Effector T Cell Egress via Afferent Lymph Modulates Local Tissue Inflammation

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Memory/effector T cells recirculate through extralymphoid tissues by entering from the blood and exiting via the afferent lymph (1). From the afferent lymph, T cells reach regional lymph nodes, which they leave via the efferent lymph, returning them back into blood. T cell migration into tissues is regulated by a multistep cascade involving adhesion and chemotactic receptors on T cells interacting with their respective endothelial ligands (2). Similarly, T cell egress from lymph nodes is controlled by chemokine-sphingosine-1 phosphate and its receptors (3). In the absence of inflammation, CD4+ and CD8+ T cells require expression of CCR7 to egress from extralymphoid tissues (4, 5). Congruently, lymphatic endothelial cells constitutively express the CCR7 ligand CCL21 in many organs (6).

T cell entry into effector sites is critical for inflammation and is a target of anti-inflammatory therapy (7). Given the large number of lymphocytes, including proinflammatory Th1 and Th17 cells, contributing to tissue inflammation. Conversely, mice with transgenic expression of Ccr7 (Ccr7TG) exhibit reduced effector responses (19). Nevertheless, in these models, it is impossible to dissect the role of T cell egress in inflammation, in particular as other anomalies contribute to the observed phenotypes, for example, regulatory T cell defects in Ccr7+− mice (14, 20) or retention of Ccr7TG T cells in splenic white pulp (19). As a result, although discussed previously (9, 16, 17, 21), the role of T cell egress in the modulation of tissue inflammation remains unresolved.

In this study, we revisited the question and demonstrated that targeting the tissue egress capacity of proinflammatory T cells modulates the local inflammatory response, enhancing our understanding of the pathogenesis of inflammation and laying the foundation for future therapies for inflammatory diseases.

Materials and Methods

All mice were on C57BL/6 background, bred in our animal facility, and used between 8 and 16 wk of age in male or female sex- and age-matched groups. CD45.1 and CD45.2 congenic mice and OTII mice were obtained from The Jackson Laboratory, Ccr7−− mice (22) were from Martin Lipp (Max Delbrück Center), Ccr7TG mice (23) from Nigel Killeen (University of California, San Francisco, San Francisco, CA [UCSF]), and pth mice (24) from Avisan Bhandosa (National Institutes of Health, National Cancer Institute, Bethesda, MD). OTII mice were crossed with Ccr7−− and with Ccr7TG mice to obtain Ccr7−−/OTII and Ccr7TG OTII mice, respectively. Ccr7−−/OTII and Ccr7TG OTII breeders, but not their offspring, were maintained on an antibiotic diet (Mouse Helicobacter MD’s 4 Drug
were preincubated with mouse and sheep IgG. Cells were stained for surface markers and analyzed as described previously (9), using fluorochrome-conjugated (FITC, Pacific blue, eFluor450, PE, and/or allophycocyanin) anti-mouse Abs from eBioscience: CD4 (RM4-5), CD45.1 (A20), and CD45.2 (104) or anti-ovine CD4 (44.38) from Serotec. CCR7 was stained with a CCL19-human Ig chimeric protein (provided by Daniel Campbell, Benaroya Research Institute, Seattle, WA) followed by biotinylated donkey anti-human IgG F(ab')2 (Jackson Immunoresearch) and PE-conjugated streptavidin (BD Biosciences). Dead cells were stained using the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit and excluded from standard analysis or used in combination with Annexin V binding to quantify dead and apoptotic cells according to the manufacturer's instructions (Life Technologies). To detect intracellular cytokines, we stimulated mouse Th1 cells with Ag-pulsed BMDCs for 5 h. In addition, mouse and sheep T cells were stimulated with ionomycin and PMA, and stained as described previously (9) with mAbs recognizing mouse IFN-γ (XMG1.2; eBioscience), IL-4 (11B11; eBioscience), and IL-10 (JES5-16E3; BD Biosciences) or ovine IFN-γ (CC302; Serotec) and IL-17A (eBio6D4EC17; eBioscience). Samples were acquired on a BD LSRII or LSFortessa using FACSDiva software (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Statistical analysis

The Mann–Whitney U test (GraphPad Prism) was used for statistical analyses, and p < 0.05 was considered statistically significant.

