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CC chemokine receptor 7 required for T lymphocyte exit from peripheral tissues

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Abstract

Lymphocytes travel throughout the body to carry out immune surveillance and participate in inflammatory reactions. Their path takes them from blood through tissues into lymph and back to blood. Molecules that control lymphocyte recruitment into extralymphoid tissues are well characterized, but exit is assumed to be random. Here, we showed that lymphocyte emigration from the skin was regulated and pertussis toxin-sensitive. CD4⁺ lymphocytes emigrated more efficiently than CD8⁺ or B lymphocytes. T lymphocytes in the afferent lymph expressed functional CCR7, and CCR7 was required for T lymphocyte exit from the skin. The regulated expression of CCR7 by tissue T lymphocytes may control their exit, acting with recruitment mechanisms to regulate lymphocyte transit and accumulation during immune surveillance and inflammation.

INTRODUCTION

Naïve T cells preferentially recirculate between blood and secondary lymphoid tissues, entering lymph nodes from the blood by crossing high endothelial venules^{1–3}. Memory and effector T cells, unlike naïve T cells, can migrate efficiently into non-lymphoid tissues and into sites of inflammation and infection (reviewed⁴), subsequently entering afferent lymphatic vessels and traveling to local lymph nodes in the afferent lymph^{3,5,6}. In parabiotic mouse models, endogenous memory T cells in most peripheral tissues reach equilibrium with immigrating blood-borne donor T cells within a week, suggesting that there is rapid turnover of T cells in peripheral tissues⁷. Indeed, blood to lymph migration through peripheral tissues such as the skin is extraordinarily dynamic with a peak transit time for lymphocytes of approximately 24 h⁸. Classic studies of recirculation in the sheep demonstrated that 1/10 of the total lymphocytes⁹ and a major fraction of memory-effector lymphocytes³ entering a given lymph node do so through the afferent lymph. Collectively, these studies indicate that

lymphocyte exit from tissues and migration via afferent lymph into the draining nodes is a critical homeostatic migration pathway for memory and effector T cells.

The afferent lymph from tissues such as the skin predominantly contains memory CD4⁺ T cells and few B cells^{5,6,10,11}, which mirrors the preferential recruitment and accumulation of CD4⁺ T cells relative to B cells in peripheral tissues. This finding and the fact that erythrocytes can enter the afferent lymph when injected into tissues, led to the current notion that lymphocyte migration into the afferent lymph is a passive and random process, determined primarily by fluid flow¹². This concept contrasts with the tight control of the various other steps of lymphocyte recirculation, from the multi-component combinatorial control of recruitment of circulating lymphocytes from the blood¹³ to the regulation of lymphocyte exit from lymph nodes by cell-cell interactions and sphingosine 1-phosphate and its receptors^{14,15}.

To directly assess the selectivity of lymphocyte exit from non-lymphoid tissues, we have established an experimental system in which the migration of adoptively transferred lymphocytes from subcutaneous tissues into draining lymph nodes is monitored. Our studies show that lymphocyte exit into afferent lymphatics is a tightly regulated process under the control of the chemokine receptor CCR7.

RESULTS

Tissue exit is non-random

Earlier studies assumed that tissue exit is a random process, and that lymphocytes within the interstitium could passively enter afferent lymph¹². To study lymphocyte exit from a peripheral site, we injected 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester- (CFSE) or PKH26 Red Fluorescent Cell Linker- (PKH26) labeled murine splenocytes subcutaneously into the footpads of recipient mice and evaluated the number and phenotype of cells that migrated into the draining popliteal lymph nodes (Fig. 1a). The frequency of migrated cells increased from 3 to 18 h; a 12-hour time point was selected for most subsequent experiments, because at this time after injection, the blood, spleen and contralateral lymph nodes remained essentially devoid of transferred cells (not shown), implying that cells that had migrated via the afferent lymph and reached the draining lymph node had not yet entered the circulation.

