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# Rho kinase is required for CCR7-mediated polarization and chemotaxis of T lymphocytes

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**Abstract** **We studied the role of Rho kinase and extracellular signal-regulated kinase (ERK)-2 in the polarization and migration of T lymphocytes in response to the CCR7 ligands EBI1 ligand chemokine (ELC; CCL19) and secondary lymphoid-tissue chemokine (SLC; CCL21). Both Rho kinase protein isoforms are expressed in T lymphocytes. Inhibition of the Rho kinases with Y-27632 strongly inhibited SLC- and ELC-induced polarized morphology and chemotaxis of T lymphocytes. Although the chemokines induced ERK-2 activation, the blockade of this signaling pathway showed no effect on polarization and migration. This study indicates an important role of Rho kinase in CCR7-mediated polarization and migration of T lymphocytes, whereas ERK-2 is not involved in these processes.**


*Key words:* Rho kinase; Extracellular signal-regulated kinase-2; Polarization; Chemotaxis; Chemokine; Chemokine receptor

## 1. Introduction

Chemokine-mediated leukocyte migration is a multi-step process involving dynamic and spatially regulated changes in the cytoskeleton and cellular adhesion [1–5]. To initiate migration, the cells have to acquire a polarized morphology by forming a leading lamellipodium and a trailing uropod [5]. When chemokines bind to their receptors, the heterotrimeric $G_i$ proteins are activated and the complex dissociates into its α and βγ subunits. The released βγ subunit activates two major signal transduction enzymes, phospholipase C, which generates two second messengers, inositol (1,4,5)-trisphosphate and diacylglycerol, and phosphatidylinositol-3-OH kinase, which generates phosphatidylinositol (3,4,5)-trisphosphate and initiates the activation of downstream targets such as protein kinase B and mitogen-activated protein kinases (MAPKs) [6,7].

Recent attention has focused on the involvement of Rho family GTPases and their downstream effectors in chemokine-elicited migration. Important Rho effectors are the Rho-activated kinases, the so called Rho-associated coiled-coil forming protein kinases (ROCK) I and II, which enhance myosin light chain (MLC) phosphorylation by both inhibiting MLC phosphatase and phosphorylating MLC, thereby regulating actin–myosin contraction [6,8]. It has been reported that the inhibition of the Rho kinases prevents MLC phosphorylation and the chemotactic peptide-induced development of polarity, rear detachment and migration in neutrophils [9,10], and blocks eotaxin-induced chemotaxis in eosinophils [11]. Very recently, the Rho–Rho kinase pathway has also been implicated in lymphocyte responses mediated by SDF-1 (CXCL12) [12]. In the present study we have explored the function of the Rho kinases and MAPK extracellular signal-regulated protein kinase (ERK)-2 on the morphology and migration of blood T lymphocytes in response to stimulation with the CCR7 ligands EBI1 ligand chemokine (ELC; CCL19) and secondary lymphoid-tissue chemokine (SLC; CCL21). CCR7 is the chemokine receptor that plays a central role in the homing and traffic of lymphocytes into and within secondary lymphoid tissues. We show that both the CCR7-mediated polarization and chemotaxis are dependent on the Rho kinases, but not on ERK-2.

## 2. Materials and methods

### 2.1. Reagents and antibodies

Chemokines were chemically synthesized according to established protocols [13]. Human interleukin (IL)-2 was kindly provided by Dr. A. Lanzavecchia (Institute for Research in Biomedicine, Bellinzona, Switzerland) and Y-27632 by Welfide Corporation (Osaka, Japan). Stock solutions of Y-27632 (10 mM) were prepared in $H_2O$ and aliquots were stored at 4°C. PD98059 was purchased from Calbiochem (La Jolla, CA, USA) and phytohemagglutinin (PHA) from Murex Biotech Ltd. (Dartford, Kent, UK). A polyclonal rabbit antibody against amino acids 2–15 of p160 ROCK I was prepared as described [14] and a monoclonal mouse anti-ROKα (ROCK II) antibody was obtained from Transduction Laboratories (Lexington, KY, USA). Alkaline phosphatase- and horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG were obtained from Bio-Rad Laboratories (Hercules, CA, USA) and anti-ERK-2 (C14) from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### 2.2. Cells and culture

