interstitial T cells from total percentage of emigrated CFSE-labeled T cells is shown in parentheses. Ab first indicates that anti–CD8α-APC mAbs were injected 2 minutes prior to T cell transfer. (**B**) Data from **A** is expressed as the percentage of transferred CD8+ T cells in the intravascular or extravascular compartment.

retained in the lungs, but within 7–10 minutes (data not shown), these cells begin to leave the lungs and migrate preferentially to the spleen. By 4 hours after transfer, more than 60% of the ^{111}In-labeled naive CD8+ T cells have left the lungs and localized primarily to the spleen (Figure 1A).

We then examined the lung retention and ultimate localization of several CD8+ T cell populations in different activation/differentiation states. These included polyclonal naive CD8+ T cells harvested 48 hours after in vitro activation (early-activated T cells), 2 sources of fully differentiated antigen-specific cytolytic effector CD8+ T cells — activated memory/effector T cells from influenza HA–immune donors harvested after 5–7 days of in vitro stimulation with specific antigen and a cloned population of influenza HA–specific CD8+ CTLs maintained in continuous culture. The latter 2 T cell populations were employed because both CD8+ T cell sources had been previously demonstrated to induce lethal pulmonary injury after adoptive transfer into HA-expressing recipients (10, 17). In preliminary studies, both the heterogeneous effector and the clonal effector T cells exhibited comparable lung retention and subsequent localization (data not shown). For convenience, as well as to minimize activation-associated heterogeneity, the cloned CD8+ T cell population was employed as the source of differentiated effector CD8+ T cells in the studies described here. The expression of cell surface activation/differentiation markers by naive and effector CD8+ T cells is summarized in Supplemental Table 1 (supplemental material available online with this article; doi:10.1172/JCI24482DS1).

Activated CD8+ T cells demonstrated a distinctly different pattern of lung retention and subsequent localization than naive CD8+ T cells. After adoptive transfer, early-activated CD8+ T cells were retained for up to 4 hours before initiating egress from the lungs (Figure 1A). Unlike naive CD8+ T cells, after egress from the lungs, the early-activated T cells localized predominantly, if not exclusively, to the anatomic distribution of the liver by 19–47 hours after transfer. A companion analysis of the lung retention time/localization of the terminally differentiated effector CD8+ T cells revealed that these cells were retained in the lungs for an extended period similar to that of the early-activated CD8+ T cells. However, the effector CD8+ T cells were retained in the lungs even longer than the early-activated T cells before their egress and localization to the liver (Figure 1, A and B). We calculated the percentage of radioactivity/labeled cells retained in the lungs over time (Figure 1, B and D) by assigning a value of 100% to the fraction of radioactivity present in the lungs at 1 minute after cell transfer.

We quantitated the retention and rate of T cell egress from the lungs by defining an anatomic region of interest (ROI) on the merged γ-camera/x-ray images, then determining the radioactivity (pixel counts) contained within that ROI. This quantitative kinetic analysis of lung retention and subsequent localization is shown in Figure 1B. The accuracy of quantitation obtained by ROI analysis was confirmed in preliminary studies by excision of individual organs and direct counting of the excised organs by the γ-camera detector (data not shown). Radioactivity/cell localization estimates defined by ROI differed from direct organ counting by 10% or less.

The prolonged retention in the lungs of both the early-activated and the antigen-specific effector CD8+ T cells suggested that the migration to and retention of activated T cells in the lungs was independent of specific antigen recognition. To further establish this point, we analyzed the homing of the HA-specific CD8+ CTLs after adoptive transfer into WT mice and HA-transgenic mice (which express the influenza HA–transgene selectively in lung alveolar type II cells). As Figure 1, C and D, demonstrates,

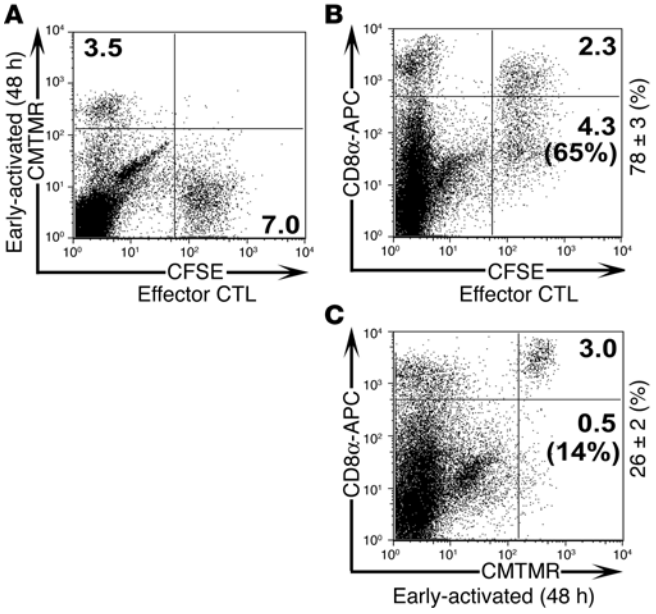

Figure 3
Effector CD8+ T cells but not early-activated CD8+ T cells transmigrate to the lung interstitium. (**A**) Effector CTLs and early-activated (48 hours) CD8+ T cells (labeled with CFSE and CMTMR, respectively) were injected into WT recipient mice. Anti–CD8α-APC mAbs were injected at 6 hours after transfer; lungs were harvested 10 minutes later. The percentages of CFSE and CMTMR-labeled cells from individual recipients are shown in dot plots. (**B** and **C**) Analysis of T cell partitioning between pulmonary vascular and interstitial compartments. The numbers in parentheses reflect the percentages of CFSE-labeled (effector CD8+ T cells) or CMTMR-labeled (early-activated CD8+ T cells) cells from individual recipients that emigrated into the interstitium from the total number of CFSE- or CMTMR-labeled cells remaining within the lungs. Results represent the mean ± SEM of 4 recipients in a total of 4 independent experiments.

the localization and retention time of the activated effector T cells in lungs of WT and HA-transgenic mice was identical, and the subsequent localization of the cells to the liver after egress from lungs was indistinguishable.