If lymphocyte exit were random, then the cells recovered from the draining lymph node would have the same cellular composition as the injected population. Instead, CD4⁺ T cells were dramatically enriched among cells recovered from the lymph node compared with the injected population (Fig. 1a). Naïve (CD44^{lo} CD45RB^{hi}) CD4⁺ T cells migrated somewhat better than memory (CD44^{hi} CD45RB^{lo}) CD4⁺ T cells as indicated by a slight enrichment of naïve CD4⁺ T cells in the migrated fraction compared with the injected cells (Fig. 1b). Although there was substantial variability in the overall numbers of migrated cells recovered from the draining lymph nodes of individual mice, CD4⁺ T cells consistently exited the footpads more efficiently than CD8⁺ T cells or B cells (Fig. 1c, left panel). CD4⁺ T cells migrated, on average, ~12-fold and ~3-fold more efficiently than CD8⁺ T cells and B cells, respectively (Fig. 1c, right panel). We conclude that exit from peripheral sites is a selective process.

Tissue exit requires G_{αi} protein-coupled receptors

To ask whether T cell exit from peripheral tissues and migration into the draining lymph node through the afferent lymph is an active process that involves chemoattractant receptor signaling, we treated mononuclear cells from spleen with pertussis toxin (PTX), which irreversibly modifies G_{αi} proteins and prevents most chemotactic responses. PTX-treated splenocytes were labeled with CFSE and injected into the footpads of recipient mice. Concurrently, equal numbers of mock-treated PKH26-labeled splenocytes were injected into

the contralateral footpads, and migration to the draining lymph nodes was assessed 12 h later. PTX pre-treatment almost completely inhibited lymphocyte migration to the draining lymph node (Fig. 2). The same result was obtained when the dyes were reversed (data not shown). At later time points after transfer (18–24 h), infrequent untreated cells, but no PTX-treated cells, could be detected in the recipients' blood (data not shown). Cells re-isolated from the injected footpad (whether PTX-treated or not) excluded propidium iodide and failed to bind annexin V, excluding a general effect on cell viability. We conclude that tissue exit is a regulated, non-random, G α_i -coupled receptor signaling-dependent process that likely involves chemoattractant receptors.

CCR7-deficient lymphocytes show impaired tissue exit

Having established a role for G protein signaling in tissue exit, we next tested whether CCR7 expression controls lymphocyte exit from peripheral tissues. To evaluate the migration of CCR7-deficient (*Ccr7*^{-/-}) cells, a mixture of CFSE-labeled splenic lymphocytes from *Ccr7*^{-/-} mice¹⁶ and PKH26⁺ wild-type lymphocytes or a control mixture of CFSE- and PKH26-labeled lymphocytes from wild-type mice were injected into the footpads of recipient mice. At 12 h after transfer, equal numbers of CFSE⁺ and PKH26⁺ wild-type cells reached the draining lymph nodes (Fig. 3a, left panel). In contrast, CFSE⁺ *Ccr7*^{-/-} cells were much less frequent in the draining lymph node (Fig. 3a, right panel): the migration of *Ccr7*^{-/-} CD4⁺ T cells was reduced by 90 % (Fig. 3b), and CCR7 deficiency inhibited the migration of CD45RB^{hi} naïve and CD45RB^{lo} memory CD4⁺ cells to the same extent (Fig. 3b). The relatively inefficient exit of CD8⁺ T cells and B cells from the skin (Fig. 1) was also largely CCR7-dependent (Fig. 3b). These differences in migration between *Ccr7*^{-/-} and wild-type lymphocyte subsets were apparent at 3 h, 6 h, and 9 h after injection as well, although fewer cells had reached the lymph nodes at these earlier time points (data not shown). As observed for wild-type lymphocytes, at these time points, and in most experiments at 12 h after injection, no *Ccr7*^{-/-} cells were detected in the spleen, the site of preferential localization of *Ccr7*^{-/-} lymphocytes¹⁶ or in the contralateral lymph nodes; and no cells were seen in blood (data not shown). These observations implicate CCR7 as a key regulator of T lymphocyte exit from the skin and subsequent migration into the draining lymph node.

