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Chemoattractant Receptors and Lymphocyte Egress from Extralymphoid Tissue: Changing Requirements during the Course of Inflammation

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Memory/effector T cells traffic efficiently through extralymphoid tissues, entering from the blood and leaving via the afferent lymph. During inflammation, T cell traffic into the affected tissue dramatically increases; however, the dynamics and mechanisms of T cell exit from inflamed tissues are poorly characterized. In this study, we show, using both a mouse and a sheep model, that large numbers of lymphocytes leave the chronically inflamed skin. Many T cells capable of producing IFN-γ and IL-17 also entered the draining afferent lymph, demonstrating that memory/effector T cells egress from sites of inflammation. Whereas efficient egress from acutely inflamed skin required lymphocyte-expressed CCR7, chronic inflammation promoted significant CCR7-independent exit as well. Lymphocyte exit at late time points of inflammation was sensitive to pertussis toxin but was only partially affected by the drug FTY720, implying the contribution of alternative chemoattractant receptors other than spingosine 1-phosphate receptor 1. Our data show that CCR7 is an important receptor for lymphocyte egress from both resting and inflamed extralymphoid tissues, but that alternative exit receptors come into play during chronic inflammation. *The Journal of Immunology, 2010, 185:4873–4882.

Inflammation is the response to microbial, physical, or chemical injury. Acute inflammation is historically characterized by polymorphonuclear leukocyte infiltration and, if not resolved, progresses into chronic inflammation. In contrast to acute inflammation, chronic inflammation is typified by a mononuclear tissue infiltrate composed mainly of lymphocytes and macrophages (7). Other hallmarks of prolonged chronic inflammation, particularly in response to foreign bodies or nonspecific adjuvants, include fibrosis with possible granuloma formation, angiogenesis, and areas of tissue necrosis. Importantly, autoimmune and chronic inflammatory diseases are characterized by a chronic infiltration of lymphocytes in extralymphoid tissues; however, the precise mechanisms that promote the development of chronic inflammation remain unknown (7). It has become clear that Th1- and Th17-polarized T cell subsets that produce the prototypical cytokines IFN-γ and IL-17, respectively, are responsible for the development and severity of inflammation in many autoimmune diseases (8, 9). Despite their importance, once inflammatory T cell subsets enter the inflamed site, it is not known whether they can subsequently exit the site of inflammation, enter the afferent lymphatics, and return to the draining lymph node and the blood circulation.

A major feature of inflammation is the drastically increased recruitment of leukocytes from blood into the affected tissue. Blood vascular endothelium regulates lymphocyte extravasation from blood into tissues via the expression of inflammation- and organ-specific chemoattractants and adhesion molecules (reviewed in Refs. 2, 10, and 11). Thus, T cell-expressed chemoattractant receptors are crucial in guiding T cells into inflamed and uninflamed extralymphoid tissues. In inflamed sites, concomitant with enhanced recruitment, there is an increase in the permeability of afferent lymphatic endothelium, the rate of lymph flow, and the numbers of cells in the regional afferent lymph (12, 13). Consequently, lymphatic endothelial cell-expressed chemoattractants may regulate T cell exit via the afferent lymph from inflamed tissues. Indeed, lymphatic endothelial cells of afferent lymphatics constitutively express the CCR7 ligand CCL21 (14–16) as well as

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Abbreviations used in this paper: E-lig, E-selectin ligand; I, injected; KO, CCR7-deficient; L-sel, L-selectin; M, migrated; PTX, pertussis toxin; S1P, spingosine 1-phosphate; WT, wild-type.

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adhesion molecules (17–19). Consistent with the lymphatic expression of CCL21, we and others recently showed that CCR7 expression on CD4 and CD8 T cells mediates their egress from resting extralymphoid tissues into the draining lymph node via the afferent lymph (20, 21). In contrast to lymphocyte exit from extralymphoid tissues via the afferent lymph, egress from lymph nodes via the efferent lymph is regulated by spingosine 1-phosphate (SIP) and its receptors (22–24). An extended role for CCR7 in lymphocyte exit from inflamed extralymphoid tissues is supported by studies demonstrating that CCL21 expression is upregulated in lymphatic endothelial cells under inflammatory conditions in vitro (25) and in vivo (26, 27). However, recent data have shown that chemokines other than CCL21 are induced in lymphatic endothelial cells upon inflammatory stimulation (19, 25, 28), and thus additional chemotactrant–chemoattractant receptor interactions may regulate the relative rate of lymphocyte egress from inflamed extralymphoid tissues.

T cell exit from extralymphoid tissues through the afferent lymph removes potentially dangerous cells and is therefore likely a crucial control point of the inflammatory response. Despite this, lymphocyte egress from inflamed extralymphoid tissues is only poorly characterized. In this study, we show that chronic inflammation promotes a large flux of T cells, including Th1 and Th17 cells, from the affected tissue into afferent lymph. Lymphocyte egress from inflamed tissue requires G protein-coupled receptor signaling. Moreover, the chronicity of inflammation determines the chemotactrant receptor expression requirements for T cell egress from the afferent site, with CCR7 being crucial in the acute but not late phases of the inflammatory response.

