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Activation of Phosphoinositide 3-Kinases by the CCR4 Ligand Macrophage-Derived Chemokine Is a Dispensable Signal for T Lymphocyte Chemotaxis

Darran G. Cronshaw,* Charles Owen,† Zarin Brown,‡ and Stephen G. Ward* *

Macrophage-derived chemokine (MDC/CC chemokine ligand 22 (CCL22)) mediates its cellular effects principally by binding to its receptor CCR4, and together they constitute a multifunctional chemokine/receptor system with homeostatic and inflammatory roles in the body. We report the CCL22-induced accumulation of phosphatidylinositol-(3,4,5)-trisphosphate (PI(3,4,5)P3) in the leukemic T cell line CEM. CCL22 also had the ability to chemotactically human Th2 cells and CEM cells in a pertussis toxin-sensitive manner. Although the PI(3,4,5)P3 accumulation along with the pertussis toxin-susceptible phosphorylation of protein kinase B were sensitive to the two phosphoinositide 3-kinase inhibitors, LY294002 and wortmannin, cell migration was unaffected. However, cell migration was abrogated with the Rho-dependent kinase inhibitor, Y-27632. These data demonstrate that although there is PI(3,4,5)P3 accumulation downstream of CCR4, phosphoinositide 3-kinase activity is a dispensable signal for CCR4-stimulated chemotaxis of Th2 cells and the CEM T cell line. The Journal of Immunology, 2004, 172: 7761–7770.

The chemokines thymus- and activation-regulated chemokine/CC chemokine ligand 17 (CCL17)† and macrophage-derived chemokine (MDC/CCL22) can be classified as both homeostatic and inflammatory chemokines and exert their effects via the chemokine receptor CCR4. CCR4 is expressed on Th2 cells (1–4), CLA+ cutaneous memory T cells (5), NK cells (6), thymocytes (7, 8), immature dendritic cells, basophils, and platelets (9, 10). Due to the fact that CCR4 was initially thought to be exclusively expressed on Th2 cells, early pathological roles for CCR4 concentrated on known Th2-mediated conditions. As a consequence, CCR4 and its ligands have perhaps been most widely investigated and implicated to varying degrees in the Th2-mediated disease of allergic inflammation of the airways. Various studies have indicated that CCR4 and its ligands may play a role in this pathological condition (4, 11–17). CCR4 and/or CCL17/CCL22 have also been implicated in atopic dermatitis (5, 18, 19), endotoxic shock (20), rheumatoid and juvenile rheumatoid arthritis (21, 22), migration of leukemia-specific T cells (23), Fulminant hepatic failure (24), Hodgkin’s lymphoma (25), and T cell non-Hodgkin lymphoma (26).

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neutrophils and subsequent migration (37–40). However, loss of PI3Kγ activity does not lead to complete abolition of the directional motility of all cells, and its requirement for migration may be restricted to the myeloid lineage (39). In addition, the class Iα catalytic isoforms have been shown to be essential in Dictyostelium chemotaxis (35, 36) as well as to play a role in macrophage and T cell migration (41, 42). Moreover, a selective phosphoinositide 3-kinase (p110δ) inhibitor used in vitro and in vivo studies has revealed an essential role for this isoform in neutrophil migration in response to fMLP (43, 44). Finally, the class II PI3K isoform, PI3K-C2α, has been shown to be activated by CCL2 stimulation of the monocytic cell line THP-1, but the functional relevance of this class is unknown (32).

In this study we show that the CCR4 ligand, CCL22, is able to activate the PI3K pathway, resulting in accumulation of PI(3,4,5)P3 and phosphorylation of the downstream effector, PKB. However, the activation of PI3K isoforms appears to be dispensable for CCL22-mediated migration of T cells.

