CCR7 binds to its cognate ligand, CCL21, to mediate the migration of circulating naive T lymphocytes to the lymph nodes. T lymphocytes can bind to fibronectin, a constituent of lymph nodes, via their $\beta_1$ integrins, which is a primary mechanism of T lymphocyte migration; however, the signaling pathways involved are unclear. We report that rapid (within 2 min) and transient phosphorylation of ERK1/2 is required for T cell migration on fibronectin in response to CCL21. Conversely, prevention of ERK1/2 phosphorylation by inhibition of its kinase, MAPK/MEK, prevented T lymphocyte migration. Previous studies have suggested that phospholipase C$\gamma_1$ (PLC$\gamma_1$) can mediate phosphorylation of ERK1/2, which is required for $\beta_1$ integrin activation. Paradoxically, we found that inhibition of PLC$\gamma_1$ phosphorylation by the general PLC inhibitor U73122 was associated with a delayed and reduced phosphorylation of ERK1/2 and reduced migration of T lymphocytes on fibronectin. To further characterize the relationship between ERK1/2 and PLC$\gamma_1$, we reduced PLC$\gamma_1$ levels by 85% using shRNA and observed a reduced phosphorylation of ERK1/2 and a significant loss of CCR7-mediated migration of T lymphocytes on fibronectin. In addition, we found that inhibition of ERK1/2 phosphorylation by U0126 resulted in a decreased phosphorylation of PLC$\gamma_1$, suggesting a feedback loop between ERK1/2 and PLC$\gamma_1$. Overall, these results suggest that the CCR7 signaling pathway leading to T lymphocyte migration on fibronectin is a $\beta_1$ integrin-dependent pathway involving transient ERK1/2 phosphorylation, which is modulated by PLC$\gamma_1$.

G protein-coupled receptors (GPCRs) are responsive to many types of stimuli, such as hormones, neurotransmitters, and chemoattractants. Ligand activation of a GPCR leads to a conformational change in the receptor, resulting in an exchange of GDP for GTP in the $\alpha$ subunit and dissociation of $\alpha$ from the $\beta\gamma$ subunits. The subunits then initiate downstream signaling events to regulate the activation state of adenyl cyclase or phospholipase C (PLC) (1). CCR7 (C-C chemokine receptor 7), a G protein-coupled receptor, is expressed on naive T lymphocytes and is required for migration into and within lymph nodes. CCR7 binds to two ligands, CCL19 and CCL21. CCL21 is expressed in high endothelial venules, the entry route into lymph nodes (2). However, it is currently unknown how T lymphocytes signal through CCR7 to mediate migration via $\beta_1$ integrins in response to CCL21.

Integrins, which are key mediators of lymphocyte adhesion and migration, are heterodimeric adhesion proteins consisting of an $\alpha$ and a $\beta$ subunit, for which they are named. $\beta_1$ integrins, which are expressed by T lymphocytes, bind to the vascular cell adhesion molecule, collagen, and fibronectin (3). Fibronectin is a major component of the lymph node and is highly expressed in the cortex in the presence of fibroblastic reticular cells, which express CCL21 (4, 5). This network provides an environment that allows CCR7-expressing T lymphocytes to migrate throughout the lymph node.

PLC$\beta$ is a downstream target of G$\beta\gamma$ signaling and is important for T cell migration (6). PLC$\gamma$ binds to and activates $\beta_1$ integrins (7). Furthermore, PLC$\gamma_1$ is required for adhesion to fibronectin and is important for $\beta_1$ integrin-mediated cell migration (8, 9). PLC$\gamma_1$ was recently shown to be phosphorylated in response to CCR7 activation in head and neck cancer cells and was necessary for the migration of squamous cell carcinoma cells (10).