Materials and Methods

Animals, lymph cannulation, and induction of cutaneous inflammation

Sex- and age-matched BALB/c wild-type (The Jackson Laboratory, Bar Harbor, ME) and CCR7-deficient mice (29), provided by Martin Lipp (Max-Delbrück Center, Berlin, Germany), were used for cell transfer experiments. Donor mice for cell transfer experiments were between 4 and 12 mo of age; recipient mice were between 6 and 12 wk old. Intact female or wether mixed breed sheep 5–10 mo of age were purchased from 3/D Livestock (Woodland, CA), the University of California, Davis (Davis, CA), or Animal Biotech Industries (Danboro, PA). Pseudolymphoid lymph vessels were induced in sheep by surgically removing the subiliac lymph nodes as previously described (30). Following lymphectomy, the afferent lymph vessels anastomose with the larger effervescent vessels, carry afferent (prenodal) lymph, and are termed “pseudolymphoid” vessels (30). Six to 12 wk after lymphectomy, pseudolymphoid lymph vessels were surgically cannulated using heparin-coated catheters (Carmeda, Upplands Väsby, Sweden), and afferent lymph was continuously collected into sterile bottles containing heparin (APP Pharmaceuticals, Schaumburg, IL) as described (30). Every 1–12 h, lymph collection bottles were changed, and leukocyte as well as lymphocyte numbers and composition were analyzed by flow cytometry. Lymphocyte output for different T lymphocyte subsets (CD4, CD8) was calculated and averaged per hour of collection. The cannulated lymph vessels drain the skin, muscles, and bones of the rear flank. In sheep, inflammation was induced by s.c. injection of 3–0.5 ml CFA emulsified with saline into two injection sites either 48 h (acute inflammatory phase) or 3 wk (chronic inflammatory phase) before experimentation as described (5). Every 1–12 h, lymph collection bottles were changed, and leukocyte as well as lymphocyte numbers and composition were analyzed by flow cytometry. Lymphocyte output for different T lymphocyte subsets (CD4, CD8) was calculated and averaged per hour of collection. The cannulated lymph vessels drain the skin, muscles, and bones of the rear flank. In sheep, inflammation was induced by s.c. injection of 3–0.5 ml CFA emulsified with saline into two injection sites either 48 h (acute inflammatory phase) or 3 wk (chronic inflammatory phase) before experimentation as described (5). In mice, inflammation was induced by s.c. injection of 10 μl nonemulsified CFA into the rear footpads using a 10-μl Hamilton syringe. Experiments were performed either 6 h (acute inflammation) or on days 10 or 21 (chronic inflammation) after induction of inflammation. All animal experiments were approved by the Institutional Animal Care and Use Committees of the University of Pennsylvania and the Veterans Affairs Palo Alto Health Care System.

Cell isolation and culture, pertussis toxin, and FTY720 treatment

Lymphocytes collected from ovine lymph were washed with RPMI 1640 medium (Invitrogen, Carlsbad, CA) with 5% newborn calf serum (HyClone Laboratories, Logan, UT). Ovine blood was collected by venipuncture and mixed with heparin. Cells were released from mouse spleens by grinding through a 40-μm cell strainer (BD Biosciences, San Jose, CA). Mononuclear cells were further separated from mouse spleen and from sheep blood by gradient centrifugation with Histopaque (Sigma-Aldrich, St. Louis, MO). For the Th1 culture, splenocytes were sorted by positive selection with CD4 microbeads (Miltenyi Biotec, Auburn, CA) to a purity of ≥96% and plated at 2 × 10^6/ml on 24-well plates (Corning Costar, Lowell, MA) coated with anti-CD3 (145-2C11; eBioscience, San Diego, CA) and anti-CD28 (37.51; eBioscience) in RPMI 1640 containing 10% FBS, 20 ng/ml IFN-γ, 5 ng/ml IL-12 (R&D Systems, Minneapolis, MN), and 5 μg/ml anti-IL-4 Ab (eBioscience). The cells were cultured for 5–6 d. The resulting Th1 cells on wild-type and CCR7-deficient background produced ≥20% IFN-γ upon restimulation with PMA and ionomycin and were used in a mixed leukocyte reaction. For pertussis toxin (PTX)-sensitive experiments, B6C3F1 hybridomas were incubated for 2 h at 37°C in RPMI 1640 medium containing 5% normal calf serum and 200 μg/ml PTX (Calbiochem, San Diego, CA) prior to extensive washing, labeling with fluorescent dyes (see below), and injection. Based on published FTY720 treatment regimens (35, 36), recipient mice were treated by i.p. injection with 1 mg/kg FTY720 (Cayman Chemical, Ann Arbor, MI) dissolved in saline containing 2% hydroxypropyl-β-cyclodextrin (Sigma-Aldrich) or carrier only 8 h prior to adoptive transfer of cells into inflamed or unflamed footpads. To determine lymphocyte numbers in blood of each FTY720- and control-treated mouse, mice were deeply anesthetized by i.p. injection with pentobarbital (Virbac Animal Health, Fort Worth, TX) and blood was collected by cardipuncture and mixed with heparin. Collected blood volume was measured and RBCs were removed using dextran cross-linking and sedimentation with subsequent lysis as described (37).

