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Airway Eosinophil Migration into Lymph Nodes in Mice Depends on Leukotriene C₄

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Abstract

Background—We previously demonstrated in mice that airway eosinophils traffic from the airway lumen into lung-draining paratracheal lymph nodes. However, mechanisms whereby eosinophils traverse from the lungs and home to paratracheal lymph nodes remain unclear. We investigated roles of cysteinyl leukotrienes in mediating eosinophil trafficking from lungs to paratracheal lymph nodes.

Methods—The expression of CCR7 was determined by flow cytometry. Transwell assays were used to test chemotactic responses of leukotriene C₄ synthase-deficient and control airway eosinophils to the chemokine CCL19 *ex vivo*. Eosinophils from the spleens of IL-5 transgenic mice, fluorescently labeled *ex vivo*, were intratracheally injected into ovalbumin-sensitized and ovalbumin-aerosol challenged leukotriene C₄ synthase-deficient and control mice. Eosinophils were identified by microscopy and flow cytometry in the lungs and paratracheal lymph nodes.

Results—Mouse eosinophils expressed CCR7, the receptor for CCL19, and responded chemotactically to CCL19. Leukotriene C₄ synthase-deficient eosinophils exhibited impaired chemotaxis to CCL19 that was restored by exogenous leukotriene C₄. The migration of intratracheally-injected eosinophils into paratracheal lymph nodes from distal alveolar lung was diminished in leukotriene C₄ synthase-deficient mice compared to wild-type mice, with increased retention of eosinophils in the lungs of leukotriene C₄ synthase-deficient mice. Exogenous

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administration of leukotriene C₄ restored trafficking of eosinophils to paratracheal lymph nodes in leukotriene C₄ synthase-deficient mice.

Conclusions—Our findings that cysteinyl leukotrienes are involved in regulating airway and lung eosinophil migration into paratracheal lymph nodes identify previously unrecognized roles for the cysteinyl leukotrienes in regulating the pulmonary trafficking of eosinophils in experimental allergic asthma.

Keywords

Asthma; CCR7 receptor; Cysteinyl leukotrienes; Eosinophils; Lymph node trafficking

Introduction

Eosinophils and cysteinyl leukotrienes (cys-LTs) both have varied and multiple roles in mediating allergic inflammation, especially in the lung and in asthma. The cys-LTs, LTC₄ and its extracellular derivatives, LTD₄ and LTE₄, are potent bronchoconstrictors, augment mucus release and enhance endothelial vascular permeability (1, 2). Within the lung, eosinophils as well as other cells are sources of cysLTs (1). CysLTs can have varied functions on eosinophils, ranging from intracrine regulators of secretion of granule-derived proteins, including the cytokine, IL-4, to paracrine roles in eosinophil chemotaxis and recruitment (2, 3).

In mouse models of asthma, eosinophils are recruited into the airways and lung tissues (4). Eosinophils in the lungs in mouse models traffic from the lung into regional paratracheal lymph nodes (pLN) (5–7). Several studies have demonstrated that migrating lung eosinophils can function as “professional” antigen-presenting cells (APCs). Eosinophils exposed to airways antigens can internalize, process and directly present antigens; and requisite eosinophil lysosomal antigen processing can be blocked by inhibitors of lysosomal acidification (including NH₄Cl for erythrocyte lysis), either employed incidentally (8) or more formally studied (7). In mice, lung-derived and migrated eosinophils in the pLN express MHC class II and co-stimulatory proteins (CD40, CD80, CD86) and in antigen-specific fashions elicit TH2-biased responses with elicited secretion of TH2 cytokines (5, 7). Within pLN, lung-recruited eosinophils localize to T-cell rich paracortical areas (5–7) and have been visualized directly interacting with antigen-specific T cells within pLN (5, 7). A recent study of human pLN and large airways in patients with fatal asthma now has provided fully comparable findings for humans (9).