Materials and Methods

Reagents

Human recombinant CCL22 was purchased from R&D Systems (Abingdon, U.K.). The R-PE-conjugated mouse anti-CCR4 mAb was purchased from BD PharMingen (Oxford, U.K.). Goat anti-PIK3 (sc-1618), rabbit anti-PI3K-δ (sc-7176), and goat anti-PI3K-γ (sc-7177) polyclonal Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p85, anti-PI3K-C2α, and anti-PI3K-C2β Abs were gifts from J. Domin (Imperial College, London, U.K.). Phospho-specific polyclonal Abs, recognizing PKB (473 catalogue no. 9271), PKB phosphothreonine 308 (catalogue no. 9275), and myosin light chain 2 (MLC2) phosphoserine 19 (catalogue no. 3671) were purchased from Cell Signaling Technologies (NEB, Hitchin, U.K.). The ECL system, [3H]orthophosphate, and [γ-32P]ATP were obtained from Amersham International (Little Chalfont, U.K.). LY294002, wortmannin, Y-27632, and pertussis toxin were purchased from Calbiochem (Nottingham, U.K.). Flo-Scint IV was purchased from Packard Bioscience (Gröningen, The Netherlands). TLC plates were acquired from Whatman (Maidstone, U.K.). Secondary Abs for immunoblotting were purchased from DAKO (Glostrup, Denmark). Cell culture reagents were purchased from Life Technologies (Paisley, U.K.). Solvents were purchased from Fisher Scientific (Loughborough, U.K.). All other reagents were purchased from Sigma-Aldrich (Gillingham, U.K.).

Cell culture

The human Caucasian acute T lymphoblastoid leukemia cell line CEM was cultured in a humidified incubator in 5% CO2 at 37°C in RPMI 1640 medium, supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

In vitro generation of Th2 cells

Heparinized blood samples from healthy adult volunteers were separated on a Ficoll-Paque (1.077) density gradient. PBMCs were removed from the gradient, and naive CD4+ T cells (CD4+ CD45RA+) were isolated using magnetic cell separation. Briefly, this involved magnetically labeling cells with CD4+ microbeads and separating (positive selection) on a column that is placed in the magnetic field of an AutoMACS separator (beads and machine from Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were washed, and the process was repeated for positive selection of CD45RA- cells within the CD4+ cell fraction. Isolated CD4+ CD45RA- cells were cultured in humidified incubators in 5% CO2 at 37°C in RPMI 1640 medium, supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Flow cytometry

Cells (1 x 10^6) were washed twice in PBS and resuspended in 1 µg of PE-conjugated anti-CCR4 mAb, or corresponding PE-conjugated IgG1, λ isotype control (BD Pharmingen), or 100 µl of PBS/20% FBS negative (vehicle) control and incubated for 30 min on ice in the dark. Cells were washed and analyzed using a FACScVantage (BD Biosciences, San Jose, CA; excitation, λ 488 nm; emission, λ 578 nm; bandpass filter 575/26).

Immunoblotting

Cells were stimulated (2 x 10^6 cells/ml; 1 ml/sample) and incubated at 37°C in RPMI 1640 and 0.1% BSA. Reactions were terminated by the addition of 200 µl of 1× sample buffer (62.5 mM Tris, 2% SDS, 5% ME (v/v), 10% glycerol (w/v), and 0.02% bromophenol blue). The samples were boiled for 5 min, and the solubilized proteins were electrophoresed through 10% polyacrylamide/SDS gels and transferred by electroblotting onto nitrocellulose membranes. Membranes were incubated for 1 h with 25 ml of blocking solution (1% nonfat milk/0.05% sodium azide in TBS (10 mM Tris (pH 7.5) and 100 mM NaCl)), then incubated overnight with 10 ml of a 1/1000 dilution (in TBS and 0.1% Tween) of the appropriate Ab, as described previously (42). When necessary, membranes were stripped for reprobing by incubation in stripping buffer (62.5 mM Tris (pH 6.8), 2% SDS, and 100 mM 2-ME) at 60°C for 30 min.