Cell labeling and transfer

Cell labeling with PKH26 was performed according to the manufacturer’s instructions (Sigma-Aldrich). CFSE (Molecular Probes, Eugene, OR) labeling was achieved by incubating cells at a concentration of 5 × 10^6 ml in HBSS containing 25 mM HEPES and 0.2 μM CFSE for 5 min at 37°C. Both the PKH26 and CFSE labeling reactions were stopped by adding FBS and washed three times in RPMI 1640 containing 5% serum and once in PBS. Inevitable transfer skin egress studies were performed as described in the text and Supplemental Table I. Briefly, between 1 × 10^6 and 5 × 10^6 cells in 10 μl PBS were injected s.c. into the inflamed or unflamed skin of mouse footpads. At 12 h after transfer, single-cell suspensions of the draining and, as a control, the nondraining popliteal lymph nodes were analyzed for transferred cells (identified by fluorescent labels), and total cell numbers were determined by flow cytometry with a fixed number of polystyrene beads (Polybead; Polysciences, Warrington, PA). In experiments comparing the relative migration efficiency of CCR7-deficient cells with that of wild-type cells of a specific lymphocyte subset, the ratio of migrated CFSE^+ cells to PKH26^+ cells of the various subsets was determined by flow cytometry. The migration of CCR7-deficient CFSE^+ cells relative to that of PKH26^+ wild-type cells was based on the ratios of migrated CFSE/PKH26 to injected CFSE/PKH26 cells in each lymphocyte subset. To control for variability in the overall recovery, results were normalized to the mean ratio of CFSE^+ wild-type cells to PKH26^+ wild-type cells for each subset. The following formulas were used: 1) ratio of migrated (M) to ratio of injected (I) cells (M/I) = (M_{CFSE/PKH26}/I_{CFSE/PKH26}); 2) mean ratio of migrated wild-type (WT) to ratio of injected WT (mean M_{WT}/IWT) = (mean M_{CFSE/WTKH26}/I_{CFSE/WTKH26}); and 3) migration of CCR7-deficient or WT CFSE^+ cells (% of mean WT) = (M_{WT}/I_{WT}) × 100.

Flow cytometry and histology

To reduce unspecific staining, mouse cells were preincubated with rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and Ab to CD16/ CD32 (2.4.4G2; BD Biosciences); sheep cells were preincubated with rat IgG (Jackson ImmunoResearch Laboratories). After blocking, the cells were labeled with the following biotinylated or fluorochrome-conjugated mAbs (FITC, PE, Alexa Fluor 647, allophycocyanin, Alexa Fluor 700): CD3 (clone 145-2C11; Biolegend), CD4 (clone RM4-5), CD8 (53-6.7), CD45RB (16A), CD19 (1D3); or mouse anti-sheep IgG (clone IM7; Biolegend). This mixture was followed with biotinylated polyclonal F(ab’2), goat anti-human IgM (Jackson ImmunoResearch Laboratories). Staining for E-selectin (CD62E) was performed according to the manufacturer’s instructions (Sigma-Aldrich). CD62L (DU1-29; VMRD, Pullman, WA). Some mouse mAbs were diluted with sheep blood by gradient centrifugation with Histopaque (Sigma-Aldrich) or carrier only 8 h prior to adoptive transfer of cells into inflamed or unflamed footpads. To determine lymphocyte numbers in blood of each FTY720- and control-treated mouse, mice were deeply anesthetized by i.p. injection with pentobarbital (Virbac Animal Health, Fort Worth, TX) and blood was collected by cardipuncture and mixed with heparin. Collected blood volume was measured and RBCs were removed using dextran cross-linking and sedimentation with subsequent lysis as described (37).

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(Jackson ImmunoResearch Laboratories). For cell surface staining,allophycocyanin- (BD Biosciences) or Alexa Fluor 405-conjugated streptavidin (Invitrogen) were used as second or third step reagents. To detect intracellular cytokines in ovine T cells, afferent lymph cells were stimulated for 4 h with 10 ng/ml PMA and 500 ng/ml ionomycin. For the last 2 h of stimulation, 10 μg/ml brefeldin A was added. Intracellular cytokine staining for IL-17A (eBiosc4DEC17; ebioscience) and IFN-γ (CC302; Serotec) of ovine T cells was performed of fixed cells in saponin buffer. Samples were acquired on a BD LSRII or FACSComp using FACSDiva or CellQuest software (BD Biosciences), respectively, and analyzed with FlowJo (Tree Star, Ashland, OR). Gates were set according to appropriate isotype control staining. Mouse feet were manually deboned and fixed in 10% neutral buffered formalin. Subsequently, the skin samples were processed into paraffin, and 6-μm sections were stained with H&E at Histotec Laboratories (Hayward, CA). Histology images were acquired on a Nikon Eclipse E600 microscope using a Nikon digital sight DS-Fi1 camera and NIS-Elements BR 3.0 software. Images with ×100 objective were acquired using oil immersion on an Olympus BX40F4 microscope with a CC12 camera.

**Statistical analysis**

The nonparametric Kruskal-Wallis test followed by Dunn’s multiple comparison post test, or the nonparametric Mann-Whitney U test, were employed using GraphPad Prism software (GraphPad Software, La Jolla, CA) analyzing combined data from all experiments performed, and p < 0.05 was considered statistically significant.

**Results**

**Chronicity of inflammation determines the relative rate of T cells entering the draining afferent lymph**

We used the sheep lymph cannulation model established by Morris and colleagues (39, 40) to analyze CD4 and CD8 T cell migration from resting and inflamed skin into draining afferent lymph at different time points of a local inflammatory response. A pseudoafferent skin-draining lymph vessel was cannulated and the output of total cells and lymphocyte subsets recorded over time (Fig. 1A). Subsequently, we induced inflammation in the drainage site of the cannulated vessel by s.c. injection of CFA. Cutaneous inflammation induced by CFA is an established model to study both acute and chronic inflammation in sheep and is a relevant model for chronic inflammatory diseases in humans (13). While acute inflammation (<48 h after induction of inflammation) increased the total output of leukocytes from the inflamed skin, the numbers of total lymphocytes (based on forward/side scatter properties) and CD4 and CD8 T cells were largely unchanged (Fig. 1A). A transient flux of neutrophils in afferent lymph was mainly responsible for the increased total cell output during the acute inflammatory response in this inflammation model (Refs. 32–34 and data not shown).