Little is known about the trafficking of eosinophils to pLN. Studies in mice have demonstrated identical trafficking of intratracheally instilled eosinophils to pLN in both normal, unsensitized mice and Ag-sensitized, airway Ag-challenged mice (6). Eosinophil trafficking to pLN is not dependent on chemokines acting via CCR3 (6) or acting via CCR1 (Wang and Weller, unpublished) since eosinophils genetically deleted of these receptors migrated equally into pLN. Hence, eosinophil trafficking is not likely over bronchial airways walls, but rather from eosinophils in more distal alveolar spaces (6). Lung emigrating mouse eosinophils have been imaged within lymphatic vessels (5). Alveolar, peripheral localizations of eosinophils are recognized in human asthma (10, 11) and leukotriene C₄

synthase has been localized in the peripheral alveolar regions (12). Mobilization from alveolar spaces into draining lymphatic vessels might be by varied peripheral lymphatic structures, likely not fully defined morphologically and of uncertain function (13, 14). The chemokine receptor CCR7 on APCs, and its chemokine ligands, CCL19 and CCL21, have central roles in orchestrating the movement of canonical APCs into proximate interactions with CD4⁺ lymphocytes in lymph nodes (15). We have previously demonstrated that human eosinophils express functional CCR7 and have multiple functional responses, including chemotaxis, to CCL19 and CCL21 (16).

Host-derived cysLTs may critically regulate the migration of immune cells. The migration of dendritic cells from the skin to lymph nodes was shown to be dependent on cysLTs, based on studies of mice genetically deficient in multidrug resistance-associated protein-1 (MRP1) that is required for the extracellular secretion of LTC₄ (17). LTC₄ promotes chemotaxis of skin epidermal dendritic cells to the chemokine CCL19 in mice, and such was blocked in MRP1^{-/-} mice that could not secrete formed LTC₄, but was restored by exogenous cysLTs (17). In contrast, lung dendritic cells that used CCR7 ligands to migrate to pLN were not dependent on cysLTs, as assessed with MRP1^{-/-} mice (18).

In the present study, we aimed to ascertain whether and how host-derived cysLTs alter the migrations of native mouse eosinophils, who retain the capacity to secrete LTC₄, as they egress from the lungs to pLNs.

Material and Methods

Mice

LTC₄S^{-/-} mice and wild-type (LTC₄S^{+/+}) littermate controls on a BALB/c background were originally generated as described (19). IL-5 transgenic BALB/c mice heterozygous for a transgene that expresses murine IL-5 under the control of human CD2 regulatory sequences (20) were obtained courtesy of Dr. Craig Gerard (Boston Children's Hospital). Protocols used were approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center.

Antibodies and Reagents

Mouse anti-CD19 and anti-CD90 magnetic microbeads were used in eosinophil purification (Miltenyi Biotec, San Diego, CA). Ovalbumin (OVA, Sigma-Aldrich, St. Louis, MO) and alum adjuvant (Thermo Scientific, Rockford, IL) were used in allergen sensitization and challenge. Allophycocyanin and phycoerythrin labeled anti-mouse CCR7 antibodies (Clone 4B12) were from Biolegend (San Diego, CA). Rabbit polyclonal anti-CysLT1 antibody was from Dr. Yoshihide Kanaoka. Anti mouse -CD11a, CD18 and α4β7 were from BioLegend.

Eosinophil Isolation from Spleens of IL-5 Transgenic Mice

Single-cell suspensions were made from spleens of IL-5 transgenic mice. Eosinophils were isolated from single-cell suspensions by negative selection as previously described (6). Purity was routinely >98% as assessed by Hema 3 staining. Viability was routinely >98% by trypan blue exclusion.

Flow Cytometry and Microscopy

The flow cytometer used was a BD Biosciences FACScan. FlowJo (TreeStar, Ashland, OR) was used for data analysis. Microscopy was performed using a motorized Olympus BX62 fluorescence microscope. To visualize fluorescently labeled eosinophils, OCT-frozen pLNs were cryosectioned (5- μ m thick sections) and collected onto Superfrost slides. Images were acquired using bandpass zero-shift TRITC and DAPI filters and overlapped using IpLab 3.6 (BD Biosciences).