Chemotaxis assay

Chemotaxis assays were conducted in 96-well chemotaxis chambers (NeuroProbes, Gaithersburg, MD) with polyvinylpyrrolidone-free polycarbonate membranes (5 µm pore size). The lower chambers of each well were filled with 365 µl of agonist at the appropriate concentration (diluted in RPMI 1640 and 0.1% BSA) and carefully overlaid with the polycarbonate membrane. Cells were washed twice and resuspended in RPMI 1640 medium and 0.1% BSA at 1 x 10^6 cells/ml and 200 µl of the cell suspension was added to the upper chambers. The chambers were incubated for 90 min in a 5% CO2-humidified incubator at 37°C, and cells migrating across the membrane into the lower chamber were determined as described previously (42) using 20 µl of Cell Titer 96 AQueous reagent (Promega, Southampton, U.K.) following the manufacturer’s instructions. Treatment of cells with the PI3K inhibitors, pertussis toxin and Y-27632, had no detectable effect on the ability of cells to bioreduce the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium compound in the CellTiter 96 AQueous One solution compared with the untreated cells (data not shown). Unless otherwise stated, results are expressed as the mean chemotactic index (±SEM; n = 5), which is the ratio of cells migrating toward chemokine vs untreated cells randomly migrating across the membrane (with or without the presence of inhibitor as appropriate). Statistical analysis was performed using ANOVA and Student’s t test, with Bonferroni correction where necessary.

Measurement of D3 phosphoinositide lipids

Cells were washed three times with phosphate-free DMEM; cells were incubated for 10 min at 37°C between washes to deplete them of phosphate. Cells were resuspended at 2 x 10^5 cells/ml in phosphate-free DMEM and 5% phosphate-free FBS and labeled with 1 µCi of [3H]orthophosphate/1 x 10^4 cells for 4 h at 37°C. 3H-labeled CEM cells were washed three times, resuspended at 2 x 10^5 cells/108 µl, and incubated with 12 µl of chomokine at the required concentration, as described in the figure legends, and phospholipids were extracted as previously described (45). The samples were deacetylated, and the glycerophoryl lipid derivatives were separated by anion exchange HPLC using a 12.5-cm Partisphere SAX column (Whatman, Maidstone, U.K.) and analyzed as described previously (45).

Results

Expression of CCR4 on human Th2 cells and CEM leukemic T cells

This study principally used the CEM cell line and human peripheral blood-derived T cells differentiated in vitro toward a Th2 phenotype. Flow cytometry revealed that CEM cells constitutively exhibited substantial amounts of cell surface CCR4 (Fig. 1A). Various studies have established the expression of CCR4 on human Th2 cells (1–3). Fig. 1B highlights the effectiveness of the method used in this study to generate CCR4-expressing Th2-like cells.
The CCR4 agonist CCL22 stimulates the accumulation of PI(3,4,5)P₃ and the phosphorylation of PKB in a concentration-dependent and transient manner in CEM cells

A method of estimating PI3K activation is to assess the accumulation of one of its major lipid products, namely PI(3,4,5)P₃. Consequently, we have used ³²P-labeled CEM cells to investigate the effect of CCL22 stimulation on the levels of this phospholipid. Treatment of CEM cells with CCL22 resulted in a transient increase in PI(3,4,5)P₃ levels, with a maximum response observed at 2–5 min (Fig. 2A), which declined toward basal levels at 10–30 min.

The major downstream effector of PI3K activity is PKB, a serine/threonine kinase, whose recruitment, phosphorylation, and subsequent activation are entirely dependent upon the lipid products of PI3K (46). Monitoring PKB phosphorylation is therefore an indirect readout of PI3K activity. Several studies have reported the activation of PKB after stimulation of chemokine receptors other than CCR4 (31, 47, 48), and PKB itself has been proposed to be involved in the polarization of neutrophils responding to a chemotactic gradient (38, 49). CEM cells characteristically display a constitutive level of phosphorylated PKB, which correlates with the lack of protein expression of the ³-phosphoinositide lipid phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) (50). However, CCL22 stimulated phosphorylation of PKB above basal levels in a concentration-dependent manner (Fig. 2B). In contrast to the rapid and transient CCL22-stimulated PI(3,4,5)P₃ accumulation, CCL22-induced increases in PKB phosphorylation were slightly more sustained, with responses reaching a maximum at 5–10 min and returning toward basal levels at 30 min poststimulation (Fig. 2C). The CEM cells may lack...
expression of the PI(3,4,5)P3-metabolizing 3' phosphatase PTEN, but they do express the Src homology 2 domain-containing inositol polyphosphate 5'-phosphatase (SHIP) (50). The activity of SHIP and/or another unknown phosphoinositide lipid phosphatase is probably responsible for the transient nature of the PI(3,4,5)P3 accumulation. Although the duration and magnitude of PI(3,4,5)P3 accumulation will influence the phosphorylation of PKB by 3'-phosphoinositide-dependent kinase-1/2, it should be noted that the duration of PKB phosphorylation will also be regulated by the activity of phosphatases such as phosphoprotein phosphatase 2A (51).