To study lymphocyte egress from the chronically inflamed skin, we first induced inflammation with CFA and then cannulated the draining pseudoafferent lymph vessel 3–4 wk later. At this time point, typical skin granulomas, 3–5 cm in diameter, had formed at the CFA injection sites (data not shown). In contrast to acute inflammation, chronic inflammation dramatically increased the numbers of exiting total lymphocytes including CD4 and CD8 T cells (Fig. 1A). To determine the phenotype of exiting T cells, we stained for the adhesion molecule 1-selectin (CD62L), which is expressed by naïve T cells and subsets of memory T cells and is required for homing into peripheral lymph nodes via high endothelial venules. Additionally, we tested T cell expression of ligands for the skin-selective endothelial adhesion molecule E-selectin; E-selectin binding ligands are expressed selectively on skin-homing memory/effector T cells (reviewed in Ref. 11). Unlike T cells in peripheral blood, most CD4 and CD8 T cells draining the skin granuloma were 1-selectin+ (28.4 ± 11%, mean ± SD) and many expressed E-selectin ligand (42.7 ± 7.8%; Fig. 1B, 1C), indicating that large numbers of memory/effector T cells exit the chronically inflamed site. Moreover, polyclonal stimulation with PMA and ionomycin revealed that the CD4 and CD8 T cells that egress from the chronically inflamed site and enter afferent lymph are capable of producing inflammatory cytokines. Specifically, depending on the individual animal, 10.5–39% of the CD4 T cells produced IFN-γ and 2–6.5% produced IL-17, and among CD8 T cells, 22–72% produced IFN-γ and 2–16% produced IL-17 (n = 7–9 animals; Fig. 1D, 1E). Thus, memory/effector T cells, including Th1 and Th17 cells, not only migrate from blood into sites of inflammation, but they also exit, leaving inflammatory sites via afferent lymph.

**Chronicity of inflammation determines the efficiency of lymphocyte egress from extravascular tissue**

Having established that chronic inflammation boosts the lymphatic exit of T cells from the affected site in sheep, it was unclear whether this finding was also true for the mouse. To test lymphocyte egress from the inflamed skin at different phases of inflammation in the mouse, we induced localized inflammation by s.c. injection of a small volume (10 μl) of CFA into the footpads. Within 30 min following injection, swelling and hyperemia, hallmarks of acute inflammation, were visible (data not shown). At 6 h after injection, the inflammation was histologically characterized by edema and polymorphonuclear infiltrates in the subcutis consistent with acute inflammation (Fig. 2B). Over time, the inflammation developed a chronic character with reduced swelling and hyperemia (not shown), and at days 10 and 21 the inflammatory response had progressed into a s.c. granuloma consisting of mostly mononuclear cells (lymphocytes and macrophages) (Fig. 2C). Thus, CFA injection into either mouse or sheep skin induced a similar inflammatory reaction.

Our data in the sheep system showed that chronic inflammation increased T cell exit from skin via the afferent lymph (Fig. 1); however, there are more tissue lymphocytes in sites of inflammation, and chronic inflammation also dramatically enhances lymphocyte migration from blood into the inflamed site (5). To test whether the efficiency of tissue egress is itself affected by chronic inflammation, or whether enhanced exit could be explained solely by increased tissue lymphocyte numbers, we turned to the mouse model. We transferred CFSE-labeled splenocytes into the CFA-inflamed footpad skin of mice at 6 h and 21 d after induction of inflammation, or into the footpads of untreated control mice. Twelve hours after cell transfer, CFSE-labeled cells were enumerated in draining and contralateral popliteal lymph nodes. During acute inflammation, transferred CFSE+ lymphocytes still exited the site and migrated to the draining lymph node, with no statistically significant difference to untreated mice for total lymphocytes, CD8 T cells, CD4 T cells, and B cells (Fig. 2D). In contrast, chronic inflammation significantly enhanced the egress of transferred total lymphocytes (p < 0.001), CD8 T cells (p < 0.05), CD4 T cells (p < 0.01), as well as B cells (p < 0.01) (Fig. 2E). Based on these findings, we conclude that chronic inflammation enhances lymphocyte exit from inflamed extralymphoid tissue in two mammalian species, and that enhanced lymphatic egress reflects in part an increase in the efficiency of exit mechanisms.

**Chronic but not acute inflammation promotes CCR7-independent tissue exit of T cells**

We have recently shown that CD4 and CD8 T cells require CCR7 for efficient egress from skin under steady-state (noninflammatory) conditions (20). To test whether T cell egress from the inflamed skin is also CCR7-dependent, we transferred CFSE- and PKH26-labeled splenic lymphocytes from wild-type and CCR7-deficient mice into inflamed skin and monitored migration into the draining...
lymph node via the afferent lymph. Twelve hours after splenocyte transfer into acutely inflamed skin (6 h after induction of inflammation with CFA), CCR7-deficient total CD4 as well as naive (CD45RB<sub>high</sub>) and memory (CD45RB<sub>low</sub>) CD4 and CD8 T cells were reduced by ~80% and B cells by ~70% in the draining lymph node compared with their cotransferred wild-type counterparts (Fig. 3A). In this setting, numbers of recovered CD8<sub>T</sub> cell were too low to be reliably analyzed (data not shown). These data indicate that CCR7 expression is critically important for lymphocyte exit from acutely inflamed skin (Fig. 3), akin to its role in lymphocyte egress from uninflamed skin (20).

In contrast, when transferred into chronically inflamed skin (day 10 after induction of inflammation with CFA), CCR7-deficient splenic total lymphocytes, total CD4, memory (CD45RB<sub>high</sub>) and naive (CD45RB<sub>low</sub>) CD4 and CD8 T cells, and B cells were reduced by only 40–60% in their ability to exit the inflamed site and access the draining node (Fig. 3B). We could detect few if any of the adoptively transferred cells in nondraining contralateral lymph nodes or the spleen, indicating that cells had entered via the afferent lymph and not the blood. The same results were obtained when recipient mice were treated with the mAb MEL-14 (anti-CD62L), which would block any residual migration from blood into lymph nodes via high endothelial venules (data not shown). Our data suggest that CCR7 acts as a dominant exit receptor for T cells during inflammation but that CCR7-independent egress mechanisms develop during late stages of the inflammatory response.