Results

Ag recognition at the effector site decreases tissue egress of effector Th1 cells

T cell-mediated inflammation involves antigen activation of proinflammatory T cells at the target site. To develop a model of T cell-mediated inflammation in which the expression of T cell exit receptors is the sole variable and is independent of cell recruitment from the blood, we modified Streilein's "local adoptive transfer assay of delayed hypersensitivity" (28). Using OVA-specific CD4+ T cells from TCR-transgenic (OTII) mice, we generated proinflammatory Th1 cells. These Th1 cells were coincubated for 1 h with OVA-pulsed APCs and transferred into the footpads of mice. The induced local tissue inflammation was transient and measurable as footpad swelling that depended on the number of transferred Th1 cells (Fig. 1A).

To test the influence of Ag recognition on egress from the inflamed site, we mixed CFSE-labeled CD45.1+ polyclonal and CD45.2+ (OVA-specific) OTII Th1 cells and coincubated them with APCs pulsed with OVA or a control protein (BSA) before transfer into the footpads of recipient mice. As done previously (4, 9, 27), we assessed T cell tissue egress by enumerating donor T cells that had left the skin and migrated into the popliteal draining lymph node (dLN) (Fig. 1B, 1C). Although at 20 h after transfer many donor Th1 cells had egressed from the inflamed site and reached the dLN, transferred cells in the contralateral lymph node or the spleen were below the level of detection (Fig. 1B, 1C, and data not shown), confirming migration via afferent lymph before transferred cells enter the blood circulation. In the presence of OVA-pulsed APCs, the number of OTII Th1 cells that left the site of inflammation and reached the dLN was drastically reduced relative to that of polyclonal Th1 cells (~75% reduction on average; p < 0.0001; Fig. 1C). We conclude that Ag recognition leads to downregulation of effector CD4+ T cell egress from inflamed tissue.

Ccr7WT enhances effector T cell egress from inflamed tissue and accelerates resolution of inflammation

To address whether tissue exit receptors can be targeted to modulate tissue egress of Ag-responding proinflammatory T cells, we generated Th1 cells from OTII mice and OTII mice whose T cells are Ccr7KO. Ccr7KO OTII Th1 cells showed a more uniformly high CCR7 expression relative to Ccr7WT OTII Th1 cells (Fig. 2A).