Activated effector CD8+ T cells preferentially transmigrate into the lung interstitium. Dual modality imaging confirmed earlier reports (15, 19) demonstrating that activated T cells are retained in lungs for an extended period after i.v. adoptive transfer. This prolonged retention could reflect nonspecific "trapping" of activated T cells within pulmonary alveolar capillaries, but the effector CTLs must also gain access to the interstitium/alveolar surface in order to initiate injury in the HA-transgenic mice — thus further implying migration of the effector T cells from the vasculature into the interstitial compartment.

To quantitate both the extent and kinetics of activated T cell partitioning between the alveolar capillaries and alveolar interstitium in situ in normal lungs, we developed a multicolor flow cytometry–based strategy. In brief, carboxyfluorescein diacetate, succinimidyl ester–labeled (CFSE-labeled) CD8+ CTLs were injected i.v. into recipients, and at different times after cell transfer, recipient mice received a single i.v. injection of anti-CD8α mAbs labeled with the fluorochrome allophycocyanin (APC). Shortly after mAb administration (~10 minutes, to allow mAb equilibration within the vascular compartment and binding to intravascular T cells), lungs were harvested and lung fragments subjected to an enzymatic digestion in the presence of unlabeled anti-CD8α mAbs. Unlabeled mAbs were included during lung digestion to prevent staining of transferred cells localized to the interstitium by labeled Abs present in the pulmonary circulation of the excised lungs. To ensure that the i.v. mAb dose administered would label all transferred T cells residing within the lung vascular compartment, we carried out control experiments in which recipient mice first received i.v. anti-CD8α mAb-APC followed by adoptive transfer of CFSE+ CTLs with harvesting of the lungs 10 minutes later. Under these conditions, more than 95% of the transferred CFSE+ T cells stained with anti-CD8α mAbs (Figure 2A). At 15 min-

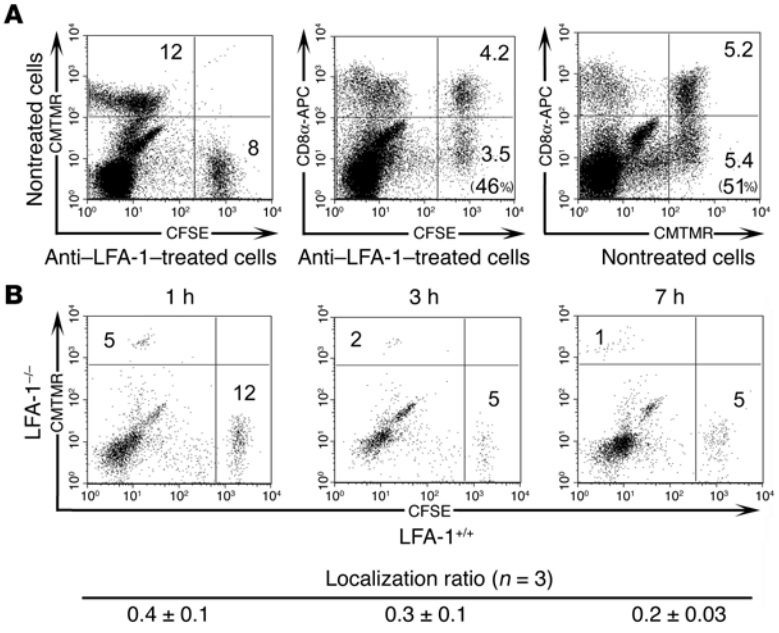

Figure 4
Regulation of the retention time within the lungs but not transmigration into the interstitium by LFA-1. (**A**) Pretreated with anti–LFA-1 mAbs, CFSE-labeled effector CD8+ T cells were mixed with CMTMR-labeled–nontreated effector CD8+ T cells and injected into WT recipient mice. Anti–CD8α-APC mAbs were injected at 6 hours after transfer; lungs were harvested 10 minutes later. The percentages of labeled CD8+ T cells from individual mice are shown in dot plots, the percentages of interstitial cells in parentheses. Results are representative of 6 recipients from a total of 6 independent experiments. (**B**) CMTMR-labeled LFA-1−/− activated CD8+ T cells were mixed with equal numbers of CFSE-labeled activated (LFA-1+/+) CD8+ T cells and injected into WT recipient mice. The percentages of labeled CD8+ T cells from LFA-1−/− and LFA-1+/+ donors localized to the lungs over time are shown. CD8+ T cells were activated by plate-bound anti-CD3/CD28 Abs. Results are representative of 4 recipient mice in a total of 4 independent experiments.