### Chronic inflammation promotes CCR7-independent exit of Th1 cells

Th1 cells are important in the maintenance of chronic inflammatory diseases and efficiently home to sites of cutaneous inflammation (41, 42). Having established that Th1 cells egress from chronically inflamed skin via lymph (Fig. 1), we asked whether this cell type required CCR7 for this process. To address this, we generated Th1 cells from wild-type and CCR7-deficient mice in vitro and labeled the cells with CFSE and PKH26. Wild-type Th1 cells expressed high levels of surface CCR7 and migrated to CCR7 ligands in an in vitro chemotaxis assay (data not shown). When transferred into the uninflamed or acutely inflamed footpad skin (6 h after induction of inflammation), CCR7-deficient in vitro-polarized Th1 cells were drastically reduced (on average by >80%) in their capacity to exit the skin and migrate to the draining lymph node (Fig. 4), showing that, similar to resting splenic T cells, Th1 effector cells require CCR7 for efficient egress from uninflamed and acutely inflamed skin (Figs. 3, 4 and Ref. 20). In contrast, when in vitro-polarized wild-type and CCR7-deficient Th1 cells were transferred into the chronically inflamed skin (day 21 after induction of inflammation with CFA), CCR7-deficient Th1 cells exited the chronically inflamed site with up to ~75% of the
efficiency of their wild-type counterparts (Fig. 4). Similar results were seen for in vitro-polarized (CD8) Tc1 cells (data not shown).

The results imply that CCR7 expression is important for Th1 cells to egress from uninflamed skin, but that during the chronic phase of inflammation alternative factors can regulate effector T cell exit via afferent lymph.

Lymphocyte exit from the chronically inflamed skin is PTX sensitive

To test whether lymphocyte egress from the chronically inflamed skin is an active process that involves Gαi protein-coupled receptor signaling, we employed PTX treatment of cells. PTX irreversibly modifies Gαi proteins, rendering chemokine receptors unresponsive to subsequent ligand binding. PTX-treated or untreated splenocytes were labeled with CFSE or PKH26 and transferred into chronically inflamed footpad skin on day 21 after induction of inflammation, respectively, or into the skin of untreated control mice (filled symbols). Twelve hours after transfer, migrated CFSE+ total lymphocytes, CD4 T cells, CD8 T cells, and B cells were enumerated in the draining popliteal lymph nodes by flow cytometry. Migration was expressed as the percentage of injected cells of each respective lymphocyte subset that migrated to the draining lymph node. A–C. One representative staining out of a minimum of five mice per condition is shown at original magnification ×10 and ×100 (insets) original magnification. Scale bars, 100 μm; insets, 5 μm. D and E. One representative out of a minimum of three experiments performed analyzing 8–10 mice per group is shown. Data points represent individually analyzed mice, and horizontal lines indicate the mean of each group.

FIGURE 2. Chronic inflammation enhances lymphocyte egress from inflamed skin in an adoptive transfer mouse model. CFA was injected s.c. into the area of footpads of mice to induce cutaneous inflammation. At different time points of inflammation, histology was assessed by paraffin sections after H&E staining in control skin (A), acutely infected skin at 6 h (B), and chronically infected skin 21 d (C) after induction of inflammation. D and E, CFSE-labeled splenic lymphocytes were transferred into acutely infected skin (D, open symbols) or chronically infected skin (E, open symbols) 6 h and 21 d after induction of inflammation, respectively, or into the skin of untreated control mice (filled symbols). Twelve hours after transfer, migrated CFSE+ total lymphocytes, CD4 T cells, CD8 T cells, and B cells were enumerated in the draining popliteal lymph nodes by flow cytometry. Migration was expressed as the percentage of injected cells of each respective lymphocyte subset that migrated to the draining lymph node. A–C. One representative staining out of a minimum of five mice per condition is shown at original magnification ×10 and ×100 (insets) original magnification. Scale bars, 100 μm; insets, 5 μm. D and E. One representative out of a minimum of three experiments performed analyzing 8–10 mice per group is shown. Data points represent individually analyzed mice, and horizontal lines indicate the mean of each group.

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T cell exit from the chronically inflamed skin is mediated in part by S1P receptors