Expression of cysLT Receptors on Mouse Eosinophils by Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from purified eosinophils or lungs of wild-type mice using commercially available kits (Qiagen, Germantown, MD). Total RNA was used as a template for RT-PCR by using OneStep RT-PCR kits (Clontech Laboratories, Mountain View, CA). The oligonucleotide primers (Clontech Laboratories) had the following sequences: CysLT₁, sense 5' - CAGGAAGAAGAGTCGAGGTCTATT -3', antisense 5' - AATCATGTATACTTGGGAAGGCTGA -3'; CysLT₂, sense 5' - ATGAAGAAGATGATCTGTTAGAGA -3', antisense 5' - GACCTTGGTAGACATCAGGCATAC -3'. Expected sizes of amplification products were 628 bp for CysLT₁ and 172 bp for CysLT₂. PCR amplification was performed on the GeneAmp PCR System 2400 (Applied Biosystems, USA) with 40 cycles comprised of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute. Lung tissues were used as positive controls for the both cys-LT receptors. PCR products were analyzed by electrophoresis on ethidium bromide-stained 1.8% agarose gels that were visualized under UV light exposure.

Ovalbumin Sensitization and Challenge

LTC₄S^{-/-} and wild-type mice were sensitized to OVA by intraperitoneal injection of 50 μ g of OVA emulsified in alum at Days 0, 7, and 14 as previously described (7). Mice were challenged by aerosol inhalation of 5% OVA in PBS for 30 minutes at Days 21, 22, and 23. Mice were sacrificed on Day 24. Lungs and (pLN) were harvested for histologic and flow cytometric analyses. For *in vitro* chemotaxis experiments, bronchoalveolar lavage (BAL) was performed prior to sacrifice of mice (21).

Fluorescent Labeling, Intratracheal Transfer of Eosinophils, and Intratracheal Administration of LTC₄

For relevant experiments, isolated eosinophils in phosphate buffered saline (PBS) were labeled *ex vivo* with the plasma membrane inserted cell tracker red-orange fluorescent dye, 1,1'-Dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiIC₁₆(3)) (Life Technologies, Grand Island, NY), by incubating at 37°C for 15 minutes. Viability of eosinophils after *ex vivo* labeling was routinely > 98%. Two hours before the last OVA aerosol challenge in OVA-sensitized, OVA-challenged mice on Day 23, 1×10^6 fluorescently labeled eosinophils were instilled by intratracheal injection. For relevant experiments, LTC₄ in PBS (1 μ g/animal, Cayman Chemical, Ann Arbor, MI) was administered by intratracheal injection 2 hours after the second OVA challenge on Day 22.

Chemotaxis Assays

Eosinophils, purified from BAL fluid of OVA-sensitized and -challenged wild-type or LTC₄S^{-/-} mice, were resuspended at a concentration of 1×10^6 cells/ml in RPMI 1640 with 0.1% OVA, 24 mM HEPES, and 2 mM glutamine and pre-incubated for 30 minutes with LTC₄ (200 nM) or control medium. Transwell permeable supports containing polycarbonate membranes with 5 μ m pores were used (Corning, Lowell, MA). The lower chambers below the transwell contained 600 μ l of RPMI-1640 containing 0.2% bovine serum albumin with or without recombinant CCL19 or CCL21 (R&D Systems, Minneapolis, MN) or *sphingosine-1-phosphate* (*Sigma*). Eosinophil suspensions (100 μ l) were added to the upper chamber. Wells were set up in triplicate for each experiment. Chemotaxis plates were incubated at 37°C for 2 hours. Migrating cells in the lower chamber were counted for 15 seconds in triplicate by flow cytometry.

Eosinophil Isolation from Bronchoalveolar Lavage

The lungs were lavaged 4 times with 1 mL aliquots of PBS containing 4 mM EDTA to obtain BAL. Eosinophils were purified on discontinuous Percoll density gradients followed by immunomagnetic purification as previously described (MACS; Miltenyi Biotec) (6).

Statistical Analyses

Unpaired *t* tests were used where indicated for analysis of flow cytometry data with the level of significance set at $p < 0.05$. Data are expressed as mean $\pm$ SD.

Results

Murine Eosinophils Express the Leukotriene Receptors CysLT₁ and CysLT₂ and the Chemokine Receptor CCR7

We have demonstrated functional effects of cysteinyl leukotrienes on the secretory capacities of human eosinophils (6, 7). In the present work, we first sought to demonstrate that mouse eosinophils express the cysteinyl leukotriene receptors, CysLT₁ and CysLT₂. The expression of CysLT₁ and CysLT₂ by mouse eosinophils from spleens of IL-5 transgenic mice was demonstrated by RT-PCR, with whole lung expression serving as a positive control (Figure 1A). BAL eosinophils from OVA-sensitized and -challenged mice, both wild-type (LTC₄S^{+/+}) and LTC₄S^{-/-}, expressed CysLT₁ on their surface detectable by flow cytometry (Figure 1B and 1C).