Cell isolation, culture, and labeling and chemotaxis assay

Lymphocytes were isolated from lymph nodes and spleens by passage through 40-μm cell strainers (BD Biosciences), as described previously (10). RBCs were lysed with 160 mM ammonium chloride, and cells were washed in RPMI 1640 with 10% FBS. Th1 cells were generated from microbead-sorting (Miltenyi Biotec) CD4+ T cells cultured on plate-bound anti-CD3 (145-2C11) UCSF Monoclonal Antibody Core (UCSF MAC) and anti-CD28 (37.51; UCSF MAC) in the presence of IL-12 (R&D Systems), IFN-γ (R&D Systems), and anti-IL-4 (11B11; BioXCell) as described previously (9). On day 5 of culture, dead cells were removed using a Nycodenz gradient (Axis-Shield). Th1 cells were labeled with 0.5 mM CFSE (Life Sciences) or 5 mM eFluor670 (eFluoro70; eBioscience) in HBSS with 25 mM HEPES (Life Technologies) for 10 min at 37˚C. Bovine serum was added to stop the reaction, and cells were washed three times. Femurs and tibias were flushed to isolate bone marrow (BM) cells. BM-derived dendritic cells (BMDCs) were generated as a source of APCs by culture of BM cells with 20 ng/ml GM-CSF (Peprotech) for 8–9 d. BMDCs were pulsed overnight with 500 ng/ml LPS (Sigma-Aldrich), 10 ng/ml mouse TNF-α (R&D Systems), and 1 mg/ml BSA (Sigma-Aldrich) or OVA protein (Sigma-Aldrich). Cells were isolated from footpad skin by mincing mouse feet followed by two 30-min enzymatic digestion steps in HBSS at 37˚C with 0.1 mg/ml DNase I (Roche) and 12.5 μg/ml Liberase TM (Roche). Subsequently, samples were passed through a 100-μm cell strainer. The chemotaxis assay was carried out and analyzed as described previously (26). In brief, 5 × 10³ lymph-borne cells in RPMI 1640 containing 10% FBS and 20 ng/ml GM-CSF were added to the upper compartment of 5-μm pore size Transwell inserts (Corning). Mouse CCL21 (R&D Systems) was added to the lower chamber at 100 nM, its optimal concentration to attract ovine CD4+ T cells (4, 27). CD4+ T cells that migrated to the lower chamber during the 90-min incubation at 37˚C were quantified by flow cytometry using a bead standard (15-μm polystyrene beads; Polysciences). Frequencies of cytokine-producing CD4+ T cells were determined in input and migrated wells by intracellular staining (described later) to calculate percentage migration of total IFN-γ+ and IL-17+ CD4+ T cells.

Induction of skin inflammation in sheep and mice, and T cell egress assay

Chronic skin inflammation was induced in sheep by s.c. injection of 0.3–0.5 ml CFA (Sigma-Aldrich), emulsified at 1:1 with saline, into two sites in the area of the flank (9). Three or more weeks later, lymph vessels draining the inflamed skin and the control side were cannulated and lymph-borne CD4+ T cells were analyzed. To elicit inflammation in mice and to test T cell egress from the site, we modified a model of delayed hypersensitivity by Streilein (28). Unless otherwise indicated, responder (OTII) Th1 cells and Ag-pulsed BMDCs generated from wild-type (WT) or CCL19-deficient pld mice were cocultured at a 2:1 ratio, keeping APC constant at 2 × 10⁵ cells/well. After 1 h, the APC–T cell mixture was injected s.c. in 10 μl PBS into the footpads of recipient mice, and tissue swelling was blindly measured over time using an engineer’s caliper (Fishier Scientific). Δ Swelling was defined as the footpad thickness after injection of cells subtracted by preinjection values for the same foot. In T cell egress assays, 20–70 h postinjection of 4–6 × 10⁵ responder Th1 cell subsets and 1 × 10⁶ Ag-loaded APCs, the draining and nondraining (control) popliteal lymph nodes and spleens of recipient mice were analyzed for donor cells based on fluorescent and/or congenic labels, as well as CD4 expression as described previously (9). Enumeration of migrated Th1 cells subsets was done by flow cytometry using a fixed number of beads (15-μm polystyrene beads) added to each sample. Cell counts and ratios were corrected for differences in input populations.