Having shown a requirement for Gαi-coupled receptors in lymphocyte egress from chronically inflamed tissue, we next tested for a role of G protein-coupled S1P receptors in the process. The drug FTY720 is phosphorylated in vivo and then acts as an agonist for the S1P receptors S1P1, S1P3, S1P4, and S1P5 and downregulates the expression and subsequent function of these receptors (43). This “functional antagonism” prevents T cell egress from lymphoid tissues and induces lymphopenia via inhibition of S1P1 (24, 43). FTY720 accumulates in tissues relative to blood (44) and can affect lymphocytes in vivo for at least 100 h following a single dose (36). As described for hematopoietic stem cells (45) and T cells (46), treatment of recipient mice with FTY720 reduced the egress of adoptively transferred wild-type CD4 (p < 0.05) and CD8 (p < 0.05) T cells from the uninflamed skin (Fig. 6A). Next, we induced cutaneous inflammation with CFA and, on day 21 of the inflammatory response, treated recipient mice systemically with FTY720 prior to adoptive transfer of wild-type splenocytes into the chroni-
analyzed for migrated CFSE+ and PKH26+ T cells. The ratio of migrated TWelve hours after cell transfer, the draining popliteal lymph nodes were induction of inflammation with CFA) footpad skin of recipient mice. via the afferent lymph (Fig. 6 and CD8 T cells (CCR7-deficient; WT, wild-type). of a minimum of three experiments analyzing each cell type is shown. KO, naive (CD45RBhigh) CD4 T cells, and total CD8 T cells and B cells. In each case, results were normalized to the mean ratio of WT CFSE+ cells to WT PKH26+ cells (internal standard cells), which was set as 100%. Data points represent individually analyzed mice of four to five mice; horizontal lines indicate the mean of each group. One representative of a minimum of three experiments analyzing each cell type is shown. KO, CCR7-deficient; WT, wild-type. cally inflamed footpads. Although the drug treatment reduced migration of adoptively transferred wild-type CD4 (p < 0.001) and CD8 T cells (p < 0.05) from the chronically inflamed skin via the afferent lymph (Fig. 6B), the inhibition of migration was less than in the absence of inflammation (Fig. 6A). Differences in B cell egress from the inflamed or uninfamed skin between drug-treated and untreated animals did not attain statistical significance (Fig. 6A, 6B). As expected, the FTY720 treatment induced lymphopenia in recipient animals, confirming activity of the drug (one example is shown in Fig. 6C). The same treatment protocol was also able to drastically reduce transferred and endogenous lymphocytes in blood compared with untreated mice when CFSE-labeled cells were transferred i.v. 12–20 h following the drug treatment and analyzed 12–20 h later (i.e., 20–28 h following the drug treatment) (Supplemental Fig. 1). This indicates that our treatment protocol is sufficient to sequester transferred lymphocytes in tissues as well as to induce lymphopenia. In additional control experiments, we continuously treated recipient mice with FTY720 via the drinking water (1 mg/kg/d) starting 32 h prior to transfer of lymphocytes from donor mice that were FTY720 treated for >24 h; no additional reduction in T or B cell egress from the chronically inflamed skin was seen when compared with the i.p. treated animals (Fig. 6A, Supplemental Fig. 2). Thus, FTY720-sensitive S1P receptors play a statistically significant but minor role in T cell exit from the chronically inflamed skin.

To determine whether inhibition of CCR7 and S1P receptors was sufficient to prevent exit, we tested the ability of FTY720 treatment to block lymphocyte egress of CCR7-deficient T cells. When fluorescent-labeled CCR7-deficient lymphocytes were transferred into the chronically inflamed skin on day 21 after induction of inflammation with CFA, FTY720 treatment led to minimally reduced egress of CCR7-deficient CD4 T cells (p < 0.01), while
the migration of CCR7-deficient CD8 T cells was not significantly affected (Fig. 6D).

The ability of PTX treatment but not combined inhibition and deficiency of CCR7 and S1P receptor signaling to abrogate lymphocyte egress suggests that S1P receptors and CCR7 can mediate exit from chronically inflamed skin, but that additional G_{a12}-coupled chemoattractant receptors must contribute as well.

Chronicity of inflammation determines the relative rate of CCR7-dependent and -independent lymphocyte egress from extralymphoid tissue

Our results showing that chronic, but not acute, inflammation enhanced lymphocyte egress from the affected skin via lymph (Figs. 1, 2), suggested that the chronicity of inflammation dictates the efficiency of lymphocyte exit from inflamed extralymphoid tissues. Moreover, our finding that CCR7 was required for lymphocyte egress from uninflamed and acutely inflamed skin (20) (Figs. 3, 4) but was less critical in T cell egress from chronically inflamed tissues (Figs. 3, 4, 6) implies that the chronicity of inflammation additionally governs the receptor requirements for exit. To test whether the capacity to exit is indeed a function of the time that inflammation has progressed, we induced cutaneous inflammation by s.c. injection of CFA into the footpad of mice at different time points (i.e., 6 h to 21 d). Next, we transferred splenic lymphocytes from wild-type and CCR7-deficient mice after labeling with PKH26 and CFSE, respectively, into the inflamed footpads and uninflamed footpads of control mice and mice with 21-d-old CFA-induced cutaneous inflammation (47, 48). Thus, chronic inflammation promoted CCR7-independent exit. Based on these findings, we conclude that the chronicity of inflammation dictates the relative rate of lymphocyte exit from the inflamed tissue as well as the mechanisms involved.

Discussion

Memory/effector T cells recirculate through extralymphoid tissues entering from the blood and leaving via the afferent lymph. During inflammation, T cell traffic into the affected tissue dramatically increases, and its mechanisms have been well studied and proven key to the inflammatory process. Intuitively, T cell egress from the inflamed site is as important as entry in determining the size and quality of the inflammatory infiltrate; however, the dynamics and mechanisms of T cell exit from inflamed tissues are poorly characterized.

Using sheep lymph cannulation and mouse adoptive transfer models, we found that chronic inflammation efficiently increased lymphocyte egress from the affected tissue via the afferent lymph. The enhanced lymphatic lymphocyte egress was not simply a reflection of enhanced entry from the blood into the tissue because, bypassing recruitment from blood, lymphocytes adoptively transferred into chronically inflamed skin emigrated more efficiently than did lymphocytes adoptively transferred into uninflamed skin (Fig. 2). This finding is in line with recent reports that chronic inflammation and infection induce expansion of draining lymphatics, mainly through lymphangiogenesis, at the site of inflammation (47, 48). Thus, in addition to facilitating transport of interstitial fluid, Ag, and APCs, such an expanded afferent lymphatic network also appears well suited to enhance egress of memory/effector T cells from inflamed sites.