Transduced CCR7 rescues the exit of *Ccr7*^{-/-} T cells

The impaired migration of *Ccr7*^{-/-} lymphocytes from the skin to the draining lymph node strongly suggested that CCR7 mediates T cell exit from peripheral sites. However, T cells from *Ccr7*^{-/-} mice could be abnormal due to their development in an organism with disrupted lymphoid architecture. Therefore, we asked if reintroducing CCR7 could restore the tissue exit of *Ccr7*^{-/-} T cells. We transduced *Ccr7*^{-/-} CD4⁺ T cells with a retroviral expression vector encoding CCR7 and an internal ribosomal entry site-green fluorescent protein (IRES-GFP) ('CCR7') or with a control vector encoding only GFP ('control vector'). To confirm expression of surface CCR7, we tested cell binding to a fusion protein consisting of the murine CCR7 ligand, CCL19, fused to human immunoglobulin G¹⁷ (CCL19-Ig). Wild-type T cells expressed high amounts of surface CCR7 (Fig. 4a, left panel), whereas *Ccr7*^{-/-} T cells, whether transduced with the control vector or not, failed to bind CCL19-Ig (Fig. 4a, middle). *Ccr7*^{-/-} T cells transduced with CCR7 (GFP⁺) bound CCL19-Ig (Fig. 4a, right panel), although less efficiently than wild-type cells. Consistent with the CCR7 surface expression, wild-type T cells migrated efficiently toward the CCR7 ligand, CCL21, in an *in vitro* chemotaxis assay, whereas *Ccr7*^{-/-} T cells responded only when transduced with CCR7 (Fig. 4b). Transducing cells with the control vector had no influence on the capacity of wild-type or *Ccr7*^{-/-} T cells to exit the skin of congenic Thy1.1 mice (Fig. 4c, left and middle panel). In contrast, transducing *Ccr7*^{-/-} CD4⁺ T cells with CCR7 rescued their ability to migrate to the draining lymph node (Fig. 4c, right panel). We conclude that the migratory defect of *Ccr7*^{-/-} T cells is due to their lack of CCR7.

T cells in the afferent lymph express functional CCR7

If physiologically recirculating T cells use CCR7 to exit from peripheral tissue sites, then T cells in the draining afferent lymph should express functional CCR7. To test this prediction, we cannulated the skin draining afferent lymph vessels (proximal to the draining lymph node) of sheep, an animal used in classical recirculation studies. We analyzed CCR7 (CCL19-Ig binding) expression by memory (CD45R⁻) and naïve (CD45R⁺) T cell subsets. Consistent with prior reports³, almost all CD4⁺ T cells in afferent lymph draining the skin were of memory phenotype (Fig. 5a). Most of these afferent lymph CD4⁺ T cells bound CCL19-Ig (Fig. 5a). Memory CD4⁺ T cells in the blood were divided into major CCL19-Ig binding and non-binding populations, as expected^{18,19}. Almost all afferent lymph CD8⁺ T cells were also of memory phenotype (CD45R⁻), and the majority of these bound CCL19 as well (Fig. 5a; range: 50–89 %; mean: 69.8 %, from 6 different experiments). Moreover, in Transwell chemotaxis assays, memory T cells from afferent lymph and from blood migrated efficiently toward the CCR7 ligands CCL21 and CCL19 (Fig. 5b). Lymph memory T cells exhibited equal or greater sensitivity toward CCR7 ligands relative to blood memory T cells as indicated by half-maximal responses at lower chemokine concentrations (Fig. 5b). These data demonstrate that shortly after tissue exit, and prior to arrival at the draining lymph node, memory T cells that recirculate through the skin express CCR7 and are enriched in responsiveness to CCR7 ligands.

DISCUSSION

Lymphocyte recirculation is a highly regulated process, controlling the distribution and targeting of immune cells throughout the body. Migration from the blood into lymphoid as well as peripheral tissues is determined by complex, combinatorially determined events involving multiple molecules that together regulate the tissue access of specialized lymphocyte subsets. Exit of lymphocytes from organized lymphoid tissues into the efferent lymphatics is controlled by distinct mechanisms, involving sphingosine 1-phosphate and its receptors^{14, 15}. The exit of lymphocytes from peripheral tissue such as the skin, however, has long been assumed to be random¹². The goal of our study was to evaluate the selectivity and mechanisms of lymphocyte exit from peripheral tissues.