GPCRs can activate the downstream effector MAPKs to regulate chemotactic migration (11, 12). In HEK293 cells, CCR7 transient transfectants stimulated with CCL19 rapidly activated ERK1/2 (extracellular signal-related kinase 1/2), whereas CCL21 had a minimal effect on ERK1/2 phosphorylation (13). In primary B lymphocytes that express endogenous CCR7, CCL21 mediated sustained activation of ERK1/2 for 10 min (11). These contrasting studies illustrate the importance of determining the role of ERK1/2 in the migration of primary T lymphocytes to CCL21. Therefore, we examined the molecular mechanisms required for $\beta_1$ integrin activation and $\beta_1$ integrin-mediated migration. We found that in primary T lymphocytes, CCL21 promotes the activation of PLC$\gamma_1$ and the transient activation of ERK1/2, which are required for $\beta_1$ integrin activation in response to CCL21. Loss of activation of either PLC$\gamma_1$ or transient phosphorylation of ERK1/2 prevented migration via $\beta_1$ integrins to CCL21. These results indicate that CCL21 activation of CCR7 promotes the activation of $\beta_1$ integrins, leading to migration following phosphorylation of PLC$\gamma_1$ and ERK1/2.
EXPERIMENTAL PROCEDURES

Mice, Primary Human Lymphocytes, and Reagents—Primary human T lymphocytes were isolated from volunteer donors under an approved protocol in accordance with the policies and procedures of the human subjects protection program at the University of Kansas Medical Center. U0126 (Calbiochem), U73122 and U73343 (Biomol), pertussis toxin (List Biological Laboratories), human PLCγ1 shRNA (OriGene), and species-specific CCL19 and CCL21 (R&D Systems) were purchased. The 12G10 anti-human activated β1 integrin antibody was generously provided by Dr. Martin Humphries (University of Manchester, Manchester, UK), and the MAB1959 function-blocking antibody was purchased (Millipore).

T Lymphocyte Isolation—Human blood was collected from volunteer donors, and peripheral blood mononuclear cells were isolated using a Ficoll-Paque gradient (GE Healthcare). Whole blood was transferred to 50-ml conical tubes and diluted at a 1:1 ratio with PBS + Ca2+/Mg2+ (Cellgro). In a separate 50-ml conical tube, 15 ml of Ficoll-Paque was layered with 35 ml of whole blood/PBS + Ca2+/Mg2+ and centrifuged for 20 min at 100 × g without the brake. The middle layer containing lymphocytes was isolated, washed, and pelleted. T lymphocytes were negatively selected using the EasySep kit (Stemcell Technologies) according to the manufacturer’s protocol. Lyophilized were maintained in RPMI 1640 medium (Invitrogen), 10% heat-inactivated FBS (Hyclone), and 2 mM L-glutamine (Invitrogen) in a humidified atmosphere at 37 °C and 5% CO2 for use within 3 days of isolation.

Chemotaxis Assays—All chemotaxis assays were carried out using a 48-well chemotaxis chamber (Neuroprobe). Lower wells were loaded with 0 or 10 nm to 2 μM CCL21 in serum-free RPMI 1640 medium and were separated from the upper wells containing the lymphocytes using a 5-μm pore nitrocellulose membrane (Neuroprobe) that had been preincubated in either serum-free RPMI 1640 medium or 10 μg/ml fibronectin (Sigma). Primary human T lymphocytes (5 × 10^5) were allowed to migrate in a humidified, 37 °C, 5% CO2 chamber for 2 h to gradients of CCL21. For β1 integrin function-blocking studies, primary human T lymphocytes were mixed with 10 μg/ml MAB1959 or an isotype control prior to loading in the migration chamber. Following migration, chambers were disassembled, and lymphocytes in the bottom chamber were collected and counted by hemocytometer or fixed in 2% paraformaldehyde and then counted using a hemocytometer. In each assay, migration of cells to serum-free RPMI 1640 medium was considered the “0” migration point, and all migration values were normalized to that point. Assays were performed in duplicate and replicated a minimum of three times.