Within hours after inflammatory stimulation with cytokines or microbes, or following antigenic rechallenge, lymphocyte output
via efferent lymph from the regional draining lymph node is temporarily decreased by up to 90% (49, 50), a phenomenon referred to as “lymph node shutdown”. Interestingly, Liao and Ruddle (51) showed that early inflammatory events transiently impair afferent lymphatic drainage, pointing to the possibility of a “shutdown” at the level of afferent lymph draining the site of inflammation. However, consistent with previous studies (13), at all time points following induction of inflammation, we did not detect a drop in lymphocyte numbers traveling in the draining afferent lymph (Fig. 1A). Consequently, throughout inflammation, T cells (along with APCs) continue to egress from extralymphoid tissue and migrate to the draining lymph node, where they can participate in the initiation or maintenance of primary and secondary adaptive immune responses. Even though we did not detect a “shutdown” at the level of afferent lymph, tissue edema at early time points of acute inflammation (Fig. 2) indicated that lymphatic drainage was temporarily insufficient to remove excess interstitial fluid and possibly large cell numbers. The process of lymphangiogenesis takes >1 wk (52), so perhaps there are no newly formed or expanded vessels that could support enhanced cellular exit during the acute phase of inflammation, explaining why the egress of adoptively transferred and endogenous lymphocytes from acutely inflamed skin was not different from egress from uninflamed skin (Figs. 1, 2).

Classic studies in the sheep showed that large numbers of lymphocytes leave the site of inflammation via the afferent lymph (5, 34). Our experiments extend these early studies by revealing that chronic inflammation dramatically boosts the egress rate of memory/effector (1-selectin<sup>low</sup>– and E-selectin ligand<sup>)</sup> CD4 and CD8 T cells from the affected site (Fig. 1). Importantly, many of the T cells leaving the chronically inflamed skin are capable of secreting the inflammatory cytokines IFN-γ and/or IL-17 (Fig. 1). The finding that effector T cell subsets actively recirculate has several implications for inflammatory diseases. For example, the fact that Th1 and Th17 cells leave the inflamed site and re-enter the circulation makes their trafficking back to inflammatory sites a promising therapeutic target, in contrast to permanently accumulating cells, which would not be affected by drugs interfering with their recruitment. By removing pro-inflammatory cells, such as Th17 cells, egress via afferent lymph may help limit the local inflammatory response. Conversely, impaired egress of inflammatory T cell subsets might exacerbate local inflammatory responses and contribute to chronic inflammation. In support of this idea, Rockson and colleagues (53) found that in a mouse model of lymph edema, both the interstitial fluid drainage and leukocyte egress from the affected site are impaired, and they suggested that reduced leukocyte exit via lymph could contribute to the development of localized inflammation that is associated with the disease.

Intriguingly, our data showed that the chronicity of inflammation not only influenced the exit rates of lymphocytes, but also the receptor requirements. CCR7, the best characterized “exit receptor” for dendritic cells (54) as well as T cells (20, 21) became less essential for lymphocyte egress from inflamed tissue during the course of inflammation. Specifically, CCR7 expression by T cells was required for their egress from acutely inflamed or uninflamed skin, while egress from chronically inflamed skin had CCR7-dependent and -independent components. Comparing the egress capacity of CCR7-deficient and wild-type lymphocytes over the course of inflammation revealed that the inflammatory response to CFA had to progress for at least 2 wk to support robust CCR7-independent egress of lymphocytes (Fig. 7). Egress at these late time points of inflammation depended on the signaling of G<sub>i</sub>-coupled receptors because cell migration was sensitive to PTX treatment (Fig. 5). Ledgerwood et al. (46) recently suggested that S1P and its G protein-coupled receptor S1P<sub>1</sub> regulate T cell egress from and retention in extralymphoid tissues. In our hands, blocking the function of S1P receptors with FTY720 significantly reduced T cell egress from the uninflamed skin (Fig. 6A). However, the drug treatment had only a small effect on the egress of wild-type T cells from chronically inflamed skin (Fig. 6B) and, under all conditions tested, did not affect the egress of B cells from the skin (Fig. 6A, 6B). Consequently, FTY720-sensitive S1P receptors are only one participant in lymphocyte egress from chronically inflamed skin. Importantly, FTY720 treatment did not abrogate exit of adoptively transferred CCR7-deficient T cells from a site of chronic skin inflammation when these were cotransferred with wild-type cells (Fig. 6D). Specifically, the drug treatment only slightly reduced migration of CCR7-deficient CD4 T cells and had no statistically significant effect on egressing CCR7-deficient CD8 T cells (Fig. 6D). Conversely, others have also observed a smaller effect of FTY720 on CCR7-deficient lymphocytes relative to their wild-type counterparts (55, 56). Thus, S1P receptors and CCR7 act, at least in part, cooperatively in the process of lymphocyte egress from inflamed skin.

**FIGURE 7.** Chronicity of inflammation determines the efficiency of CCR7-dependent and -independent lymphocyte egress from extralymphoid tissue. Different stages of cutaneous inflammation were induced by s.c. injection of CFA into the footpads of mice at different time points. CCR7-deficient and WT splenocytes were labeled with CFSE and PKH26, respectively, mixed, and injected into inflamed footpad skin of mice at the different indicated time points of a CFA-induced inflammation or into footpads of untreated mice (Control). Twelve hours after cell transfer, migrated CFSE<sup>+</sup> and PKH26<sup>+</sup> lymphocytes were enumerated in the draining popliteal lymph nodes. One out of two experiments with similar results, analyzing four to five mice per time point, is shown. KO, CCR7-deficient; WT, wild-type.
Collectively, these findings imply that alternative (G_{ia}) chemoattractant receptors mediate CCR7-independent, S1P receptor-independent lymphocyte egress from the chronically inflamed skin. Most likely, additional chemoattractants that are expressed by lymphatic endothelium at the inflamed site support the removal of CCR7⁻ cell subsets, allowing for more efficient flux of lymphocytes through the site of inflammation.