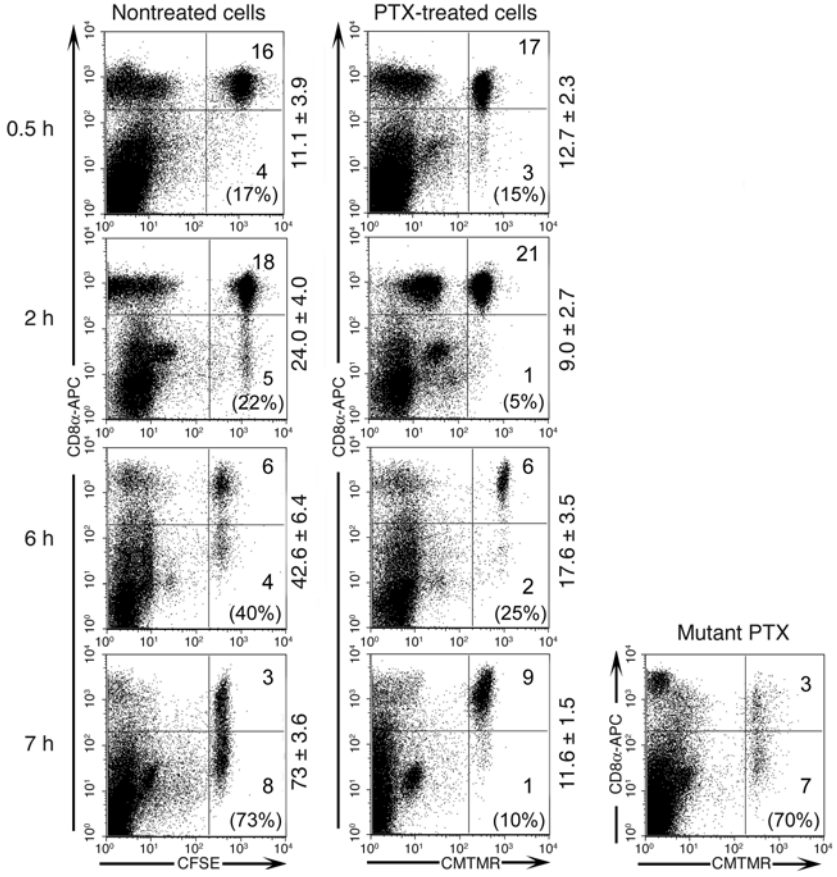

Figure 5
Migration of effector CD8+ T cells into the lung interstitium is PTX-sensitive. PTX-pretreated effector CD8+ T cells or nontreated effector CD8+ T cells were labeled with CFSE and CMTMR, mixed equally, and injected into WT recipient mice. Anti–CD8α-APC mAbs were injected at indicated time points after transfer, and lungs were harvested 10 minutes later. The percentages of total labeled, PTX-treated or control T cells retained in the lungs over time from individual recipient are indicated. The percentages of cells localized to the interstitium ($CFSE^+/CD8\alpha\text{-}APC^-$, $CMTMR^+/CD8\alpha\text{-}APC^-$) are shown in parentheses. Results represent the mean ± SEM from 3 to 9 recipient mice for each time point. In control experiments, cells were treated with mutant PTX (PT9K 129G, provided by E. Hewlett, University of Virginia, Charlottesville, Virginia, USA).

utes after transfer of labeled T cells, essentially all T cells ($CFSE^+$ $CD8\alpha\text{-}APC^+$) were intravascular. By 1 hour after cell transfer, the labeled cells began to migrate into the interstitium — with 22% of cells exhibiting a $CFSE^+$ CD8a-APC^- phenotype. Over the ensuing 2–6 hours, the cell migration from the vascular compartment into the interstitium progressively increased, with approximately 70% of the transferred cells migrating into the interstitium by 6 hours after transfer. As previously reported (17), there was no incremental loss of CFSE staining intensity due to cell division by the transferred effector $CD8^+$ T cells in situ, as these cells were transferred into HA WT recipients. The kinetics of effector $CD8^+$ CTL transmigration into the interstitium is summarized in Figure 2B. As predicted from the imaging analysis (Figure 1B), the total number of $CFSE^+$ effector $CD8^+$ T cells present in the lungs decreased over time after cell transfer. The kinetics of total effector cell transit out of the lungs (Supplemental Figure 1) paralleled the decline in radioactivity determined by ROI analysis using ^{111}In-labeled T cells (Figure 1B). In companion studies, we also analyzed the distribution of CFSE-labeled T cells to other organs at times up to 6–7 hours after cell transfer (liver, 1–5%; blood, < 1%; kidney, < 0.4%; spleen and bone marrow, < 0.1%; LNs, 0%) and confirmed that the transferred effector $CD8^+$ T cells were retained preferentially in the lungs up to 6–7 hours after transfer (Supplemental Figure 2).

Effector CTLs but not early-activated $CD8^+$ T cells transmigrate from the alveolar capillaries to the alveolar interstitium. In order to directly compare the lung-retention time of early-activated and effector T cells as well as the partitioning of the cells between the vascular and interstitial compartments in the same recipient mice, we used a dual-labeling strategy. In preliminary studies, we established that T cell labeling did not affect T cell migration to and retention in the lungs (data not shown). As Figure 3A demonstrates, 6 hours after transfer, the localization ratio of CFSE-labeled effector to CellTracker Orange (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine)–labeled (CMTMR-labeled) early-activated $CD8^+$ T cells recovered from the lungs increased to 2.03 ± 0.2 (n = 3, P < 0.01). This finding was consistent with the dual-modality kinetic analysis of lung-retention time (Figure 1B) and suggested that effector CTLs were retained in the lungs longer than early-activated $CD8^+$ T cells. More importantly, at 6 hours, 65% or more of the effector T cells exhibited a CD8α-APC negative phenotype indicative of migration from the vascular compartment into the interstitium (Figure 3B), while a substantially smaller number of early-activated $CD8^+$ T cells (14%) were able to migrate into the interstitium (Figure 3C). Identical results were obtained when the dye labeling was reversed between 2 different populations of analyzed T cells (data not shown). These results suggest that only $CD8^+$ T cell effectors efficiently transmigrate into the interstitium of noninflamed lungs. Therefore, the decline in the number of early-activated $CD8^+$ T cells in the lungs over time reflects the migration of these intravascular T cells out of the pulmonary circulation directly into the systemic circulation whereas effector $CD8^+$ T cells preferentially migrate into the interstitium, then leave the interstitial compartment, reenter the circulation, and finally localize to the liver.