**PI3K inhibitors abrogate the CCL22-stimulated accumulation of PI(3,4,5)P3** and PKB phosphorylation, but have no effect on CEM cell chemotaxis.

One functional response of chemokines is to elicit directed cell migration. The involvement of PI3K in chemokine-mediated T cell chemotaxis has been reported for various receptors (31, 39, 42). Hence, we investigated whether CCL22 could stimulate chemotaxis of CEM cells and whether this was sensitive to the structurally unrelated PI3K inhibitors, wortmannin and LY294002, which do not exhibit any degree of selectivity toward individual PI3K isoforms. The fungal metabolite wortmannin causes irreversible inhibition of PI3K isoforms by covalent modification of the catalytic sites, whereas LY294002 acts as a competitive inhibitor at the ATP binding site of PI3K (52), so both drugs can elicit long-lasting inhibitory actions in the conditions used in the following experimental procedures.

Several studies have demonstrated the ability of CCL22 to elicit the migration of a variety of CCR4-expressing cells (53–55). Accordingly, CCL22 stimulated the chemotaxis of CEM cells in a concentration-dependent manner with classic bell-shaped characteristics (Fig. 3A), although we could detect no effect on chemo-

**FIGURE 3.** CCL22-stimulated PI3K/PKB activation is sensitive to PI3K inhibitors. A, CEM cells (2 × 10⁷ cells/200 μl) were added to the upper chambers of 96-well chemotaxis plates above lower chambers containing varying concentrations of CCL22, as described in Materials and Methods. Chemotaxis across a 5-μm pore size membrane was determined after 1.5-h incubation at 37°C in 5% CO₂. The data were derived from a single experiment with quintuplicate replicates that is representative of three other experiments. Data were analyzed by ANOVA and Student’s t test, with Bonferroni correction, for significance of CCL22-stimulated migration compared with randomly migrating cells (**, p < 0.0005; ***, p < 0.0001; ****, p < 0.00005). The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels. B, ³²P-labeled CEM cells (2 × 10⁷) were pretreated for 30 min with the indicated concentrations of LY294002 (top graph) and for 15 min with wortmannin (bottom graph) before stimulation with CCL22 (10 nM) for 5 min at 37°C. Phospholipids were extracted and deacylated, and the glycerophosphorylinositol derivatives of PI(3,4,5)P3 were analyzed by HPLC, as described in Materials and Methods. Data are representative of at least two separate experiments. C and D, CEM cells (2 × 10⁶ cells/ml) were left untreated (C) or were treated with LY294002 (30-min pretreatment) or wortmannin (15-min pretreatment) at the concentrations indicated. Cells were then stimulated with CCL22 (10 nM) at 37°C for the times indicated, and lysates were generated. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-phospho-PKB (Thr473) and anti-phospho-PKB (Ser308). The blots were stripped and reprobed with anti-PKB Ab to verify equal loading and efficiency of protein transfer.
kinesis (data not shown). Approximately 20% of the total number of cells added to the upper chamber of the chemotaxis plate migrated across the 5-μm pore size membrane within the 1.5-h period (Table I). Pretreatment of cells with wortmannin had no detectable impact on CCL22-mediated CEM cell chemotaxis, even at concentrations as high as 300 nM (Table I). Curiously, pretreatment of the cells with LY294002 significantly reduced the levels of basal CEM cell migration by ∼50% at 10–30 μM (Table I). However, only a modest inhibition of the total number of cells migrating in response to CCL22 was observed in the presence of 30 μM LY294002, a concentration at the upper limit of its selectivity toward PI3K. The disparity between wortmannin and LY294002 in terms of their effects on basal migration probably reflects differences in the secondary targets of these drugs, with those inhibited by LY294002 being important for cell motility.