Peripheral blood lymphocytes were isolated from buffy coats of donor blood (Central Laboratory of the Swiss Red Cross, Bern, Switzerland) by centrifugation on Ficoll-Paque and Percoll gradients [15]. The cells were activated for 4–6 days by culturing in the presence of 1 μg/ml PHA and 200 U/ml IL-2 in RPMI 1640 supplemented with 1% glutamine, non-essential amino acids, sodium pyruvate, 50 U/ml

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*Abbreviations:* CCL, CC chemokine ligand; CCR, CC chemokine receptor; ELC, EBI1 ligand chemokine; SLC, secondary lymphoid-tissue chemokine; ERK, extracellular signal-regulated kinase; ROCK, Rho-associated coiled-coil forming protein kinase

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penicillin, 50 mg/ml streptomycin, 0.05 mM β-mercaptoethanol and 5% human serum (Swiss Red Cross Laboratory, Bern, Switzerland). These cells are referred to as 'T lymphocytes'. CEM cells, a human lymphoblastoid $CD4^+$ T cell line, were cultured in RPMI containing 10% fetal calf serum. Human platelets were isolated as previously described [16].

### 2.3. Polarization

Cells ($3\times10^6$/ml) were fixed in 1% glutaraldehyde for 30 min at 37°C, washed once in phosphate-buffered saline (PBS) and resuspended in PBS containing 1 mg/ml $NaN_3$. Cell morphology was determined with differential interference contrast microscopy using a Zeiss IM35 microscope with a 100× objective. Cell shape was determined by counting at least 100 cells per sample and classified according to the following shape categories: spherical cells with a smooth surface, non-polar cells and polarized cells [17].

### 2.4. Chemotaxis

Cell migration was assessed in 48-well chambers (Neuro Probe Inc., Cabin John, MD, USA) using polyvinylpyrrolidone-free polycarbonate membranes (Poretics Corp., Livermore, CA, USA) with 3-μm pores [15]. Migration was allowed to proceed for 1 h and migrated cells were counted at a 1000× magnification in five fields per well. All determinations were performed in triplicate.

### 2.5. Immunodetection of ROCK I and II

Total protein samples of T lymphocytes, CEM cells and platelets were prepared by TCA precipitation and separated by 11% SDS–PAGE followed by transfer to Immobilon-P membrane. The membranes were incubated with polyclonal rabbit anti-ROCK I or monoclonal mouse anti-ROCK II antibodies overnight. Enhanced chemiluminescence was used for detection of horseradish peroxidase-conjugated secondary antibodies.

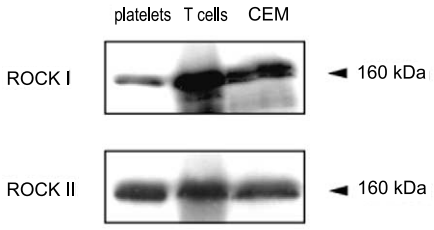


Fig. 1. Immunodetection of ROCK I and II expression. Protein extracts of T lymphocytes, CEM cells and platelets were separated by SDS–PAGE followed by transfer to Immobilon-P membrane and immunodetection of the ROCK isoforms. The specific antibodies to ROCK I and ROCK II react with bands corresponding to a molecular weight of about 160 kDa.

### 2.6. ERK-2 phosphorylation

T lymphocytes were serum-starved overnight in medium supplemented with 200 U/ml IL-2 [18]. The cells were then washed twice and resuspended in HBSS containing 20 mM HEPES, pH 7.4. Stimulation with the indicated chemokines was terminated after 2 min by the addition of TCA. Total protein samples were separated by 11% SDS–PAGE followed by transfer to Immobilon-P membrane. The membrane was incubated with anti-ERK-2 antibodies overnight. Enhanced chemiluminescence was used for detection of alkaline phosphatase-conjugated secondary antibodies.