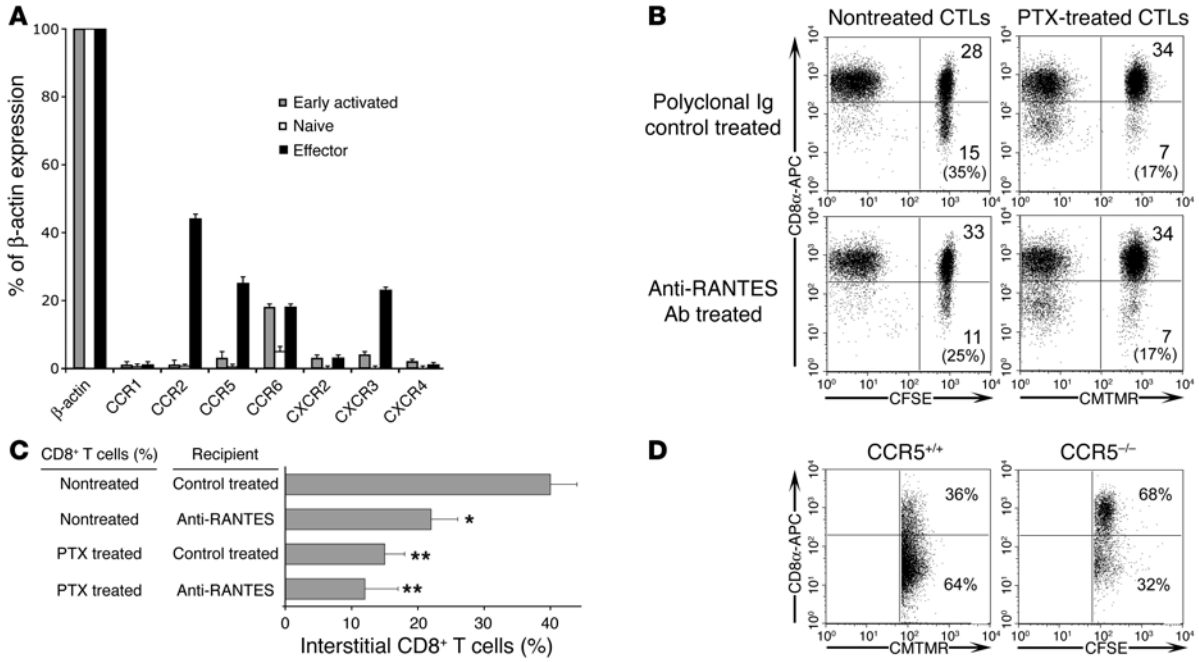

Figure 6

RANTES/CCR5-dependent transmigration of activated effector CD8+ T cells to alveolar interstitium. (**A**) Naive, early-activated, and effector CD8+ T cells were analyzed for expression of chemokine receptors. Results show the percentages (± SEM) of chemokine receptor expression for naive (white bars), early-activated (gray bars), and effector (black bars) CD8+ T cells from total expression of β-actin as a control. (**B**) WT recipients were injected with 1 of the following: polyclonal goat anti-RANTES Ab, polyclonal goat Ig, or PBS. After 1 hour, equal numbers of PTX-treated and nontreated effector CD8+ T cells labeled with CMTMR and CFSE were injected into recipient mice. After 4 hours, anti–CD8α-APC mAbs were injected, and 10 minutes later, lungs were harvested. Counterplots represent cells gated on CFSE+ and CMTMR+ T cells. The percentages of emigrated T cells in vascular (CFSE+/CD8-APC+, CMTMR+/CD8-APC+) and interstitial (CFSE+/CD8-APC–, CMTMR+/CD8-APC–) compartments from individual mice are shown. The percentage of emigrated interstitial labeled cells is shown in parentheses. The results are representative of 6 independent experiments. (**C**) Percentage of interstitial PTX-treated and control CD8+ T cells in the indicated recipients. *$P < 0.05$, **$P < 0.001$ between the percentages of interstitial nontreated T cells in nontreated mice and all other groups. (**D**) Effector CD8+ T cells from CCR5−/− mice and CCR5+/+ littermates were labeled with CFSE and CMTMR, respectively, mixed in ratio 1:1, and injected i.v. into recipient mice. After 7 hours, anti–CD8α-APC mAbs were injected, and 10 minutes later, lungs were harvested. Flow cytometry plots represent cells gated on CD45+ dye-labeled (CD45+/CFSE+ or CD45+/CMTMR+) cells. The percentages of lung-resident transferred CD8+ T cells in vascular (CFSE+/CD8-APC+, CMTMR+/CD8-APC+) and interstitial (CFSE+/CD8-APC–, CMTMR+/CD8-APC–) compartments are shown.

LFA-1 controls the retention of activated CD8+ T cells within the pulmonary vascular bed. At present, little information is available on the factors that control activated T cell migration to and retention in noninflamed peripheral sites such as the alveolar capillary bed and the associated alveolar interstitium. Integrins are likely candidates to play an important role in this process. In particular, the β_2 integrin, LFA-1, has been implicated in regulating T cell migration both to lymphoid tissues and sites of inflammation (20). We examined the impact of in vitro pretreatment of the effector CTLs with blocking anti–LFA-1 mAbs on the pulmonary retention and egress of these cells into the interstitium after adoptive transfer into normal mice.

LFA-1 blockade reduced T cell accumulation in the lungs by approximately 30% (i.e., a localization ratio of anti–LFA-1–treated to untreated cells of 0.7 ± 0.1) (Figure 4A and Supplemental Figure 3). Importantly, LFA-1 blockade had no effect on T cell partitioning between the vascular and interstitial compartments (Figure 4A and Supplemental Figure 4). These results suggest that the LFA-1 expressed at high levels (Supplemental Table 1) on these activated CD8+ T cells had little or no role in T cell transmigration and functions primarily within the vascular compartment to promote the T cell–vascular endothelial cell interaction and the retention of the activated T cells within the alveolar capillary bed.

Since LFA-1 blockade only partially reduced effector CD8+ T cell retention time in the lungs (in part due to the combined effects of blocking antibody dissociation and de novo LFA-1 synthesis during the analysis period in vivo; data not shown) and since early-activated CD8+ T cells also exhibited a prolonged pulmonary retention time, we next tested whether a deficiency in LFA-1 would likewise decrease the retention time of early-activated CD8+ T cells. For this determination, we used in vitro–activated CD8+ T cells from LFA-1–deficient (LFA-$1^{-/-}$) and LFA-$1^{+/+}$ littermates 48 hours after in vitro stimulation with anti-CD3/CD28 Abs. Since LFA-1 signaling contributes to optimum T cell activation by CD3/CD28 crosslinking (21), we examined these activated T cells for expression levels of activation- associated markers (i.e., CD62L, CD44, and CD69) prior to adoptive transfer. No significant difference in the level of cell-surface expression of these markers was noted between LFA-$1^{-/-}$ and control WT T cells (data not shown).

As Figure 4B indicates, the retention of LFA-$1^{-/-}$ CD8+ T cells in the lungs was reduced by up to 65% compared with WT cells with the localization ratio of approximately 0.35 ± 0.1 for the LFA-$1^{-/-}$

cells at each time point examined. This finding supports the view that LFA-1 plays an important role in activated CD8$^+$ T cell migration to and retention within the pulmonary vascular compartment. It should be noted however, that the absence of LFA-1 on T lymphocytes did not completely abolish retention of activated CD8$^+$ cells within lung compartments. Thus, it is likely that other adhesion molecules may also contribute to T cell localization and retention in the normal noninflamed lungs. Blockade of very late antigen-4 (VLA-4; expressed at high levels on activated CD8$^+$ T cells; Supplemental Table 1) had no effect on T cell–retention time (16), and T cell–retention time in normal lungs was likewise unaffected by CD44 blockade (data not shown).