We have also shown that human eosinophils express the chemokine receptor CCR7, which has a central role in coordinating leukocyte trafficking from tissues to lymph nodes, including from the lungs into mediastinal lymph nodes (16). We sought here to establish that mouse eosinophils express CCR7 as well. Eosinophils isolated from spleens of IL-5 transgenic mice were assessed by flow cytometry for the presence of CCR7, the receptor for CCL19. CCR7 was present on the surface of eosinophils (Figure 1D). These data are representative of three independent experiments. Exogenous administration of LTC₄ with concentrations up to 200 nM did not alter the surface or total intracellular expression of CCR7 by eosinophils isolated from the spleens of IL-5 transgenic mice, from the BAL of OVA-sensitized and -challenged mice, and from the BAL of OVA-sensitized and –

challenged LTC₄S^{-/-} mice (Supplemental Figure 1). Eosinophils isolated from the BAL of OVA-sensitized and -challenged wild-type and LTC₄S^{-/-} mice also demonstrated surface expression of CCR7 (Figure 1E and 1F). There was no difference between wild-type and LTC₄S^{-/-} mice in the surface expression by BAL eosinophils of the adhesion molecules CD11a, CD18 and α4β7 (Supplemental Figure 2).

Full *In Vitro* Migratory Capacity of Eosinophils to CCL19 is Dependent on LTC₄

Transwell assays were performed to assess chemotaxis of isolated lung eosinophils from OVA-sensitized and -challenged LTC₄S^{+/+} and LTC₄S^{-/-} mice to CCL19, a cardinal chemokine ligand of CCR7. LTC₄ synthase-deficient (LTC₄S^{-/-}) mice are incapable of synthesizing LTC₄ due to the lack of this enzyme. Chemotaxis to CCL19 was robust in wild-type lung eosinophils, without any further augmentation when eosinophils were pretreated with exogenous LTC₄ prior to chemotaxis assays. Lung eosinophils from LTC₄S^{-/-} eosinophils, however, displayed substantially reduced chemotaxis to CCL19 that was almost fully restored when they were pretreated with LTC₄ prior to chemotaxis assays (Figure 2A). The results are compiled from two independent experiments. The restoration of impaired chemotaxis to CCL19 with LTC₄ pretreatment was observed across all concentrations of CCL19 that were tested (Figure 2B). We also tested chemotaxis to CCL21, the other known ligand of CCR7, and did not observe differences in migration to CCL21 between wild-type and LTC₄S^{-/-} BAL eosinophils at the concentrations tested (Supplemental Figure 3). We did not observe migration by wild-type or LTC₄S^{-/-} eosinophils to S1P, a lipid mediator involved in immune cell trafficking (Supplemental Figure 4).

Eosinophil Trafficking from Lungs to Draining Paratracheal Lymph Nodes in LTC₄S^{-/-} Mice

We studied the *in vivo* effect of cys-LTs on airway eosinophil trafficking by using LTC₄ synthase-deficient (LTC₄S^{-/-}) mice. We utilized an OVA sensitization and inhalational challenge model in LTC₄S^{+/+} and LTC₄S^{-/-} mice with intratracheal instillation of DiIC₁₆(3)-labeled eosinophils prior to the last inhalational OVA challenge on Day 23 (Figure 3A). We previously used a similar model of allergic airway inflammation with intratracheal adoptive transfer of fluorescent eosinophils to demonstrate that eosinophils migrate to pLN, where they interact with CD4⁺ lymphocytes and act as antigen presenting cells (7). Intratracheal instillation of PBS alone served as a control. Twenty-four hours after the last OVA challenge, draining pLN were harvested and examined by fluorescence microscopy. While a strong fluorescent signal from migrating eosinophils was noted in pLN from LTC₄S^{+/+} mice, minimal fluorescence from migrating eosinophils was noted in pLN from LTC₄S^{-/-} mice (Figure 3B). Flow cytometry of cells from pLN yielded a similar result; in the representative data shown, DiIC₁₆(3)⁺ cells comprised 3.34% of the total pLN population in the LTC₄S^{+/+} mouse shown, while they comprised 1.12% of the total pLN population in the LTC₄S^{-/-} mouse shown (Figure 3C). Aggregate data from 6 mice per group over three separate experiments show this decrease in DiIC₁₆(3)-labeled eosinophils to the pLN to be a consistent finding ($p < 0.01$, Figure 3D).