Our findings reveal that lymphocyte exit into afferent lymphatics, far from being random, is controlled by specific chemotactic mechanisms, and that CCR7 is an essential mediator of this process. Memory as well as naïve CD4⁺ T cells exit more efficiently compared to CD8⁺ T cells or B cells, but CCR7 is important in each case. The enhanced efficiency of exit into lymph by CD4⁺ T cell relative to CD8⁺ T cells or B cells is mirrored in the predominance of CD4⁺ T cells in afferent lymph^{5,6,10,11}. The differential emigration also suggests that additional signals may be required for tissue exit, because CD4⁺ and CD8⁺ T cells from murine spleen have similar CCR7 expression¹⁷. Additional control mechanisms might include interactions with adhesion molecules expressed by lymphatic endothelium such as Clever-1²⁰. The results are in line with previous reports of a higher capacity of CD4⁺ relative to CD8⁺ T cells to recirculate from blood through tissues such as the peripheral and mesenteric lymph nodes²¹, intestine, and skin²². The reason for these T cell subset-specific differences in recirculation remains to be elucidated.

The critical role of CCR7 in T cell recirculation at the stage of exit from peripheral tissues may be considered surprising in light of concepts of CCR7 as a marker for “central memory T cells” that circulate exclusively through secondary lymphoid organs^{18,23}. Recent studies, however, have shown that CCR7 is expressed by T cells that exert immediate effector functions^{17,24, 25}, and by many memory-effector T cells that infiltrate extralymphoid tissues, including the skin, synovium, and lung^{19,24,26}.

Moreover, the CCR7 ligand, CCL21, is constitutively expressed by lymphatic endothelial cells within extralymphoid tissues²⁷⁻²⁹, where it is known to mediate the CCR7-dependent migration of mature antigen-presenting dendritic cells into the afferent lymph^{16,28,30,31}. CCL21-expressing lymphatics can be visualized immunohistochemically within skin, primarily adjacent to venules involved in lymphocyte recruitment or immediately underlying the epithelium^{32,33}; thus, they are well positioned to mediate the CCR7-dependent exit of T cells from physiologic sites of lymphocyte infiltration. In this context, the efficiency of short term exit of footpad-injected cells is expected to depend not only on their expression of CCR7, but also on their proximity to these lymphatic-rich microenvironments; the distribution of injected cells to the deep connective tissues versus the superficial dermis may thus underlie the relatively low overall efficiency and may also explain the animal to animal variability of T cell exit in our experiments. In addition, in normal skin, in the absence of inflammatory stimuli, CCL21 expression by lymphatic endothelial cells is relatively low^{32,33}, perhaps paralleling the low rate of homeostatic lymphocyte recirculation through this site. Interestingly, the efficiency of CD4⁺ T cell migration we observed (range, 0.3 to 1.7 percent of injected cells; mean of 5 experiments = 0.81) is similar to that reported for dendritic cells in similar studies (0.01 to 3% migration, 48 h after injection³²). CCR7-CCL21 interaction also controls lymphocyte migration from the blood into secondary lymphoid tissues through high endothelial venules^{16,34,35}. Our results thus extend our understanding of the roles of CCR7, suggesting a unifying mechanism for the control of lymphocyte access to secondary lymphoid tissues, whether via high endothelial venules from the blood or via lymphatic endothelium and afferent lymph from peripheral tissues.

Exit is likely to be as important as entry in controlling the accumulation of lymphocytes at peripheral sites. In this regard, the importance of CCR7 in lymphocyte transit may help explain observations of lymphocyte accumulation in the gastrointestinal tract, lung, and body cavities of *Ccr7*^{-/-} mice (Uta E. Höpken and M.L., unpublished data). In a mouse model of influenza, most influenza virus-specific interferon- γ -positive (IFN- γ ⁺) CD4⁺ T cells in the lungs of actively infected mice are CCR7-negative, but the majority of bystander effector T cells (which produce IFN- γ in response to polyclonal stimuli) are CCR7-positive²⁶. Moreover, antigen recognition transiently down-regulates CCR7 function on T cells, at least in lymph nodes³⁶. Together, these data suggest that down-regulation of CCR7 on actively responding effector T cells may help antigen-specific T cells accumulate at the peripheral sites of antigen-recognition³⁷, while allowing bystander memory and effector T cells to exit rapidly and continue their immune surveillance elsewhere. Consistent with this concept, *in vitro* activated CCR7-transgenic effector T cells show a deficit in pulmonary accumulation in asthma models associated with enhanced exit from the lung and migration into the draining mediastinal lymph nodes compared to control (CCR7-negative) Th2 cells (Shannon Bromley and Andrew Luster, personal communication).