To verify that the PI3K inhibitors are having the intended inhibitory effect on PI3K activity, parallel experiments examined the effects of wortmannin and LY294002 on PI(3,4,5)P3 accumulation and PKB phosphorylation. Pretreatment of the CEM cells before CCL22 stimulation with either LY294002 or wortmannin for 30 and 15 min, respectively, diminished chemokine-induced PI(3,4,5)P3 accumulation in a concentration-dependent manner (Fig. 3B). Additionally, phosphorylation of PKB on both Ser473 (Fig. 3, C and D, top blots) and Thr308 (Fig. 3C, bottom blots) in response to CCL22 was abrogated in a concentration-dependent manner by both LY294002 and wortmannin. The inhibitory effect of LY294002 on CCL22-stimulated PI3K/PKB activation was sustained for at least the 30 min post-CL22 stimulation (Fig. 3D).

The Gaα protein inhibitor, pertussis toxin, abrogates the chemotaxis of CEM cells in response to CCL22

Currently, all chemokine receptors have been shown to couple to Gaα, with some chemokine receptors also demonstrating the ability to couple to other G proteins as well, such as Gaq, Gar16, and Gar11 (56, 57). Although the G inhibitor, pertussis toxin (PTX), had no effect on basal cell migration, it did completely abrogate CCL22-mediated CEM cell chemotaxis (Fig. 4A). The phosphorylation/activation of PKB in response to CCL22 are also dependent upon Gaαi, coupling to CCR4 (Fig. 4B). PTX treatment did not reduce the basal levels of PKB phosphorylation, demonstrating that the treatment did not have an adverse affect on levels of PKB activation in general.

**CCL22 is able to elicit PKB phosphorylation in differentiated human Th2 cells**

To determine whether the effects we witnessed in a leukemic T cell line are an accurate reflection of the events in peripheral blood-derived T lymphocytes, we examined CCR4 in Th2 cells, where the expression of CCR4 is well documented (1–3). As was observed in CEM cells, CCL22 stimulated increases in PKB phosphorylation in a time-dependent manner (Fig. 5). Similar phosphorylation of PKB was also detected after stimulation with

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**Table 1. CCL22-mediated CEM cell migration in the presence of PI3K inhibitors**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vehicle</th>
<th>LY294002 (μM)</th>
<th>Wortmarin (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Control</td>
<td>3650 ± 253</td>
<td>2820 ± 120</td>
<td>2125 ± 131</td>
</tr>
<tr>
<td></td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>CCL22 1 nM</td>
<td>38260 ± 2735</td>
<td>33475 ± 1892</td>
<td>38340 ± 1056</td>
</tr>
<tr>
<td></td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

*CEM cells were incubated with either vehicle or the indicated concentrations of LY294002 or wortmarin for 30 and 15 min, respectively. Cells (2 × 10^5/200 μl) were added to the upper chambers of a 96-well chemotaxis plate, above lower chambers containing 1 nM CCL22 or medium alone (control). Chemotaxis across a 5-μm pore size membrane was determined after 1.5-h incubation, as described in Materials and Methods. Data are expressed as cell number migrated ± SEM. Data were analyzed by ANOVA and Student’s t test with Bonferroni correction to compare responses in the presence and the absence of PI3K inhibitors within control and MDC-stimulated groups. The data are derived from a single experiment (n = 3–5), representative of two others.

*p < 0.01.