To visualize dynamics of eosinophil egress from lungs, we adoptively transferred fluorescently DiIC₁₆(3) labeled eosinophils into the lungs of wild-type host mice. We observed that the fluorescently-labeled eosinophils in lungs decreased quickly over time

(Figure 4A). To quantify the fluorescently-labeled eosinophils in lungs, we made single cell suspensions from lungs of host mice harvested at various time points after the last OVA inhalation challenge, and assessed by flow cytometry to determine the kinetics of fluorescently-labeled eosinophil egress from the lungs. We compared 24-hour and 72-hour time points in wild-type and $\text{LTC}_4\text{S}^{-/-}$ mice to determine if the decrease in eosinophil trafficking to draining pLN of $\text{LTC}_4\text{S}^{-/-}$ mice correlated with increased numbers of eosinophils in the lungs (Figure 4B). We observed this to be the case at both the 24-hour and 72-hour time points, with $\text{LTC}_4\text{S}^{-/-}$ mice retaining a greater percentage of $\text{DiIC}_{16(3)}^+$ eosinophils in the lungs than their $\text{LTC}_4\text{S}^{+/+}$ counterparts (3 mice per condition for each time point, $p < 0.01$ for 3-day time point).

Restoration of Mobilization of Lung Eosinophils to Draining Paratracheal Lymph Nodes by Exogenous LTC_4

$\text{LTC}_4\text{S}^{-/-}$ mice were sensitized and challenged with OVA with intratracheal injection of $1\ \mu\text{g}$ of LTC_4 or PBS control after the second aerosol challenge at Day 22 (Figure 5A). No exogenous intratracheal eosinophils were introduced in these experiments. Eosinophils were identified in pLN by flow cytometry as $\text{SSC}^{\text{high}}/\text{Siglec-F}^+$. Results from $\text{LTC}_4\text{S}^{+/+}$ mice served as a control. $\text{LTC}_4\text{S}^{-/-}$ mice treated with exogenous LTC_4 had partial restoration of eosinophil migration to draining paratracheal lymph nodes at the dose used (Figure 5B). Aggregate data from 4 mice per group over two separate experiments are displayed in Figure 5C.

Discussion

The data presented here build upon our prior observations in mouse models of allergic airways inflammation that eosinophils migrate from lung to pLN and interact with CD4^+ lymphocytes (6, 7). We establish that mouse eosinophils express CCR7, which is the ligand for CCL19 and a key factor in leukocyte migration to lymph nodes, and also express CysLT_1 and CysLT_2 , the appropriate receptors for LTC_4 . Our observations of *in vitro* chemotactic behaviors to CCL19 of eosinophils from LTC_4 synthase deficient animals indicate that LTC_4 has a direct effect in facilitating chemotaxis, as pre-incubation of these eosinophils with LTC_4 restores their full chemotactic potential to CCL19. Furthermore, the restoration of chemotactic activity to eosinophils that cannot produce their own LTC_4 suggests the possibility of a paracrine effect by LTC_4 in augmenting chemotaxis. We observed *in vivo* that eosinophil migration to the paratracheal lymph nodes is substantially diminished in mice lacking LTC_4 synthase undergoing an OVA-sensitization and -challenge model of asthma, compared to wild-type controls. Furthermore, eosinophils are retained in the lungs of mice devoid of LTC_4 due to the absence of LTC_4 synthase. These data indicate that egress of eosinophils from the lungs to lymph nodes is dependent on the presence of LTC_4 in the local environment. Furthermore, migration of eosinophils out of the lungs can be at least partially restored when exogenous LTC_4 is reintroduced via the airways. Dissimilar to *in vitro* chemotaxis experiments, the intratracheally transferred eosinophils in both $\text{LTC}_4\text{S}^{+/+}$ and $\text{LTC}_4\text{S}^{-/-}$ conditions were eosinophils that had functional LTC_4 synthase. The possible paracrine effect of eosinophil-generated LTC_4 observed *in vitro* may have been insufficient in $\text{LTC}_4\text{S}^{-/-}$ OVA-sensitized and -challenged mice to restore eosinophil migration, when

accounting for the relatively small cell numbers distributed over an otherwise LTC₄-deficient cellular milieu. While these *in vivo* experiments do not fully delineate the mechanism by which the presence of LTC₄ facilitates the movement of eosinophils from lung to lymph node, the *in vitro* data presented suggest that augmentation of eosinophil chemotaxis to CCL19 is a significant contributor.