Of course, a substantial fraction of normal circulating memory and effector T cells lack detectable CCR7 expression even in the blood; these cells may be destined to enter but not leave tissue sites, or may be able to up-regulate the CCR7 'exit pass' after tissue entry. It will be important in future studies to characterize the regulation of CCR7 expression on different lymphocyte subsets during peripheral immune responses, and to evaluate the relevance of CCR7-regulated lymphocyte exit to the course of inflammation and the defense against pathogens.

In conclusion, we have shown that lymphocyte exit from tissues into lymph is selective and active, and that CCR7 is key in this process. These findings fill a major gap in our understanding of the cycle of lymphocyte recirculation, and reveal tissue exit as a novel potential control point for the modulation of local inflammatory and immune responses.

METHODS

Animals, surgery, and lymph cannulation

For cell transfers, sex and age-matched BALB/c *Ccr7*^{-/-} and wild-type mice were used. Donor mice for experiments comparing memory T cell exit were 6–9 months of age; those used for transduction were 6 weeks old. As recipients, 6–12 week-old recipient BALB/c mice (Jackson Laboratories) or Thy1.1 congenic BALB/c mice (a gift from I. Weissman, Stanford University) were used. 8–10 month old randomly bred sheep (Central Animal Facility, University of Guelph, Ontario, Canada) or Cheviot wethers (Ovis, South Dakota) were used. For the collection of pseudoafferent lymph³⁸, subiliac lymph nodes were surgically removed >4 weeks before lymph cannulation as described³⁸. Afferent (popliteal) and pseudoafferent lymph vessels were cannulated, and lymph was collected over 12-h periods as described³⁸. The cannulated lymph vessels drained the skin, muscles, and bones of the hind leg or the rear flank and abdomen³⁹. All animal experiments were approved by local review boards (Veterans Affairs Palo Alto Health Care System Institutional Animal Care and Use Committee (IACUC), Stanford University's Administrative Panel on Laboratory Animal Care, South Dakota State University IACUC, and the University of Toronto Animal Care Committee), and performed according to institutional, federal, and state guidelines.

Cell isolation, PTX treatment, cell labeling, and transfer

Cells were isolated from murine lymph nodes and spleens as described²⁶. Ovine lymphocytes from lymph were washed with RPMI 1640 containing 5% FCS and 25 mM HEPES, pKa 7.55 (cRPMI). Ovine blood was collected from the jugular vein and mixed with anti-coagulant. Mononuclear cells were isolated from murine spleen and ovine blood by gradient centrifugation using Histopaque (Sigma). In some experiments, CD8⁺ T cells were enriched 5-fold in the injected population by negative selection using CD19 microbeads (Miltenyi Biotec). For PTX treatment, splenocytes were incubated for 2 h at 37°C with 200 ng/ml PTX (Sigma) in cRPMI. Cell labeling with PKH26 was performed according to the manufacturer's instructions (Sigma). For CFSE (Molecular Probes) labeling, cells at 5×10⁶/ml were incubated in 0.2 μM CFSE in HBSS containing 25 mM HEPES for 5 min at 37°C. PKH26 and CFSE labeling reactions were stopped by adding serum. Cells were subsequently washed three times using cRPMI followed by one PBS wash. 1–5×10⁶ cells in 10 μl PBS were injected into the footpads of recipient mice. 12 h after transfer, single cell suspensions of the draining popliteal lymph nodes were analyzed for transferred cells (identified by fluorescent labels or Thy1.2 congenic marker), and total cell numbers were enumerated by flow cytometry using a fixed number of polystyrene beads (Polybead, Polysciences). In experiments comparing the relative migration efficiency of *Ccr7*^{-/-} to wild-type cells of specific lymphocyte subsets (total lymphocytes, total CD4⁺ T cells, naïve (CD45RB^{hi}) and memory (CD45RB^{lo}) CD4⁺ T cells, CD8⁺ T cells, and B cells), the ratio of migrated CFSE⁺ (*Ccr7*^{-/-} in test mice; wild-type in control mice) to PKH26⁺ cells (admixed wild-type cells as a common internal standard) of the indicated subset was determined by flow cytometry. The relative migration of *Ccr7*^{-/-} to wild-type cells was determined by calculating the ratios of migrated CFSE/PKH26 to injected CFSE/PKH26 cells within each lymphocyte subset. To control for variability in overall recovery, results were normalized to the mean ratio of CFSE⁺ wild-type to PKH26⁺ wild-type cells for each subset (set as 100%). In different experiments, the injected wild-type and *Ccr7*^{-/-} populations consisted of 18–25 % CD4⁺ with 22–40 % of these being CD45RB^{lo}, 48–67 % CD19⁺ B cells, and 10 % CD8⁺ (after enrichment).