Western Blots—Lymphocytes were pretreated in serum-free medium with U0126 (1 μM) for 90 min, U73122 (2 μM) for 20 min, or pertussis toxin (100 ng/ml) for 2 h at 37 °C. Lymphocytes were then stimulated with 8 nm CCL19 or CCL21 for 0, 2, 5, and 10 min. Lymphocytes were then lysed in radio immunoprecipitation assay buffer (100 mM Tris, pH 8.0, 150 mM NaCl, 2% Nonidet P-40, 1% sodium deoxycholate, and 0.2% SDS) supplemented with a protease inhibitor mixture (Sigma), 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 10 mM sodium fluoride (Sigma), and 1 mM β-glycerophosphate (Sigma) for 15 min on ice. Lysates were sheared, and insoluble material was removed by centrifugation at 14,000 × g for 20 min. Supernatants (5 × 10^6 cell equivalents per time point) were mixed with Laemmli sample buffer, fractionated on 7.5% SDS polyacrylamide gels, and transferred to PVDF membranes. Membranes were preincubated in 5% milk/Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.5, 150 mM NaCl), 0.1% TBS with Tween 20 (TBST), or 5% BSA/TBST (Sigma) and probed with anti-phospho-ERK1/2, anti-total ERK1/2, anti-total PLCγ1, or anti-phospho-PLCγ1 antibody (Cell Signaling Technology). Primary antibodies were detected with HRP-conjugated anti-species-specific IgG (Pierce), and immune complexes were visualized with SuperSignal West Femto substrate (Pierce). Membranes were stripped using Blotfresh (SignaGen Laboratories), rinsed in 1 × TBST, blocked, and re-probed. Protein bands were quantified using ImageJ software.

PLCγ1 shRNA Transfection—Primary human T lymphocytes were transiently transfected with human PLCγ1 shRNA (OriGene TF302445) or control shRNA (OriGene TR30015) using Lipod293 (SignaGen) according to the manufacturer’s instructions. Briefly, PLCγ1 or control (8 μg) shRNAs and Lipod293 were diluted into DMEM. Diluted Lipod293 was added to the diluted shRNA and incubated for 15 min, and the Lipod293-shRNA complexes were added to the lymphocytes. Lymphocytes were incubated with the complexes in complete medium for 72 h at 37 °C and 5% CO2 prior to assay.

β1 Integrin Activation Assay—Lymphocytes were treated with U0126 or pertussis toxin or transfected with PLCγ1 shRNA, as described previously. Lymphocytes were unstimulated (0 min) or stimulated (0.5–5 min) with 400 nm CCL19 or CCL21. Following stimulation, lymphocytes were submerged in 1 ml of ice-cold 1× PBS. Lymphocytes were labeled with 0.1 μg/ml 12G10 on ice for 30 min (or overnight), washed in 1 × PBS two times, incubated with FITC-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories) for 30 min, and washed in 1 × PBS two times. Lymphocytes were fixed in 2% paraformaldehyde and analyzed with the FACSCalibur flow cytometer. All integrin levels were normalized to the mean fluorescence level of integrins found on vehicle control cells (control) for each experiment.

Statistics—All migration and β1 integrin activation assays were analyzed by two-tailed, unpaired Student’s t test. A p value of <0.05 was considered statistically significant.

RESULTS

Primary Lymphocytes Differentially Migrate to CCL21 on Fibronectin—Naïve T lymphocytes, which express CCR7, enter the lymph nodes in response to CCL21 found in high endothelial venules. To define the molecular mechanisms that control CCL21-mediated T lymphocyte migration, we used chemotaxis assays. Naïve T lymphocytes were induced to migrate over a physiological range of CCL21 concentrations (10 nm to 2 μM) or in the absence of CCL21. We found that naïve T lymphocytes migrated maximally to 2 μM CCL21 on polycarbonate membranes (Fig. 1A). To understand how T lymphocytes migrate via β1 integrins, we preincubated the polycarbonate membranes with the β1 integrin ligand fibronectin. Lymphocytes were
induced to migrate over a wide range of physiological concentrations (10 nM to 2 μM) to CCL21, with the greatest migration occurring at 400 nM (Fig. 1B). To confirm that the cells were migrating via β1 integrins, we examined the migration in the presence of β1 integrin function-blocking antibodies. Under these conditions, the cells failed to migrate across the fibronectin-coated membranes. Isotype controls had no effect on migration (data not shown). These results implicate β1 integrins in human T lymphocyte migration to CCL21.