Migration of effector CTLs into the alveolar interstitium is PTX sensitive. Migration of naive as well as activated lymphocytes is regulated by adhesion receptors and chemokines that involve Gαi protein–coupled signaling pathways. To explore the potential role of a Gαi protein–coupled signaling event in initiating migration of effector T cells into the alveolar interstitium, we examined the impact of T cell treatment with the selective Gαi protein–coupled receptor inhibitor, PTX. In preliminary studies, we demonstrated that the PTX treatment did not affect the initial distribution of the transferred cells into other organs (Supplemental Figure 2); therefore, after transfer, equivalent numbers of nontreated and PTX-treated CD8$^+$ T cells localized within the lungs. As shown in Figure 5, after PTX pretreatment, most effector CD8$^+$ T cells (CMTMR$^+$ CD8α-APC$^+$) were retained within the vasculature. Even as late as 7 hours after transfer, a time point at which more than 70% of the untreated CTLs (CFSE$^+$ CD8α-APC$^-$) transmigrate into the interstitium, the majority of the PTX-treated T cells failed to localize in the interstitium (Figure 5). This suggests that, rather than being merely physically trapped within the alveolar capillaries and passively migrating into the interstitium space, the activated CD8$^+$ T cells actively transmigrate into the alveolar interstitium involving a PTX-sensitive Gαi protein–coupled signaling event.

Activation of LFA-1 has been reported to be essential for the initiation of firm adhesion and the subsequent migration of leukocytes out of the vascular compartment into the interstitium; it occurs through a Gαi protein–coupled receptor–mediated cascade (22). It is therefore noteworthy that PTX treatment of effector CD8$^+$ T cells in vitro did not alter either the total number of effector T cells within the lungs (Supplemental Figure 2) or the cell-retention time within the pulmonary vascular compartment, as the localization/retention ratio of PTX-treated and control T cells was essentially equivalent (Figure 5). This finding implies that activation of LFA-1 though Gαi protein–coupled PTX-sensitive signaling pathways may not be crucial for localization and retention of activated effector CD8$^+$ T cells within the alveolar capillary network. Rather, PTX pretreatment preferentially inhibited the T cell transmigration from the vascular compartment into the alveolar interstitium.

Regulation of transmigration of activated effector CD8$^+$ T cells into the alveolar interstitium by RANTES and CC chemokine receptor 5. The finding that early-activated T cells and effector CTLs differed in transmigration capacity offered us the opportunity to explore whether this difference might be linked to the differential expression of 1 or more chemokine receptors for constitutively expressed lung chemokines. To evaluate this possibility, we isolated mRNA from early-activated and effector CD8$^+$ T cells along with RNA from freshly isolated naive CD8$^+$ T cells and subjected the RNA to expression array analysis. As summarized in Figure 6A, both the early-activated and fully differentiated effector T cell populations expressed comparable levels of mRNA for CXC chemokine receptor 2 (CXCR2) and CC chemokine receptor 6 (CCR6) relative to the β-actin control. In contrast, only the effector CTL population expressed high levels of mRNA encoding the CCR2, CCR5, and CXCR3 receptors. Elevated cell surface CCR5 expression at the protein level was confirmed for CTL effectors (Supplemental Figure 5).

This finding raises the possibility that 1 or more chemokine ligands for these receptors may be controlling the transmigration process. Chemokine expression within the lung parenchyma has been examined in numerous murine models of pulmonary inflammation where a diverse array of chemokines has been reported to be upregulated in response to lung inflammation/injury (23, 24). In normal/noninflamed lungs, a limited array of constitutively expressed chemokines could serve as ligands for the chemokine receptors expressed in effector CD8$^+$ T cells. RANTES/CCL5 serves as a ligand for CCR5, CCR3, and CCR1 (25) and has been reported to be a dominant if not exclusive chemokine constitutively expressed within the normal lungs (22) on airway epithelium and at lower levels on vascular endothelium (see Supplemental Figure 6).

To explore the potential role of RANTES in the regulation of effector CTL transmigration from the pulmonary vascular compartment into the pulmonary interstitium, we examined the impact of the RANTES blockade in vivo on the T cell egress from the vascular to the interstitium compartments in adoptive transfer following the administration of polyclonal anti-RANTES Abs into recipients. As an internal positive control, we used PTX-treated effector CD8$^+$ T cells to compare the effects of RANTES blockade with the effects of blockade of G protein–coupled signaling pathway in the same recipient mice. CFSE-labeled/nontreated and CMTMR-labeled/PTX-treated effector CD8$^+$ T cells were transferred into anti-RANTES–treated or either polyclonal goat Ig or PBS-treated recipient mice. As Figure 6B and Supplemental Figure 7 demonstrate, anti-RANTES Ab administration did not affect the effector CTL retention within the lungs but reduced effector T cell transmigration from the vascular to the interstitium by up to 40% compared with control-treated recipients. As exemplified in Figure 6B, only 25% of the lung-associated T cells localized within alveolar interstitium in anti-RANTES Ab-treated recipients while 35% of T cells migrated into the interstitium in polyclonal goat Ig-treated recipients (Figure 6B). It is noteworthy that, in the same experiment, PTX-pretreated effector T cells showed a similar degree of interstitial transmigration whether transferred into anti-RANTES or control recipients (Figure 6B and as summarized in Figure 6C). Taken together, these results suggest that RANTES plays an important role in effector CD8$^+$ T cell transmigration from alveolar capillaries into the pulmonary interstitium.