Retroviral transduction

Human CCR7 upstream of an internal ribosomal entry site-green fluorescent protein (IRES-GFP) construct in MSCV 2.2β retroviral vector⁴⁰ was provided by D. Campbell, University of Washington. Virus was produced as described⁴¹. Thy1.2⁺ CD4⁺ T cells were isolated from

spleens of wild-type and *Ccr7*^{-/-} mice using CD4 microbeads according to the manufacturer's instructions (Miltenyi Biotec) and stimulated with plate-bound Abs to CD3 and CD28. 24 h after stimulation, the cells were infected with retrovirus containing either CCR7 and IRES-GFP or only IRES-GFP (control vector), as described⁴¹. On day 4 or 5 of culture, the cells were used for experiments.

Flow cytometry

Murine and ovine cells were pre-incubated in rat IgG plus anti-CD16/CD32 (2.4G2, BD Pharmingen) or sheep IgG plus donkey IgG, respectively. Purified IgGs were purchased from Jackson Immunoresearch. The following biotin- or fluorochrome- (phycoerythrin, peridinin chlorophyll-protein-cyanine 5.5, allophycocyanin, Alexa Fluor 647, phycoerythrin-cyanine 7, or fluorescein isothiocyanate) conjugated rat monoclonal antibodies recognizing murine surface markers, obtained from BD Pharmingen, were used: CD4 (RM4-5), CD45RB (16A), CD44 (IM7), CD19 (1D3), CD8 (53-6.7), Thy1.2 (30-H12). Alexa Fluor 405-labeled streptavidin (Molecular Probes) was used as a second step reagent. The following fluorochrome-conjugated mouse monoclonal antibodies recognizing ovine surface markers, obtained from Serotec, were used: CD4 (44.38), CD8 (CC63), CD45R (20.96). Where indicated, unlabeled monoclonal antibodies were labeled with Zenon mouse antibody labeling kits according to the manufacturer's instructions (Molecular Probes). CCL19-Ig¹⁷ binding²⁶ was detected using biotinylated multi-species adsorbed F(ab')₂ donkey anti-human IgG (Jackson Immunoresearch) followed by phycoerythrin-labeled streptavidin (BD Pharmingen). Specificity of the staining was shown by blocking with excess unlabeled recombinant murine CCL19 (R&D Systems) or staining with control Ig chimera (not shown).

Chemotaxis assay

Single cell suspensions were incubated for 1 h at 37°C prior to the assay. Recombinant murine CCL21 and human CCL19, obtained from R&D Systems, were titrated in triplicate between 1 and 300 nM. The assay was performed using 5 µm pore-size, 24-well Transwell plates (Corning Costar, obtained through Fisher Scientific) and analyzed as described²⁶.

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Figure 1. Tissue exit is non-random

Splenocytes were labeled with CFSE or PKH26 and injected into the footpads of mice. 12 h after transfer, the draining popliteal lymph nodes were analyzed for migrated CFSE⁺ or PKH26⁺ lymphocytes. **(a)** Flow cytometric analysis of the total injected and migrated populations. **(b)** Flow cytometric analysis of gated CD4⁺ T cells in the injected and migrated populations. **(c, left panel)** Migration of total cells and CD8⁺, CD4⁺, and B (CD19⁺) lymphocytes is presented as the percent of injected cells of a given subset recovered from the isolated lymph nodes of individual mice. **(c, right panel)** The migration of CD8⁺ T cells and CD19⁺ B cells, normalized to that of CD4⁺ T cells in each analyzed mouse. One representative experiment (**a**, **b**, and **c** left panel) of 4 independent experiments using 4–10 mice each, or data points representing individual mice and the median of all experiments (**c**, right panel) are shown.