**ERK1/2 Is Required for Migration to CCL21—GPCRs can activate ERK1/2 to mediate the migration of human primary macrophages and human osteosarcoma cells (12, 14). To examine the role of ERK1/2 in the migration of naïve T lymphocytes to CCL21 via their β1 integrins, we examined migration to CCL21 in the presence of the MEK inhibitor U0126. Under these conditions, naïve T lymphocytes failed to migrate on fibronectin compared with controls treated with an equivalent dilution of vehicle (dimethyl sulfoxide; \( p = 0.031 \)) (Fig. 2A). We used Western blotting to confirm that U0126 blocked MEK activation of ERK1/2. As expected, U0126 blocked ERK1/2 phosphorylation in cells treated with CCL21 (Fig. 2B). PLCγ1 can mediate the phosphorylation of ERK1/2 (15, 16). To examine the contribution of ERK1/2 to activation of PLCγ1, we probed for PLCγ1 phosphorylation. Unexpectedly, we found that phosphorylation of PLCγ1 was also decreased (Fig. 2B). From these results, we concluded that rapid ERK1/2 activation is required for migration to CCL21. Furthermore, PLCγ1 phosphorylation may be mediated by activated ERK1/2 to regulate CCL21-mediated migration.

Cell migration requires regulated integrin adhesion, which is mediated by activation, followed by integrin de-adhesion (17, 18). Therefore, we questioned the role of ERK1/2 in β1 integrin activation. To this end, we pretreated lymphocytes with U0126 or an identical dilution of the vehicle (dimethyl sulfoxide) and measured the levels of β1 integrin activation compared with lymphocytes pretreated with U0126 (C).

From these data, we concluded that phosphorylation of ERK1/2, as determined by a Western blot, was inhibited under these conditions. To examine the role of ERK1/2 in the activation of β1 integrins, lymphocytes were stained with an activation-specific antibody, 12G10 (19). Levels of activated β1 integrins were analyzed by flow cytometry. We found that lymphocytes treated with U0126 displayed similar β1 integrin activation compared with vehicle controls (control) (Fig. 2C). From these data, we concluded that β1 integrin activation in T lymphocytes in response to stimulation of CCR7 with CCL21 is not regulated by ERK1/2 phosphorylation.
**CCR7/CCL21 Migration via β1 Integrins Is Mediated by PLCγ1**

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**PLC Is Required for Migration on Fibronectin to CCL21**—As mentioned previously, PLCγ1 binds to and activates β1 integrins (7). Because the loss of ERK1/2 phosphorylation correlated with decreased PLCγ1 phosphorylation and reduced migration to CCL21, we questioned if PLC was necessary to control migration to CCL21. To this end, we used chemotaxis assays in the presence of U73122, a universal PLC inhibitor, or the U73343 control (Fig. 3A). To confirm that U73122 blocked activation of PLCγ1 in T lymphocytes in response to CCL21 stimulation, we assayed for phosphorylation of PLCγ1 by Western blotting. Lymphocytes pretreated with U73122 displayed decreased phosphorylation of PLCγ1 compared with the U73343-treated controls (Fig. 3B). Although U73122 blocked all migration, it was unclear why the U73343 control shifted the migratory response of cells. In contrast to the U73343-treated cells, phosphorylation of ERK1/2 was delayed in the U73122-treated cells (Fig. 3B). When T lymphocytes were preincubated with U73122, migration to CCL21 was reduced compared with U73343-treated controls (Fig. 3A). From these results, we concluded that rapid phosphorylation of PLC and/or ERK1/2 mediates the migration of T lymphocytes to CCL21.

**PLCγ1 Is Necessary for β1 Integrin Migration and Activation**—Because U73122 is a universal inhibitor of PLC, to determine the role of PLCγ1 in the migration of naive T lymphocytes via β1 integrins, we used PLCγ1 shRNA to specifically target and knockdown levels of PLCγ1 in primary human T lymphocytes. Lymphocytes were transiently transfected with PLCγ1 shRNA or control shRNA and assayed for β1 integrin activation and migration. Using NIH Image-Quant software, we found that total PLCγ1 levels were reduced by ~85% in the shRNA-transfected lymphocytes (Fig. 4A). T lymphocytes that were depleted of PLCγ1 failed to migrate to 400 nM CCL21 on fibronectin-coated membranes, as opposed to T lymphocytes treated with control shRNA (Fig. 4B). In addition, the loss of PLCγ1 led to a failure to activate β1 integrins in response to stimulation with 400 nM CCL21, as determined by flow cytometry (Fig. 4C). Because we observed delayed ERK1/2 phosphorylation in response to U73122, we questioned whether this was due to an inhibition of PLCγ1. We observed that the knockdown of PLCγ1 resulted in a trend toward a rapid and sustained increased ERK1/2 phosphorylation compared with controls (Fig. 4, D and E). From these results, we concluded that PLCγ1 is required for rapid ERK1/2 phosphorylation and activation of β1 integrins, resulting in the migration of naive T lymphocytes to CCL21 on fibronectin.
CCR7/CCL21 Migration via β1 Integrins Is Mediated by PLCγ1