Flow cytometry

To reduce nonspecific staining, we preincubated mouse cells with rat IgG (Jackson ImmunoResearch), anti-CD16/CD32 (2.4G2; UCSF MAC), and, if necessary, donkey and mouse IgG (Jackson ImmunoResearch). Sheep cells
These Th1 cell subsets were differentially labeled with fluorescent dyes and, after coincubation with OVA-pulsed APCs, coinjected into the footpads of recipient mice. Strikingly, Ccr7\textsuperscript{TG} OTII Th1 cells showed a significantly enhanced capacity to egress from the inflamed skin and enter the dLN 20 h after transfer relative to Ccr7\textsuperscript{WT} OTII Th1 cells (\( p = 0.0002 \); Fig. 2B, 2C). At this time point after cell transfer, both Ccr7\textsuperscript{TG} and Ccr7\textsuperscript{WT} Th1 cells were below the level of detection in spleens and contralateral lymph nodes (data not shown), confirming that cells had reached the dLN through the afferent lymph. Migratory differences were independent of the cell labels, because there were no differences in the migration of differentially labeled Ccr7\textsuperscript{WT} OTII Th1 cells (Fig. 2B). At this time point, we could not detect differences in cell proliferation or survival between Ccr7\textsuperscript{TG} and Ccr7\textsuperscript{WT} OTII Th1 cells in skin or dLN (Supplemental Fig. 1A–C). The Ccr7 transgene did not affect differentiation into Th1 cells as indicated by similar cytokine expression profiles after stimulation with OVA-pulsed APCs or PMA and ionomycin before injection (Fig. 2D and data not shown) and after restimulation of transferred Th1 cells recovered from skin (Supplemental Fig. 1D). Strikingly, although both Th1 cell subsets initially induced a similar magnitude of inflammation, resolution of inflammation was accelerated in mice that had received Ccr7\textsuperscript{TG} OTII Th1 cells (Fig. 2E). We conclude that enhancing exit receptor expression is able to dislodge...
Ag-sequestered effector T cells from inflamed tissue, translating into faster resolution of inflammation.

**Lack of T cell CCR7 enhances local tissue inflammation**

Next, we analyzed in vitro polarized Th1 cells from Ccr7−/− OTII and Ccr7WT OTII mice (Fig. 3A), which produced cytokines at similar frequency after stimulation with OVA-pulsed APCs or PMA and ionomycin (Fig. 3B and data not shown). Consistent with CCR7 as a major exit receptor (4, 5, 9), Ccr7−/− Th1 cells were drastically reduced in the dLN relative to Ccr7WT Th1 cells (p = 0.0159) and below the level of detection in the spleens of most recipients even 3 d after transfer together with OVA-loaded APCs into footpad skin (Fig. 3C and data not shown). These results are in line with data showing that lack of T cell CCR7 promotes T cell retention at effector sites (11, 16, 17, 29). Importantly, the reduced egress of Ccr7−/− OTII Th1 cells from the site of inflammation translated into enhanced and prolonged inflammation in our hypersensitivity model (Fig. 3D). Together with our findings that enhancing T cell egress alleviates the inflammatory response (Fig. 2E), we conclude that the tissue egress capacity of effector T cells is a critical determinant of the course of inflammation and that tissue exit receptors can be targeted to modulate inflammation.

**The CCR7-CCL21 axis is active in tissue egressing proinflammatory T cells**

If the CCR7-CCL21 axis is a physiological target relevant to the egress of proinflammatory T cells, then effector T cells that have entered the afferent lymph draining inflamed sites should be responsive to CCR7 ligand. Because analysis of effector lymph T cells is not feasible in mice and humans, the sheep has emerged as a classical model of effector lymph cannulation, allowing for the analysis of tissue-recirculating lymphocytes (1, 4, 8, 30). Afferent lymph vessel cannulation in sheep revealed that lymph-borne IFN-γ+ Th1 and IL-17+ Th17 cells egressing from uninfamed or chronically inflamed skin migrated toward CCL21 in an ex vivo chemotaxis assay (Fig. 4). We conclude that the CCR7-CCL21 axis is not only active in adoptively transferred effector T cells but is also a physiological target in proinflammatory T cells that recirculate through extralymphoid sites.

**Discussion**

In this article, we demonstrate that the magnitude and duration of T cell–mediated inflammation is a function of the capacity of proinflammatory T cells to leave the inflamed tissue via the afferent lymph. This fills an important gap in our knowledge regarding the regulation of T cell–driven inflammation and highlights the importance of studies delineating the mechanisms that regulate T cell egress versus retention.