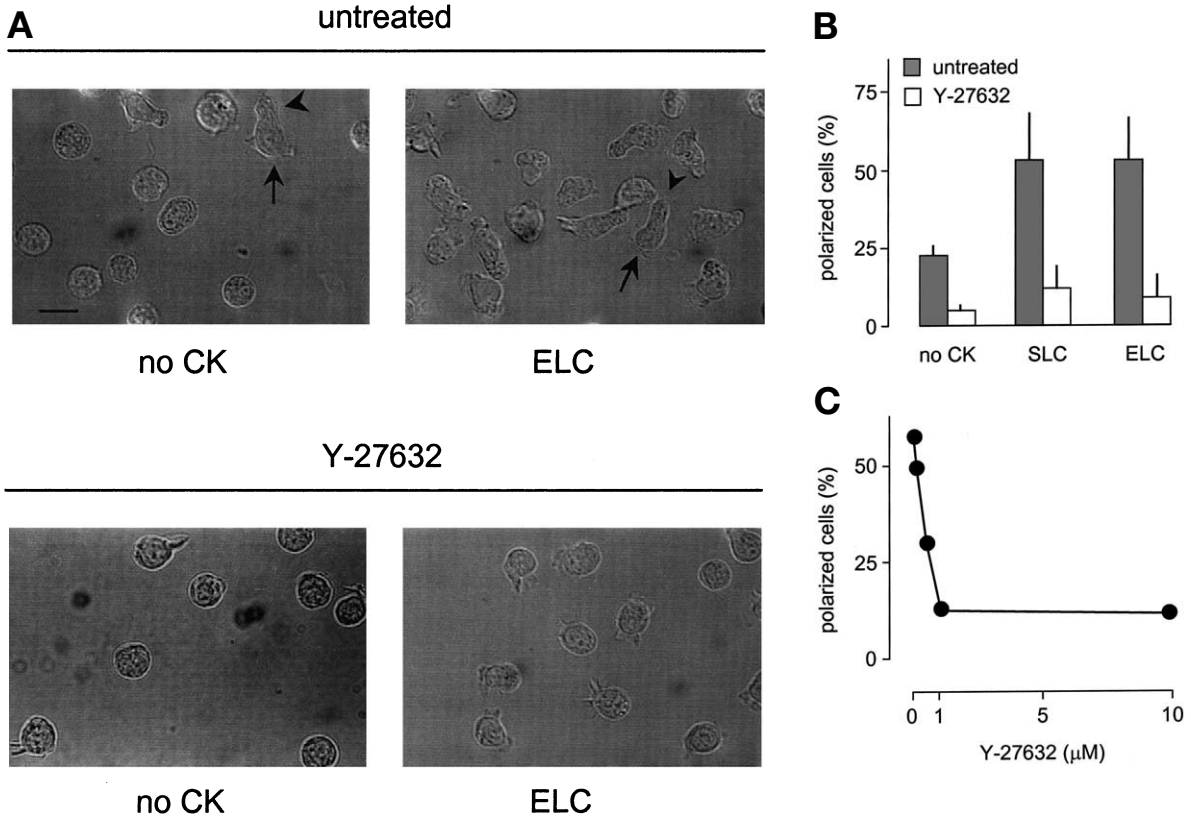


Fig. 2. Effect of ROCK inhibition on chemokine-induced polarization of T lymphocytes. T lymphocytes were left untreated or exposed to 10 μM Y-27632 for 2 h at 37°C and then further incubated for 30 min without addition (no chemokine, no CK) or in the presence of 300 nM SLC or ELC. Cells were subsequently fixed with 1% glutaraldehyde and the polarity was determined. The photographs in panel A were obtained with a differential interference contrast Zeiss IM35 microscope. Arrowhead indicates the uropod and arrow the leading front of a typical polarized cell (bar: 10 μm). The data in panel B are mean values ± S.D. from three independent experiments. C: The cells were exposed to increasing concentrations of Y-27632 for 2 h at 37°C and then further incubated for 30 min in the presence of 300 nM ELC and the polarity of the fixed cells was determined. The data of one out of two independent experiments are shown.

## 3. Results

### 3.1. Expression of ROCK I and II in T lymphocytes

To observe the presence of ROCK I and ROCK II in T lymphocytes we performed Western blot analysis using specific antibodies. Protein extracts from T lymphocytes and CEM cells were separated by SDS–PAGE. Antibodies against p160 ROCK (ROCK I) reacted with a band corresponding to a molecular weight of about 160 kDa (Fig. 1, upper panel) and an antibody against ROCK II with a band of a similar molecular weight (Fig. 1, lower panel). A protein extract of human platelets which were shown to express the Rho kinases [9,14] was included as positive control.