Ga<sub>i</sub> Mediates Migration to CCL21—To examine the signaling events upstream of PLCγ1, we investigated the role of G proteins in the activation of PLCγ1. CCR7 couples to Ga<sub>i</sub> to initiate downstream signaling events and is required for migration to CCL21 (2). Therefore, to examine signaling events more proximal to the membrane that mediate activation of PLCγ1, we pretreated lymphocytes with pertussis toxin. As expected, lymphocytes pretreated with pertussis toxin failed to migrate to any concentration of CCL21 compared with controls (Fig. 5A). Therefore, to determine the role of Ga<sub>i</sub> in signaling through CCR7 to mediate activation of PLCγ1 and ERK1/2 in the presence of pertussis toxin, we assayed for changes in phosphorylation of PLCγ1 and ERK1/2 in response to stimulation with 400 nM CCL21. In pertussis toxin-treated lymphocytes, phosphorylation of ERK1/2 and PLCγ1 was reduced following treatment with CCL21 compared with controls (Fig. 5B). These results indicate that CCR7 coupling to Ga<sub>i</sub> mediates PLCγ1 and ERK1/2 phosphorylation and β1 integrin deactivation, leading to the migration of T lymphocytes on fibronectin.

To determine the extent to which Ga<sub>i</sub> affects β1 integrin activation in naive T lymphocytes, we pretreated lymphocytes with pertussis toxin, stimulated with 400 nM CCL21, and assayed by flow cytometry for activated integrins on the cell surface. In the presence of pertussis toxin, CCL21 induced the same level of β1 integrins as in the CCL21-stimulated vehicle control cells (p = 0.365) (Fig. 5C), although cell migration to CCL21 in the presence of pertussis toxin was lost compared with control cells. In addition, there was a significant elevation in the levels of activated β1 integrins when lymphocytes were treated with CCL21 in the presence (p = 0.0014) or absence (p = 0.0236) of pertussis toxin compared with unstimulated controls in the presence or absence of pertussis toxin, respectively. There was no significant difference in the levels of activated integrins with or without pertussis toxin prior to treatment with ligand (p = 0.365). Because cell migration requires the control of integrin adhesion (activation) and de-adhesion (deactivation), from these studies, we concluded that signaling through Ga<sub>i</sub> leads to cycling of adhesion and de-adhesion states. To test this hypothesis, we examined the activation state of the β1 integrins over time (Fig. 5D). We found that although the levels of activated β1 integrins varied over time in both pertussis toxin-treated cells and control cells, the levels of activation overlapped throughout the time course. These results suggest that Ga<sub>i</sub> is important for migration to CCL21, but not for the initial activation of β1 integrins in response to CCL21 stimulation.

DISCUSSION

CCR7 is expressed on B lymphocytes, dendritic cells, natural killer cells, T regulatory cells, and naive T lymphocytes and is required for their migration into and within lymph nodes. CCR7 binds to two ligands, CCL19 and CCL21. Although many studies have described CCL19 signaling through CCR7, little is known about how CCL21 signals through CCR7, leading to migration on fibronectin (20–23).

High endothelial venules and lymph nodes express substantial amounts of fibronectin (1). As lymphocytes enter into and migrate through lymph nodes, they use β1 integrins for adhesion and migration (2). Because CCL21 is required for lymph node entry as well as migration within the lymph nodes, it was important to define the CCL21-mediated mechanisms that control β1 integrin-mediated migration on fibronectin. In this study, we have provided evidence that CCL21 induces β1 integrin activation and promotes migration on fibronectin via PLCγ1 and ERK1/2 phosphorylation.