To assess the role of CCR5 receptor in the CD8$^+$ T cell transmigration into the interstitium, we generated activated effector CD8$^+$ T cells in vitro from both CCR5$^{-/-}$ mice and CCR5$^{+/+}$ littermates and analyzed the partitioning of these populations between pulmonary vascular and interstitial compartments in the same recipient mice. As Figure 6D demonstrates, CCR5$^{-/-}$ CD8$^+$ T cells showed approximately 50% reduction in the transmigration into the interstitium compared with WT CD8$^+$ T cells. Interestingly, neither anti-RANTES Ab administration nor CCR5 deficiency

completely reproduced the PTX-effect, suggesting that other chemokines/chemokine receptors may also be involved in the transmigration of the effector CD8+ cells from pulmonary vascular into the interstitium compartments.

Discussion

The orchestration and implementation of an effective immune response is dependent on the T cell's ability to reach the site of inflammation, recognize antigen, and orchestrate effector functions. The pattern of circulation of both naive and effector/memory T cells is largely dictated by the regulated expression and function of adhesion molecules and chemokine receptors on T cells that interact with their specific ligands expressed on endothelial cells of vessel walls and in the target tissues. Although effector CD8+ T cells enter the lungs during both chronic and acute infections, the factors governing CD8+ T cell traffic to either the normal or infected lungs are not well defined. Also, to date there has been no information evaluating whether the activated CD8+ T cells that are transiently retained in the lungs reside in the vascular compartment or in the interstitium.

It has been previously shown that naive T cells are unable to enter the lungs and that only activated and memory T cells can do so (8, 26). More importantly, naive T cells fail to accumulate in the lungs even during an ongoing respiratory infection, suggesting that these cells lack the combinatorial code of adhesion molecules and/or chemokine receptors that would permit their entry into the infected lungs (9). This implies that the circulating naive T cells would rapidly transit through the pulmonary microcirculation without being retained in the lungs. The significantly shorter transit time for the naive CD8+ T cells supports this view. Our data are also consistent with the migration kinetics reported for adoptively transferred naive CD4+ T cells through normal lungs (19), which, like the naive CD8+ T cells, transit the lungs with similar kinetics and migrate preferentially to the spleen, suggesting that both naive CD4+ and CD8+ T cells may have similar homing patterns.

In contrast, after naive T cells have been activated by TCR engagement, they are retained in the lungs for an extended period, but the pattern of retention is dictated by the activation/differentiation stage of CD8+ T cells. Thus, T lymphoblasts harvested after short-term stimulation (48 hours) of their TCRs are retained in the lungs but remain primarily intravascular. However, fully differentiated CTLs are retained in the lungs for a longer time and efficiently egress from the vascular compartment into the pulmonary interstitium. As discussed below, these distinct patterns of activated CD8+ T cell migration/retention within normal noninflamed lungs are in part controlled by specific adhesion receptor/ligand and chemokine receptor/chemokine interactions in lung compartments.

One of the unresolved questions concerning activated T cell homing is whether the presence of cognate antigen drives effector T cell recruitment/retention at sites of inflammation. Recently, it was reported that, while antigen was necessary to activate CD8+ T cells, the migration of activated T cells to the lungs was independent of the antigen presence in the lungs in a virus infection model (26). However, in that study, the effect of pulmonary viral infection on cytokine/chemokine production on the activation of the pulmonary vascular endothelium and the impact of the presence or absence of antigen on the residence time of the recruited CD8+ T cells were not addressed. We explored this issue by employing a transgenic model where a CTL target antigen, influenza HA, was expressed in the lungs in the absence of virus infection–induced inflammation. Our results using this model as well as previous results (16) suggest that the initial migration and retention of activated CD8+ T cells in the lung is independent of both specific antigen recognition and inflammation-induced chemotactic mediators. However, as reported (17), once the effector CD8+ T cells have entered the interstitium, T cell encounters with specific antigens result in inflammatory mediator release and marked lung inflammation/injury (with additional antigen-dependent effector CD8+ T cell recruitment) by 48–72 hours after T cell transfer. In contrast, in WT recipients of effector CD8+ T cells, no corresponding release of inflammatory mediators (from T cells or alveolar type II cells) is detected (17) even after high-dose T cell transfer, and subsequent recruitment of effector CD8+ T cells into the normal lungs is observed.

Although numerous studies have shown the preferential migration to and prolonged retention of activated T cells within the inflamed lungs, few studies have documented the partitioning of the activated T cells between the pulmonary circulation and the interstitium, and to date, the localization of activated CD8+ T cells within the noninflamed lungs has been unexplored. Our data suggest, we believe for the first time, that CD8+ T cells actively migrate from the vascular compartment into the pulmonary interstitium as a normal consequence of the cell transit through the pulmonary microcirculation. We found that upwards of 70% of the transferred effector CTLs egressed into the interstitium by 7 hours after transfer. Furthermore, the finding that, after leaving the lungs, fully activated CD8 + T cells were preferentially retained in the liver not only supports earlier data implicating the liver as the ultimate site of activated effector CD8+ T cell migration (19, 27) but also suggests an undefined pathway of CD8+ T cell migration from the pulmonary interstitium to the liver.

Leukocyte trafficking/transmigration within the postcapillary venules is a well-regulated process requiring rolling, firm adhesion, and subsequent transmigration. In contrast, pulmonary microcirculation is an extensive network of alveolar capillaries that must maintain close proximity to the external environment in order to carry out gas exchange. Available evidence suggests that the initial capture and rolling events necessary for leukocyte retention and transmigration in the peripheral are not necessary to retain leukocytes within the alveolar microcirculation. This difference is generally attributed to the narrow diameter of the alveolar capillaries, which is typically smaller than the average diameter of the leukocytes (28). However, selectins as well as LFA-1 are involved in firm adhesion of T cells within the lungs (29).

LFA-1 is expressed on all leukocytes and has been implicated as an important accessory molecule for homing to the lungs (19). The results reported here confirm and extend earlier observations (e.g., ref. 16). Specifically, we observed a critical role for LFA-1 in the prolonged residence of T lymphoblasts within the pulmonary compartment. Thus, our findings suggest that the retention of activated T cells within the lungs is not simply passive trapping of T lymphoblasts within pulmonary microcirculation but rather an active LFA-1–dependent process of cell retention within the pulmonary microvasculature. However, as noted above, while we were unable to assign a role for several other adhesion receptors (e.g., VLA-4 and CD44) in the retention of activated CD8+ T cells within the normal lungs, other as yet undefined adhesion receptor/ligand pairs probably also contribute to this process. Furthermore, although LFA-1 likely plays a dominant role in the reten-

tion of activated CD8+ T cells within the pulmonary vasculature, it appears to have minimal impact on activated CD8+ T cell transmigration into the pulmonary interstitium. In addition, our results suggest that the egress of activated T cells out of the lungs and the ultimate homing of the cells to the liver are not dependent on LFA-1 expression/upregulation.