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**Figure 4.** CCL22-mediated CEM cell chemotaxis is abrogated in PTX-treated cells. A. CEM cells were incubated with either vehicle or 100 ng/ml PTX for 16 h at 37°C. Cells (2 × 10^6/200 μl) were added to the upper chambers of a 96-well chemotaxis plate, above lower chambers containing 1 nM (■) or 3 nM CCL22 ( []). Chemotaxis across a 5-μm pore size membrane was determined after 1.5-h incubation at 37°C in 5% CO2 as described in Materials and Methods. The data are derived from a single experiment with quintuplicate replicates that is representative of three other experiments. Data were analyzed by ANOVA and Student’s t test, with Bonferroni correction, to compare responses to CCL22 in the presence and the absence of PTX ( **, p < 0.0001; $$$, p < 0.00005). The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels. B. A sample of CEM cells (2 × 10^6 cells/ml) from those untreated or treated with PTX was taken and stimulated with CCL22 (10 nM) for the indicated times, and lysates were generated. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-physiopKB (14) Ab. The blot was stripped and reprobed with anti-PKB Ab to verify equal loading and efficiency of protein transfer.
another known CCR4 ligand, CCL17 (Fig. 5). Interestingly, the kinetics of PKB phosphorylation were more rapid and transient than those observed for CCL22 stimulation of CEM cells, with detectable increases in PKB phosphorylated on serine 473 residue (PKB\(^{\text{Ser473}}\)) occurring between 0.5–2 min and returning to basal levels within 5 min.

**CCR4-mediated Th2 cell migration is insensitive to PI3K inhibitors**

CCL22 stimulated the chemotaxis of Th2 cells in a concentration-dependent manner (Fig. 6A). Comparable levels of directional Th2 cell migration were also stimulated by CCL17 (Fig. 6B). As observed with CEM cells, CCL22-stimulated Th2 cell migration was unaffected by pretreatment with either LY294002 or wortmannin (Fig. 6B). Similarly, CCL17-stimulated Th2 chemotaxis was unaffected by these two PI3K inhibitors (Fig. 6B). However, CCL22-stimulated phosphorylation of PKB\(^{\text{Ser473}}\) was inhibited by concentrations of PI3K inhibitors LY294002 (3–30 μM) and wortmannin (30–100 nM) that were without effect on cell migration (Fig. 6C). As expected, CCL22-mediated Th2 cell chemotaxis is also abrogated after pretreatment of the cells with PTX (Fig. 6D).

**CCR4-mediated CEM cell chemotaxis is sensitive to the Rho-associated coiled-coil-forming protein kinase (ROCK) inhibitor Y-27632**

Recent attention has focused on the role of Rho GTPase and its downstream effector ROCK in chemokine-elicited migration, as it appears to be required for CCR7 and CXCR4 T lymphocyte migration in response to CCL19/CCL21 and CXCL12, respectively (58, 59). Pretreatment of CEM cells with the ROCK inhibitor Y-27632 had no effect on basal cell migration (data not shown), yet markedly inhibited CCL22-induced chemotaxis in a concentration-dependent manner (Fig. 7A). ROCK is known to inhibit myosin L chain phosphate and to directly phosphorylate myosin L chain (60). Hence, we verified that Y-27632 did indeed inhibit the intended ROCK target by monitoring cellular levels of phospho-MLC2 phosphorylated on serine 19 residue (phospho-MLC2\(^{\text{Ser19}}\)) in lysates derived from resting and CCL22-stimulated CEM cells. CCL22 induced a sustained phosphorylation of MLC over a 30-min period, which was completely abrogated by pretreatment with Y-27632 (Fig. 7B).