In support of a role of alternative chemoattractants in lymphocyte egress from extrafollicular lymphoid tissues, lymphatic endothelium expresses chemokines other than CCR7 ligands upon inflammatory stimulation in vitro (19, 25) and in vivo (28, 57). However, the induction of chemokines in lymphatic endothelial cells occurs rapidly (within hours to days) following inflammatory stimulation (19, 25), whereas it took weeks until we detected maximal egress of CCR7⁻ deficient lymphocytes (Fig. 7). Thus, besides alternative chemoattractors, structural changes at the site of inflammation may also contribute to CCR7⁻ independent lymphocyte exit. Some of the possible structural changes supporting CCR7⁻ independent egress could be due to the changing leukocytic infiltrate; that is, from granulocytes at early time points to mononuclear cells at late time points of the inflammatory response (Fig. 2). For example, due to their expression of subset-specific cytokines and other mediators, infiltrating leukocytes could induce different repertoires of chemoattractants by lymphatic endothelial cells as well as influence the expression and/or function of chemoattractant receptors on infiltrating lymphocytes. It is also conceivable that, as a result of the ongoing lymphangiogenesis at the inflamed site, newly formed lymph vessels have a different profile of chemoattractant expression relative to “old” vessels. Finally, lymphocyte or lymphatic endothelial cell contact with other factors characteristic of chronic inflammation, such as tissue fibrosis or necrosis, could influence the expression and subsequent usage of chemoattractant receptors and chemoattractants.

Interestingly, during the course of inflammation, receptor requirements for lymphocyte recruitment from the blood into lymphoid and extrafollicular tissue change too. For instance, the blood vascular endothelium of chronically inflamed skin often expresses molecules that are usually absent in skin but are associated with homing into lymphoid tissues such as peripheral node addressin (58) or CCL21 (59, 60). Similar to our observations of CCR7⁻ independent lymphocyte migration from inflamed extrafollicular tissue into lymph nodes via afferent lymph (Figs. 3, 4), effector CD8 T cell migration from blood into reactive lymph nodes via high endothelial lymph nodes becomes also independent of CCR7 with inflammation, and T cells require CXCR3 instead (61).

The finding that chronic inflammation supports robust lymphatic egress of CCR7⁻ deficient lymphocytes is highly relevant for the receptor requirements in the tissue egress of cell types other than lymphocytes. For example, CCR7⁻ dendritic cells or malignant cells could, through usage of alternative exit receptors, present Ag in draining lymph nodes maintaining the chronic inflammatory response or efficiently metastasize via the lymphatics, respectively. We are presently testing alternative chemoattractant receptors that may mediate CCR7⁻ independent egress from chronically inflamed extrafollicular tissue. Based on their induction in lymphatic endothelial cells by inflammatory stimuli (19, 25, 28, 57, 62), and the expression of corresponding receptors by lymphocytes, the following chemokine receptor–ligand pairs are potential candidates for mediating CCR7⁻ independent exit: CCR2–CCL2, CCR5–CCL3/CCL5, CCR6–CCL20, CCR10–CCL27, CXCR4–CXCL12, and, possibly, CXCR3–CXCL9/CXCL10 (63). As already established for lymphocyte entry into sites of inflammation, it is likely that chemokines expressed by lymphatic endothelium exert redundant roles in mediating lymphocyte egress from inflamed tissues. Additionally, future studies are needed to address how individual components of chronic inflammation influence trafficking of different leukocyte subsets into and out of the inflamed site.

In summary, the phase of inflammation in extralymphoid tissue determines the magnitude of lymphocyte exit from the site and the mechanisms employed in the process. Potentially deleterious Th1 and Th17 cells egress from sites of chronic inflammation via different lymph, suggesting T cell exit as a key regulator of inflammation and a promising future therapeutic target.

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Disclosures
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Supplemental Figure 1. FTY720 treatment used in Figure 6 of the manuscript is active for a period of >20h and sufficient to induce lymphopenia of endogenous as well as IV transferred lymphocytes. Recipient mice were treated with FTY720 IP as described in the manuscript. 8h after drug treatment, CFSE-labeled splenic lymphocytes from untreated donor mice were transferred IV. 12-20h later (20h shown here), the blood of recipient mice was analyzed for transferred and endogenous lymphocytes. Data points represent individually analyzed recipient animals. Horizontal lines represent the mean of each group of 5 mice. One of three experiments with similar results is shown. *, p<0.05; **, p<0.01; ***, p< 0.001; NS, not significant (p>0.05).

Supplemental Figure 2. Continuous oral treatment of both recipient and donor mice does not abrogate lymphocyte egress from the chronically inflamed skin. Naïve donor mice and recipient mice with 21d-old CFA-induced cutaneous inflammation in both hind footpads were treated orally in drinking water with 1mg/kg/day FTY720 or carrier only. >24h post treatment of donor mice, donor lymphocytes from spleen and skin lymph nodes were harvested, CFSE labeled, and injected into the chronically inflamed footpad skin of FTY720-treated mice. For the control group, lymphocytes from untreated donors were injected into chronically inflamed footpads of carrier-only treated mice. Recipient mice were kept on treatment for the entire length of the experiment. 12h after cell transfer, CFSE-labeled lymphocytes that migrated into the draining popliteal lymph node were enumerated by flow cytometry. Data points represent individually analyzed recipient animals with 8-10 mice per group, and horizontal lines indicate the mean of each group. One out of two experiments analyzed is shown. NS, not significant (p>0.05)
Donor Lymphocytes in Blood

Endogenous Blood Lymphocytes

Cells per mL of Blood

Control, FTY, Control CD4, FTY CD4, Control CD8, FTY CD8, Control CD19, FTY CD19