We found that CD4+ effector T cells that recognize Ag at the inflamed site greatly reduce their egress from the tissue (Fig. 1). This is in agreement with data demonstrating that upon antigenic stimulation in situ, virus-specific CD8+ T cells decrease their egress from the lung during influenza virus infection (10) and/or develop into long-term TRM in the vesicular stomatitis virus–infected brain (31). Because T cell entry into tissues is independent of Ag specificity (32), enhancing the retention of Ag-specific T cells by decreased egress from sites of inflammation and infection ensures continued T cell effector functions, while allowing

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**FIGURE 3.** Lack of CCR7 impairs effector T cell egress and enhances local inflammation. Ccr7WT and Ccr7−/− (CD45.2+) OTII Th1 cells were separately coincubated with OVA-pulsed DCs before s.c. injection into the footpads of two groups of CD45.1+ congenic WT recipients. (**A**) Representative CCR7 staining of Th1 cells before injection. (**B**) Intracellular cytokine profile of Ccr7WT OTII and Ccr7−/− OTII Th1 cells before injection after stimulation with OVA-pulsed APCs. (**C**) Seventy hours after transfer, recovered donor Th1 cells were quantified in the dLN. (**D**) Footpad swelling induced by Ccr7WT and Ccr7−/− OTII Th1 cells was measured over time. Data points depict individual recipients (C) and/or the mean ± SEM of each group (C and D). One experiment of ≥2 with similar results using five recipients per group. *p < 0.05.

**FIGURE 4.** Inflammation exiting Th1 and Th17 cells chemotax to CCR7 ligand. Chronic skin inflammation was induced in sheep by s.c. injection of CFA. Three or more weeks later, affeer lymph vessels draining the inflamed and uninfamed control skin were cannulated and skin-egressing T cells were collected. Chemotaxis of lymph-borne CD4+ T cells toward CCL21 was tested ex vivo in a Transwell chemotaxis assay. (**A**) Representative intracellular cytokine staining of inflammation-draining CD4+ T cells by decreased egress from sites of inflammation and infection ensures continued T cell effector functions, while allowing
affector lymphatics fulfill network and enhancing the drainage of fluid and inflammatory VEGF-D) decrease inflammation by expanding the lymphatic phatic vessel function and activation (e.g., by VEGF-C and 2 (D6), which scavenges inflammatory chemokines while pre-

The finding that the CCR7-CCL21 axis is active in physiologically recirculating effector T cells that have entered the inflammation-draining afferent lymph (Fig. 4) highlights the potential importance of this receptor-ligand pair as a therapeutic target. Although many inflammatory signals upregulate lymphatic CCL21 at effector sites, its expression levels, as well as that of other lymphatically expressed chemokines and adhesion mole-

Dampening inflammation by guiding proinflammatory T cells out of the inflamed site adds to the various anti-inflammatory functions of lymphatic endothelial cells (reviewed in Ref. 21). One example of counterregulation of inflammation by lymphatic endothelial cells is the expression of atypical chemokine receptor 2 (D6), which scavenges inflammatory chemokines while pre-

In conclusion, our data show a proof-of-principle that the ca-

References

References

Disclosures
The authors have no financial conflicts of interest.
SUPPLEMENTAL FIGURE 1. Transgenic CCR7 expression does not influence Th1 cell apoptosis, proliferation or IFN-γ production in short-term tissue egress assay. (A-D) CD45.2+ Ccr7WT and Ccr7TG OTII Th1 cells were co-cultured with OVA-pulsed APCs for one hour before transfer into the footpads of two groups of CD45.1+ congenic WT recipients. 20 h later, lymphocytes were isolated from dLN and footpad skin. (A and B) Percentage of apoptotic and dead donor Th1 cells recovered from dLN (A) and skin (B) assessed by annexin V binding and LIVE/DEAD fixable dead cell staining. (C) Proliferation of CFSE-labeled CD45.2+ donor Th1 cells from dLN and skin was measured as percent of total donor Th1 cells with diluted CFSE label. (D) IFN-γ expression of donor Th1 cells from skin without stimulation (left) and after re-stimulation for 4 hours with PMA and ionomycin (right) assessed by intracellular staining. Data points depict individual recipients and the mean ± SEM of each group. One out of 2-3 experiments with similar results analyzing 5 mice per group. NS, not significant by Mann Whitney U test.