Figure 2. Tissue exit involves $G_{\alpha i}$ protein-coupled receptor signaling

Splenocytes were treated with PTX or mock-treated and labeled with CFSE and PKH26, respectively. Untreated PKH26⁺ cells were injected into one footpad and an equal number of CFSE⁺ PTX-treated cells into the contralateral footpad. **(a)** Flow cytometric analysis of injected and recovered cells. **(b)** Cell counts for recovered CFSE⁺ and PKH26⁺ cells from the draining popliteal lymph nodes of individual mice. One representative staining **(a)** or experiment **(b)** of 4 independent experiments using 5–10 mice each are shown.

Figure 3. T cells require expression of CCR7 to exit the skin and reach the draining lymph node
 PKH26⁺ wild-type spleen cells were mixed with equal numbers of either CFSE⁺ *Ccr7*^{-/-} cells or CFSE⁺ wild-type cells, and injected into the footpads of mice. 12 h after transfer, the draining popliteal lymph nodes were analyzed for migrated CFSE⁺ and PKH26⁺ cells. **(a)** Cell counts for total recovered CFSE⁺ and PKH26⁺ cells from the draining popliteal lymph nodes of individual mice injected with wild-type PKH26⁺ and wild-type CFSE⁺ cells (left panel), or wild-type PKH26⁺ mixed with CCR7-deficient CFSE⁺ cells (right panel). **(b)** The effect of CCR7 deficiency on the migration of total lymphocytes, total CD4⁺ T cells, naïve (CD45RB^{hi}) and memory (CD45RB^{lo}) CD4⁺ T cells, CD8⁺ T cells, and B cells. In each case, the ratio of migrated CFSE⁺ to PKH26⁺ cells (admixed wild-type internal standard cells) of the indicated subset was determined by flow cytometry. Results are normalized to the mean ratio of CFSE⁺ wild-type to PKH26⁺ wild-type cells for each subset (set as 100%). Each data point represents an individually analyzed mouse from a group of 4–5 mice; the mean migration of each group is indicated. One representative experiment of a minimum of three performed is shown.

Figure 4. Transduction of CCR7 rescues *Ccr7*^{-/-} CD4⁺ T cell exit from the skin and migration to the draining lymph node

CD4⁺ T cells from spleens of wild-type or *Ccr7*^{-/-} (KO) mice were transduced with a retroviral vector encoding GFP from an IRES (control vector) or CCR7 and IRES-GFP (CCR7). A mixture of GFP⁻ (non-transduced) and GFP⁺ (transduced) cells was analyzed. **(a)** Binding of CCL19-Ig by wild-type (left panel) and CCR7-deficient T cells (middle panel) transduced with control vector or *Ccr7*^{-/-} cells transduced with CCR7-encoding vector (right panel) was assessed by flow cytometry. **(b)** Chemotaxis of wild-type (left panel) and *Ccr7*^{-/-} cells transduced with control vector (middle panel) or *Ccr7*^{-/-} cells transduced with CCR7-encoding vector (right panel) toward CCL21 was tested in a Transwell chemotaxis assay. Results are expressed as the percentage of GFP⁻ and GFP⁺ Thy1.2⁺ CD4⁺ T cells that migrated to the lower chamber, and data points represent the mean $\pm$ SD of triplicate wells at each concentration. **(c)** The percent migration to the popliteal lymph node of injected GFP⁻ and GFP⁺ wild-type (left panel), *Ccr7*^{-/-} cells transduced with control vector (middle panel), or *Ccr7*^{-/-} cells transduced with CCR7-encoding vector (right panel) was determined 12 h after injection into the footpad in individual mice. Note, a different y-axis scale is shown in the left panel. Data are representative of three independent experiments (**a–c**) analyzing 4–5 individual mice in each group (**c**).

Figure 5. Ovine T cells in the afferent lymph express functional CCR7

Peripheral blood and afferent lymph cells were collected after venopuncture or catheterization of skin draining afferent or pseudoafferent lymph vessels of sheep, respectively. **(a)** The binding of CCL19-Ig by CD45R⁺ naive and CD45R⁻ memory CD4⁺ and CD8⁺ T cells was assessed by flow cytometry. **(b)** Chemotaxis of CD4⁺ and CD8⁺ T cells toward human CCL19 or murine CCL21 was tested *ex vivo* in a Transwell chemotaxis assay. Results are expressed as the percentage of T cells of the respective subset that migrated to the lower chamber, and data points represent the mean $\pm$ SD of triplicate wells at each concentration. Data are representative of four individually analyzed sheep **(a)** and one out of two independent experiments with similar results **(b)**.