Chemokines are involved in the regulation of homeostatic recirculation of naive lymphocytes as well as in homing of antigen-experienced T cells into nonlymphoid tissues (7). Currently, the best understood pathways of tissue-specific migration of naive T cells are in the LNs and Peyer patches (2, 30) and in certain nonlymphoid tissues such as skin and intestine (1). However, the role of chemokines in regulating activated T cell–trafficking pathways to other noninflamed peripheral sites such as lungs, liver, and kidney has not been investigated.

To define the mechanism by which activated effector CD8+ T cells preferentially transmigrate into the pulmonary interstitium and to determine the contribution of chemokines and chemokine receptors in this process, we blocked Gαi-heterotrimeric G protein–coupled receptor signaling in T cells with PTX. PTX treatment of the T cells prior to transfer did not affect T cell retention time within the lungs but markedly inhibited T cell egress from the vascular compartment into the interstitium. Since chemokine-dependent and chemokine receptor–mediated signaling occur via a PTX-sensitive pathway (31), this finding provides initial evidence suggesting a role for chemokine-dependent signaling in T cell transmigration.

There have been a number of studies analyzing chemokine production in the respiratory tract in response to pulmonary infection or inflammation, with the upregulation or induction of the chemokines RANTES, macrophage inflammatory protein-1α (MIP-1α), MIP-1β, IFN-γ–inducible protein-10 (IP-10), and monocyte chemoattractant protein-1 (MCP-1). This upregulation represents a prominent feature of the pulmonary chemokine response throughout the course of infection (32). However, only RANTES has been reported to be constitutively expressed at significant levels in the normal lungs (32). RANTES/CCL5 is a C-C chemokine that binds CCR1, CCR3, and CCR5 and promotes the migration of monocytes, activated T cells, and eosinophils (25). RANTES is constitutively produced by a variety of cell types, including airway epithelial cells (33), and is readily detectable on airway epithelial cells and at lower levels on vascular endothelia of normal lungs. Since the chemokine ligands for 2 of the chemokine receptors exclusively expressed on effector CTLs (i.e., Mig, IP for CXCR3, and MCP-4, MCP-5 for CCR2) have not been reported to be constitutively expressed within normal lungs, the CCR5 ligand RANTES was the most likely candidate chemokine to orchestrate the T cell transmigration process. Indeed, we observed that the administration of polyclonal Abs to RANTES partially inhibited CD8+ T cell transmigration into the normal lung interstitium. This finding implicate RANTES as a likely regulator of activated T cell homing and migration from the pulmonary vascular compartment into the interstitium. Although RANTES is unlikely to be the sole stimulus for T cell transmigration, we have to date been unable to identify other chemotactic stimuli (e.g., MCP-1, LTB4, etc.) that are expressed in the normal/noninflamed lungs and for which appropriate receptors are expressed on activated CD8+ T cells.

T cells isolated from the intestines, skin, liver, and lung preferentially express CCR5 and CXCR3, suggesting that these receptors provide homing capacity for these cells to nonlymphoid tissues or that they may contribute to T cell retention within tissues (34). Skin lymphocytes are enriched in CLA expression; intestinal T cells express $\alpha_4\beta_7$ whereas lung T cells have high LFA-1 levels. Thus, the unique combination of adhesion receptor/ligand interactions directs homing of activated T cells into different tissues, and chemokine/chemokine receptor interactions provide a signal for migration within the peripheral site. For effector CD8+ T cell homing to the normal lung and migration into the noninflamed interstitium, this adhesion/chemokine receptor combination appears to be LFA-1^{high}/CCR5+. The inability of PTX to inhibit LFA-1–dependent retention of activated T cells within the pulmonary vasculature was unexpected and may simply reflect the simultaneous elevated display of LFA-1 by the T cells and its ligand (ICAM-1) by the noninflamed pulmonary capillaries. However, we cannot exclude the possibility of a PTX-insensitive mechanism of LFA-1 activation (35) accounting for this result, as the LFA-1 expressed by the activated CD8+ T cells can undergo divalent cation–dependent increased binding to ICAM-1 (data not shown). Trafficking studies with activated CD8+ T cells from CCR5$^{-/-}$ mice also suggest that migration of activated CD8+ T cells to the normal pulmonary interstitium requires, at least in part, CCR5-dependent signaling.

Recent evidence suggests that upon systemic infection, effector/memory CD8+ T cells are widely disseminated into a number of peripheral sites, including the lungs (3). Our observations suggest that the trafficking of effector CD8+ T cells to peripheral tissues may be an active process that is partially dependent on LFA-1 expression by T cells for migration to and retention in the pulmonary microvasculature and CCL5/CCR5-dependent signaling for T cell transmigration into the noninflamed pulmonary interstitium. It will be interesting to investigate whether or not the small fraction of effector T cells which are retained in the pulmonary interstitium (i.e., do not transit from the lungs to the liver after i.v. transfer) may be the source of peripheral memory CD8+ T cells found in this site.

In conclusion, we have described in this report a novel mechanism for the peripheral homing and retention of effector CD8+ T cells in the respiratory tract that is not dependent on inflammation/infection of the respiratory tract and would function independently of the initial site of infection. Since the respiratory tract is the major portal of entry of microorganisms in the body, such a mechanism may ensure that adequate numbers of memory T cells are available in the lungs after vaccination or infection at a distal site to respond to subsequent infection of the respiratory tract.

Methods

Mice. HA-transgenic mice (10), LFA-1$^{-/-}$ mice (20) on BALB/c background, CCR5$^{-/-}$ and CCR5$^{+/+}$ littermates (36), and BALB/c mice (Jackson Laboratory) were used in this study. Animals were bred and maintained under specific pathogen-free conditions and used at 8–12 weeks of age. The plan for these studies was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Virginia Medical Center.