**Discussion**

This study has investigated the role of PI3K in the regulation of CCL22/CCR4-mediated chemotaxis of T cells. With the use of human Th2 cells and the leukemic T cell line CEM, we have demonstrated that CCL22-mediated CCR4 ligation leads to the accumulation of PI(3,4,5)P\(_3\) and phosphorylation of the major PI3K effector, PKB. However, with the use of two chemically distinct PI3K inhibitors, wortmannin and LY294002, used at concentrations that inhibit PI(3,4,5)P\(_3\) accumulation and PKB phosphorylation (at both Thr\(^{308}\) and Ser\(^{473}\) residues), there is no negative effect on CCL22-mediated Th2 or CEM cell chemotaxis. Although it appears that neither CEM cells nor Th2 cells require PI3K activity for chemotaxis, it would seem that there are some fundamental differences between their responses to the CCR4 ligands. For example, the kinetics for PKB phosphorylation are far more rapid and transient in Th2 cells. This difference could be partially explained by differences between Th2 cells and CEM cells in the expression of the lipid phosphatases SHIP and PTEN. Hence, peripheral blood-derived Th2 cells express both SHIP and PTEN (D. G. Cronshaw and S. G. Ward, unpublished observations), whereas CEM cells lack expression of the PI(3,4,5)P\(_3\)-metabolizing 3’ phosphatase PTEN (50). The predicted more efficient removal of PI(3,4,5)P\(_3\) by SHIP and PTEN in Th2 cells is probably responsible for the transient nature of PKB phosphorylation in Th2 cells vs CEM cells. It is worth noting that the duration of PKB phosphorylation will also be determined by the activity of phosphatases such as phosphoprotein phosphatase 2A, and the differential expression of such phosphatases by Th2 and CEM cells cannot be ruled out (51).

PI3K isoform knockout studies and the use of pharmacological inhibitors of PI3K have primarily concentrated on the chemotaxis of granular leukocytes and *Dictyostelium*, but there is increasing evidence to suggest that perhaps PI3K isoforms do not provide the directional cues for T lymphocyte migration. Recent studies have indicated that T lymphocyte migration is not dependent upon the activation of PI3K pathways (47, 61). Our studies have demonstrated that PI3K inhibitors have no effect on CCR4-mediated chemotaxis despite being able to inhibit CCR4-mediated PI3K/PKB activation. Similar resistance of CXCR3-mediated chemotaxis of T cells was reported by Smit et al. (47). Smit suggested that class II PI3K isoforms may still be involved. Indeed, PI3KC\(_{2\alpha}\) has been demonstrated to be activated downstream of the CCR2 receptor (32). However, we found that both the class II PI3KC\(_{2\alpha}\) and C\(_{2\beta}\) isoforms are sensitive to the concentrations of wortmannin and LY294002 used in this study (D. G. Cronshaw and S. G. Ward, unpublished observations). It would therefore be expected that if PI3KC\(_{2\alpha\beta}\) isoforms were involved in the migratory responses observed, there would be a detectable inhibitory effect on T cell chemotaxis at the concentrations of PI3K inhibitors used in this study.

A variety of studies have demonstrated a role for the 3’-phosphoinositide phosphatase PTEN in localizing to the trailing and lateral edges of migrating cells, with exclusion from the leading edge, a region where PI3K, its products, and their effectors have been observed to localize (35, 36, 62). Overexpression or deficiency of PTEN attenuates or ameliorates leukocyte motility, respectively, whereas deficiency of SHIP, another key regulatory phosphatase, brings about enhanced motility (63, 64). There are, however, several lines of evidence to suggest that relocation of PTEN to the posterior of the cell is not a prerequisite for migration of all cell types (see Ref. 65 for review). Firstly, the CEM cell line used in this study lacks PTEN, yet the mechanisms of cell motility, with respect to PI3K requirements, appear to be identical with...
those of CCL22-induced migration of Th2 cells, which contain both PTEN and SHIP. Secondly, Jurkat cells lack both PTEN and SHIP, yet polarize and migrate with apparent ease in response to chemokines (31, 42). Third, posterior localization of PTEN is not observed in migrating HL60 cells (66).