GPCRs are well known for their ability to activate MAPKs (24, 25). MAPKs, which are involved in many cellular processes, including migration (26–28). Several groups have reported that stimulation of CCR7 by CCL21 mediates phosphorylation of the MAPK ERK1/2 in primary murine T lymphocytes, B lymphocytes, and HEK293 CCR7-transfected cells (11, 13, 29). However, from these studies, it is difficult to interpret what if any role ERK1/2 plays in the migration of T lymphocytes. In this study, similar to previous studies, we have reported that...
ERK1/2 is rapidly and transiently phosphorylated in response to CCR7 activation by CCL21 in primary human T lymphocytes. In addition, we found that rapid and transient phosphorylation of ERK1/2 is required for migration to CCL21 via CCR7 on the β1 integrin ligand fibronectin. Transient activation of ERK1/2 has been shown to be important for aortic smooth cell migration (30). We used the MEK inhibitor U0126 to demonstrate that phosphorylation of ERK1/2 is important for T cell migration in response to CCL21. In addition, using PLCγ1 shRNA, we found that ERK1/2 phosphorylation was sustained, which correlated with decreased migration to CCL21. Taken together, these results suggest that rapid and transient phosphorylation of ERK1/2 is important for migration to CCL21 in T lymphocytes.

Stimulation of human epidermoid carcinoma cells with 12(S)-hydroxylipoxygenase-5 (12(S)-HETE) results in PLCγ1 phosphorylation and downstream activation of ERK1/2 via pertussis toxin-sensitive signaling events (31). In our study, we found that transient phosphorylation of ERK1/2 is lost following treatment with PLCγ1 shRNA, which results in decreased migration to CCL21. Taken together, these results suggest that transient phosphorylation of ERK1/2 is important for control of T cell migration to CCL21.

In the absence of Gαi, activation was not necessary for β1 integrin activation, even though pertussis toxin inhibited migration to CCL21. Furthermore, we found that pertussis toxin alone did not increase levels of activated β1 integrins, and although the levels of activated β1 integrins increased in the presence of CCL21, similar to pertussis toxin-treated cells, we found that in the presence of U0126, β1 integrins could be reactivated, migration to CCL21 was inhibited. Because pertussis toxin led to reduced levels of phosphorylation of ERK1/2, we speculated that Gαi and possibly ERK1/2 are not required for the initial activation of β1 integrins; however, they are required for de-adhesion (inactivation) of β1 integrins. Gαi is bound to the resting CCR7. Inhibition of Gαi, with pertussis toxin, however, inhibits rapid ERK1/2 phosphorylation and phosphorylation of PLCγ1, but leads to activation of β1 integrins. In contrast, a reduction in PLCγ1 levels following treatment with PLCγ1 shRNA blocked integrin activation. One possible explanation is that separate pools of PLCγ1 may be activated by CCR7. In that case, one could control β1 integrin activation, whereas the second pool would control the phosphorylation state of effectors such as PLCγ1 and ERK1/2. In the absence of Gαi, β1 integrins would be activated; however, the lymphocytes would be unable to regulate PLCγ1 activity to allow for de-adhesion of the β1 integrins, and the integrins would remain activated. Alternatively, following treatment with shRNA, other PLCs could be depleted. We concluded that it is the de-phosphorylation of ERK1/2 that is important for α1 integrin recycling and migration to CCL21. Studies are currently under way in our laboratory to sort out these hypotheses.

T lymphocytes are crucial mediators of inflammation, autoimmune disorders, allergic disease, and cancer. Migration is not only important for a normal, functioning immune system, but is also important in disease. Therefore, it is important to understand the molecular mechanisms of how T lymphocytes migrate into and throughout lymph nodes to become effector cells to carry out immune responses in the periphery. This study is an important step in further understanding the molecular mechanisms that regulate T lymphocyte migration to CCL21 and provide the basis for pharmacological targets for controlling T cell entry and migration within lymph nodes to regulate the immune response.

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