^{111}In labeling and dual modality imaging. On day 5 following stimulation with irradiated syngeneic splenocytes that were infected with A/Japan/305/57 influenza, cloned influenza HA–specific CD8+ CTLs (used as a defined source of differentiated effector CD8+ CTLs) separated from stimulators by density gradient centrifugation (17). In preliminary studies, splenocytes from influenza HA–immune donors were stimulated in vitro with A/Japan/305/57-infected splenocytes, harvested at days 5–7 of culture, and after CD8+ T cell purification, used as a source of dif-

ferentiated effector CTLs. Purified effector CD8+ T cells were adoptively transferred into HA-transgenic and control recipients (17), then analyzed by cytotoxicity in vitro. Isolated splenic naive CD8+ T cells were analyzed directly or stimulated with 5 μg/ml plate-bound anti-CD3/CD28 Abs (BD Biosciences — Pharmingen) for 48 hours (early-activated CD8+ T cells). These early-activated CD8+ T cells and cloned HA-specific CTLs were analyzed for cell surface expression of CD25, CD69, CD44, L-selectin, CCR5, LFA-1, and VLA-4 (all Abs and isotypes from BD Biosciences — Pharmingen) by flow cytometry. For labeling with ^{111}In, 5×10^6 CD8+ T cells were resuspended in 100 ml HBSS containing 50 μg tropolone (Sigma-Aldrich) and 200 μCi ^{111}In (PerkinElmer) and incubated for 15 minutes at room temperature, washed, resuspended in 300 μl RPMI and injected i.v. in mice following administration of ketamine (125 μg/g body weight; Ketalar; Parke Davis) and xylazine (12.5 μg/g body weight; Phoenix Scientific). The efficiency of ^{111}In uptake by the cells ranged from 25 to 30% in multiple experiments. Cell viability after ^{111}In labeling was always greater than 95%. Dual modality whole-body imaging was done at indicated time points following cell transfers using a charge coupled device–based x-ray detector and γ-camera. The x- and γ-ray detectors were both designed and built with spatial resolution that is appropriate for small animal research (0.05 mm and 1.8 mm pixel sizes). Following image acquisition, the Interactive Data Language imaging software (version 5.6; Research Systems Inc.) was used to merge the x-ray and γ-ray images to obtain a single coregistered image containing both structural (x-ray) and functional (γ-ray) information. Quantitative analysis of radioactivity in the lungs was done on the merged images by defining the ROI, using the rib cage as a reference to identify the lungs.

Chemokine receptor gene expression. Total RNA from naive, early-activated, and effector CD8+ T cells was extracted by TRIzol reagent kit (Invitrogen Corp.), and chemokine receptor gene expression was determined using the mouse chemokine receptor GEarray kit (SuperArray).

In vivo trafficking experiments. To study the kinetics of CD8+ T cell partitioning between vascular and interstitium compartments, 4×10^6 CFSE-labeled activated effector CTLs were used in adoptive transfer. The homing of different populations of CD8+ T cells was compared directly in individual mice using differently fluorescent dyes. In brief, different CD8+ T cell populations were incubated with either 0.1 μM CFSE (Invitrogen Corp.) in PBS or 7 μM 5 CMTMR (Invitrogen Corp.) in RPMI at 37°C for 20 minutes, then washed twice with 1 % FBS in PBS or with RPMI, respectively. Labeled cells were mixed in a ratio of 1:1, and 12×10^6 labeled cells were injected i.v. into recipient mice. We injected 20 μg anti-CD8α-APC mAbs (clone 5H10; CALTAG Laboratories) i.v. at different time points; 10 minutes later, mice were sacrificed, and lungs were harvested and digested with 125 U/ml collagenase type XI, 60 U/ml hyaluronidase type I-s, and 60 U/ml DNAse1 (all Sigma-Aldrich) in the presence of saturating amounts of unlabeled anti-CD8α mAbs (60 μg/ml) at 37°C for 30 minutes. The percentages of CFSE and CMTMR-labeled cells in the total population of gated live lymphocytes in different organs were determined by flow cytometry on a FACSCalibur (BD) with WimMDI software, version 2.8 (Joseph Trotter, Scripps Research Institute). The localization ratio from the percentages of CFSE and CMTMR-labeled cells in each organ was calculated. If the ratios in injected populations were not exactly 1, a correction coefficient was applied to normalized localization ratios.

To analyze the role of LFA-1 in the retention of activated CD8+ T cells within lungs, the combinations of LFA-1$^{-/-}$ and LFA-1$^{+/+}$ CD8+ T cells activated for 48 hours by anti-CD3/CD28 mAbs or effector CD8+ T cells pretreated with 300 μg of anti–LFA-1 mAbs (TIB-217; ATCC) for 30 minutes on ice and nontreated effector CTLs were used for adoptive transfer. To block the Gαi function, activated effector CTLs were preincubated with 200 ng/ml of PTX (Sigma-Aldrich) at 37°C for 2 hours, washed, mixed with nontreated effector T cells, and used for adoptive transfer. To assess the RANTES implication in the migration of activated CD8+ T cells to the lungs interstitium, 50 μg of polyclonal goat anti-RANTES Abs or either polyclonal goat Ig (both Santa Cruz Biotechnology Inc.) or PBS was injected i.v. followed by adoptive transfer of CD8+ T cells 1 hour later. To generate effector CD8+ T cells, splenocytes and cells from peripheral LNs from both CCR5$^{-/-}$ mice and CCR5$^{+/+}$ littermates were obtained. Pooled cell suspensions (5×10^6 cells/ml) were stimulated with 5 μg/ml plate-bound anti-CD3/CD28 Abs for 5 days and then kept in media containing murine IL-2 (10 IU/ml) for the next 9 days. On day 14, CD8+ T cells were purified (purity > 95%) and restimulated (2×10^5 cells/ml) with plate-bound anti-CD3/CD28 Abs. Activated CD8+ T cells were collected on day 5 after restimulation and used for adoptive transfer experiments.

Statistics. All data represent means ± SEM. The localization ratio was compared with the theoretical ratio by paired 2-tailed Student's *t* test. Statistical significance was set at $P < 0.05$.

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