Additionally, although the kinase activity of these PI3K isoforms may be severely diminished with the use of PI3K inhibitors, we cannot rule out the possibility that the associated adapter subunits (i.e., p55, p85, and p101) may play important functional roles, participating in protein-protein interactions distinct from...
The small GTPases, Rho, Ras, Rac, and cdc42, have been shown to play pivotal roles in the remodeling of the actin cytoskeleton and, consequently, cell polarization and migration. ROCK, an effector of Rhos, enhances MLC phosphorylation and thereby regulates actin-myosin contraction (72), and it has been shown to be involved in uropod function at the rear of migrating neutrophils (66). A recent study demonstrated a ROCK inhibitor-attenuated, CCR7-mediated polarization and migration of T lymphocytes (58). Moreover, CXCL12 has been observed to activate the Rho-ROCK-myosin light chain kinase-MLC pathway in PBLs (59). This correlates with our observations that inhibition of ROCK also attenuates CCL22-mediated T lymphocyte migration and MLC phosphorylation. In neutrophils, both PI3K-dependent and PI3K-independent pathways have been demonstrated to be involved in pseudopodia formation in response to the chemoattractant fMLP (73). It is possible that T lymphocytes migrating in response to CCR4 ligation (and perhaps CXCR3) do not rely upon a PI3K-dependent pathway, but upon at least one or more PI3K-independent pathways, one of which involves Rho-ROCK-MLC activity. The action of ROCK on MLC phosphorylation may involve direct phosphorylation, indirect phosphorylation via myosin light chain kinase, or inhibition of MLC phosphatase (59, 74). In addition to phosphorylation of MLC, ROCK may also phosphorylate and activate LIM kinase 2, leading to inactivation of cofilin and subsequent stabilization of actin filaments within actin:myosin filament bundles (75, 76).

Additional evidence of cell-specific migration mechanisms occurring within the leukocyte family is highlighted with downstream of Crk-180 homolog-2 (DOCK)−/− mice. Upstream of the Rac small GTPases lies the scaffolding protein DOCK2. Lymphocytes from DOCK2−/− mice revealed a severe lack of a migratory response to CXCL12, CXCL13/BLC, CCL19/ELC, and CCL21/SLC, yet monocyte/macrophages migrated normally to CXCL12 and CCL2/MCP-1, indicating cell-specific mechanisms. It is unclear how DOCK2 is recruited, although there is evidence of PI3K dependence in CXCR3 signaling (79). This suggests a possible mechanism for the involvement of both DOCK2- and ROCK-dependent pathways in T lymphocyte-directed cell migration without the need for PI3K activity. There could be at least two pathways that are required for signaling to the front and back of the cell with a negative feedback interaction between these pathways to obtain a coordinated crawling movement of the cell (80).

The data presented in this paper indicate that CCR4-mediated T lymphocyte cell migration is independent of detectable PI3K catalytic activity. The ability of CCL22 to activate class I PI3K isoforms (data not shown) suggests a role for them in currently non-characterized, CCR4-mediated functional responses (such as cell proliferation/antiapoptosis). These data suggest, along with the other studies mentioned, that the involvement of PI3K in chemokine-mediated migration of T lymphocytes is, in all probability, context dependent. The abrogation of CCR4-mediated chemotaxis with the ROCK inhibitor has indicated at least one PI3K-independent pathway that is required for CCL22-induced T cell chemotaxis. Some other possible PI3K-independent mechanisms have also been discussed, but these pathways remain very ambiguous. Future studies making use of the recently increased accessibility of small interfering RNA technology to target individual PI3K isoforms would perhaps offer more conclusive evidence for the role of PI3Ks downstream of CCR4.

FIGURE 7. The ROCK inhibitor, Y7632, abrogates CCL22-induced CEM cell chemotaxis. A. CEM cells (2 × 10⁶ cells/200 μl) were preincubated with the indicated concentrations of Y7632 for 60 min at 37°C and then added to the upper chambers of a 96-well chemotaxis plate, above lower chambers containing 10 nM CCL22. Chemotaxis across a 5-μm pore size membrane was determined after 1.5-h incubation at 37°C in 5% CO₂ as described in Materials and Methods. The data are derived from a single experiment with quintuplicate replicates that is representative of three other experiments. Data were analyzed by ANOVA and Student’s t test, with Bonferroni correction, to compare responses in the presence and the absence of the ROCK inhibitor (*, p < 0.0005; **, p < 0.00005; ***, p < 0.000005). The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels. B. Th2 cells (5 × 10⁵ cells/500 μl) were pretreated with Y-7632 as described above. Cells were then stimulated at 37°C with CCL22 (10 nM) for the indicated times before cells were lysed by the addition of 1× sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-MLC2ser19 Ab, and protein was visualized with ECL. The blots were stripped and reprobed with anti-β-actin Ab to verify equal loading and efficiency of protein transfer.