Leukotrienes have long been recognized to have an integral role in allergic airways inflammation and eosinophil immunobiology (1, 22). CysLTs have paracrine effects on eosinophil chemotaxis and recruitment, as well as regulatory function in the secretion of stored cytokine products by eosinophils (1–3). Cys-LTs are important mediators in the airway inflammation, mucous production, and bronchial hyperreactivity characteristic of asthma (23). Mice with augmented cys-LT production through the overexpression of LTC₄S exhibit a phenotype that models human aspirin-exacerbated respiratory disease (24). Leukotriene receptor blockade, specifically of CysLT₁, has become a staple of asthma management (1, 25–27). In a murine model of chronic asthma, the CysLT₁ receptor antagonist montelukast effectively reversed airway remodeling compared to corticosteroids (28).

Exposure to lipid mediators, including leukotrienes, has been demonstrated to modulate immune cell migration (29). Lipid mediators have specifically been observed to influence the migration of dendritic cells toward CCL19 and CCL21, the known chemokine ligands of CCR7. The CCR7 axis has an integral role in directing leukocyte movement into draining lymph nodes (15). Prostaglandin E₂ (PGE₂) enhances chemotaxis of dendritic cells in response to CCR7 ligands, as well promoting CCR7 surface expression on dendritic cells and signaling through CCR7 (30–33). The interplay between lipid mediators and CCR7 function in dendritic cells extends to leukotrienes. Leukotriene B₄ is a promoter of chemotaxis and CCR7 expression in murine dendritic cells (34). Relevant to our current findings with respect to eosinophil migration, the presence of LTC₄ with dendritic cells leads to increased stimulation of *in vitro* lymphocyte responses compared to PGE₂ and improved migration of dendritic cells to CCL19 (17, 35).

While the presence of CCR7 on mouse eosinophils had not been described prior to the current report, we have previously demonstrated that human eosinophils isolated from the peripheral blood and cultured in IL-5 do express CCR7 (16). Furthermore, the expression of CCR7 is functionally significant, with eosinophils showing directed chemotaxis to CCL19 and CCL21 in a dose-dependent fashion with concomitant activation of downstream signaling pathways upon stimulation of CCR7 (16). Our current data extend these observations to an *in vivo* murine model of allergic airways inflammation, while also integrating previous lines of evidence described above that suggest the integral importance of lipid mediators, specifically LTC₄, in the responses of leukocytes to CCR7. These data also build on the recognition that eosinophils traffic to the T-cell rich paracortical areas of draining lymph nodes in allergic airways inflammation, where they display immunoregulatory capacities, including the ability to function as antigen presenting cells (6, 7, 21, 36). Eosinophils also have the ability to differentially and rapidly secrete multiple preformed Th1 and Th2 cytokines to discrete stimuli, further bolstering the possibility that

they may have as yet unrecognized immunoregulatory function at the level of lymph node in allergic airways disease (37).

The present findings demonstrate that LTC₄ is necessary for optimal egress of lung eosinophils to draining mediastinal lymph nodes and that LTC₄ is needed for optimal chemotaxis of eosinophils to CCL19. Distal lymphatics may play an important function as the site of egress from the lung parenchyma, though further investigation is required (38). While our data suggest that CCR7 is central to the migration of eosinophils from lung to lymph node, there remains the possibility that CCR7 is not the only chemokine receptor that participates in the migration of eosinophils from lung to lymph node. Of note, CCR7^{-/-} mice that have undergone OVA sensitization and challenge do show migration of eosinophils to draining lymph nodes (39). As eosinophils are themselves a major source of LTC₄, there may be a significant autocrine component to the effect of LTC₄ on eosinophil migration to the draining lymph node. In our experimental model, the fluorescent eosinophils from IL-5 transgenic mice that were adoptively transferred to wild-type and LTC₄S^{-/-} mice were likely insufficient in number in the inflamed lung to provide enough LTC₄ to restore directed chemotaxis out of the lung. This was supported by the proportionally small number of fluorescent eosinophils that ultimately were recovered from the paratracheal lymph nodes even in wild-type mice (3.34%, Figure 3C).

In the aggregate, these data add to the understanding of the eosinophil trafficking from the lungs to draining lymph nodes in allergic asthma, as well as the functional importance of cys-LTs in facilitating of the migration of eosinophils to chemokines, including CCL 19. These findings may further explain some of the beneficial effects of cys-LT receptor antagonism as an effective therapy for asthma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Mouse eosinophils express CysLT₁ and CysLT₂ receptors and CCR7

A, Total RNA was extracted from eosinophils, from the spleens of IL-5 transgenic mice or lungs of mice (positive control) and analyzed for the expression of CysLT₁ and CysLT₂ mRNA by RT-PCR. 15S housekeeping gene (361 bp) amplified in parallel from samples to verify the integrity of cDNA. **B, C,** Eosinophils from the BAL of OVA-sensitized and –challenged wild-type mice (**B**) and from the BAL of OVA-sensitized and –challenged $LTC_4S^{-/-}$ mice (**C**) were stained with anti-CysLT₁ polyclonal Ab (solid line) or isotype Ab (dashed line) and analyzed by flow cytometry. **D,E,F,** Eosinophils from the spleens of IL-5 transgenic mice (**D**), from the BAL of OVA-sensitized and –challenged wild-type mice (**E**), and from the BAL of OVA-sensitized and –challenged $LTC_4S^{-/-}$ mice (**F**) were stained with anti-CCR7 mAb (solid line) or isotype Ab (dashed line) and analyzed by flow cytometry.

Figure 2. LTC₄ pre-treatment of LTC₄S^{-/-} eosinophils fully restores their migratory capacity to CCL19 *ex vivo*

A, Migration of LTC₄ pre-treated LTC₄S^{-/-} eosinophils to CCL19 compared with medium-treated LTC₄S^{-/-} eosinophils. The data represent mean ± SD of chemotaxis from two independent experiments. **B**, Chemotactic responses of LTC₄ pre-treated (30 min pre-incubation) and medium-treated LTC₄S^{-/-} eosinophils toward increasing concentrations of CCL19 (0, 10, 100, or 500 ng/ml).

Figure 3. Impaired airway eosinophil migration into draining paratracheal lymph nodes in LTC₄S-deficient mice

A, Mice were sensitized and challenged with OVA as depicted. Eosinophils (1×10^6) from the spleens of IL-5 transgenic mice, labeled *ex vivo* with DiIC₁₆(3), were intratracheally injected into wild-type (WT) or LTC₄S^{-/-} mice before last OVA aerosol challenge. Control mice intratracheally received the same volume of PBS. **B, C, D,** Twenty-four hours after injection, the pLN were harvested, and the fluorescently positive eosinophils in the pLN (as a percentage of total cells) were determined by either fluorescence microscopy (**B**) or flow cytometry (**C, D**). **D,** Mean $\pm$ SD from six mice per group over three separate experiments (* $p < 0.01$).

Figure 4. Retention of eosinophils in the lungs of LTC₄S^{-/-} mice

(A) DiIC₁₆(3) eosinophils in the lungs of wild-type mice at 1, 4, 8, 12, 24, and 48 hours after intratracheal instillation. DiIC₁₆(3) eosinophils labeled in red. Hoechst-stained lung parenchyma in blue. (B) Percentages of donor DiIC₁₆(3) eosinophils (as a percentage of total cells) in the lungs 24 hours and 72 hours after intratracheal instillation. Data represent mean $\pm$ SD from three mice for each condition.

Figure 5. Exogenous LTC_4 partially restores eosinophil mobilization to pLN *in vivo* in $LTC_4S^{-/-}$ mice

A. $LTC_4S^{-/-}$ mice received exogenous LTC_4 or PBS intratracheally 2 hours after the 2nd OVA-aerosol challenge. **B.** The eosinophils in pLN were gated as $SSC^{\text{high}}/\text{SiglecF}^+$ cells. **C.** Percentages of $SSC^{\text{high}}/\text{SiglecF}^+$ eosinophils in pLN cells are shown as mean $\pm$ SD from four mice per group over two separate experiments (* $p < 0.01$ compared to $LTC_4S^{-/-}$).