### 3.2. Effect of ROCK inhibition on chemokine-induced polarization of T lymphocytes

Polarization of T lymphocytes in response to stimulation with the CCR7 ligands ELC (CCL19) and SLC (CCL21) was determined by differential interference contrast microscopy (Fig. 2A). About 20% of the T lymphocytes displayed a bell-like, polarized morphology with a leading front and a trailing uropod. On treatment with 300 nM SLC and ELC, the extent increased to 55% (Fig. 2A,B). Pretreatment with the selective ROCK inhibitor Y-27632 [19] markedly reduced the number of polarized cells. Spontaneous and chemokine-induced polarization was inhibited by 80–90%. Cells treated with Y-27632 alone exhibited a round shape with ruffles and short protrusions that were observed in about 30% of the cells (Fig. 2A), similar to effects of Y-27632 on the morphology of neutrophils [9]. As shown for the inhibition of ELC-induced polarization of T lymphocytes in Fig. 2C, the effect of Y-27632 was concentration-dependent and already clearly detectable at a concentration of 0.5 μM and reached maximal levels at 10 μM.

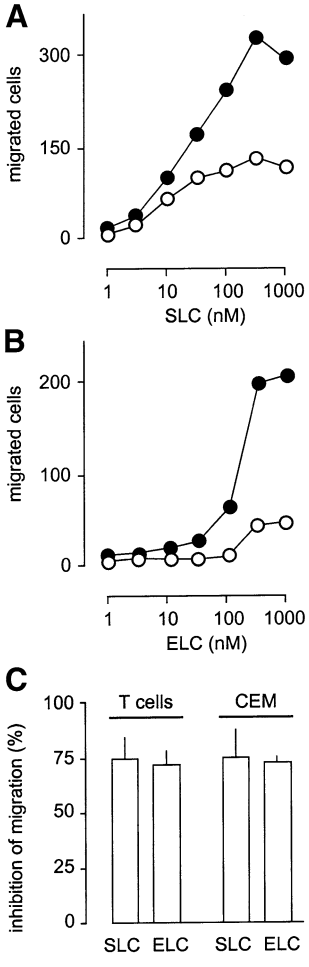


Fig. 3. Effect of ROCK inhibition on chemokine-induced chemotaxis of T lymphocytes. T lymphocytes were left untreated (closed circles) or exposed to 20 μM Y-27632 (open circles) for 2 h at 37°C. Chemotaxis was then determined in response to increasing concentrations of SLC (A) and ELC (B). Shown are the average numbers of migrated cells per five high-power fields in triplicate wells. The data are representative of three to five independent experiments. Relative inhibition of migration after pretreatment with 10 μM Y-27632 in response to 300 nM SLC and ELC in T lymphocytes and CEM cells (C). Mean values ± S.D. from three to seven independent experiments.

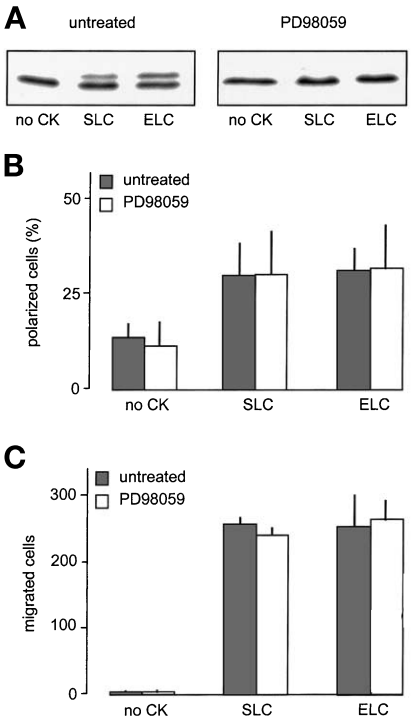


Fig. 4. Effect of ERK inhibition on chemokine-induced polarization and chemotaxis of T lymphocytes. T lymphocytes were left untreated or exposed to 50 μM PD98059 for 30 min at 37°C and then tested for ERK-2 activation (A), polarization (B) and chemotaxis (C). A: After pretreatment, the cells were incubated for 2 min at 37°C without addition (no chemokine, no CK) or in the presence of 100 nM SLC or ELC, and whole cell lysates were separated by SDS–PAGE followed by transfer to Immobilon-P membrane and immunodetection. Activated ERK-2 migrates with a slower electrophoretic mobility than non-activated ERK-2. The data are representative of three independent experiments. B: After pretreatment, the cells were incubated for 30 min at 37°C without addition (no chemokine, no CK) or in the presence of 300 nM SLC, ELC. Cells were subsequently fixed with 1% glutaraldehyde and the polarity was determined. Shown are the mean values ± S.D. from four independent experiments. C: After pretreatment, chemotaxis in response to medium (no chemokine, no CK), 300 nM SLC, 300 nM ELC was determined. Shown are the average numbers ± S.D. of migrated cells per five high-power fields in triplicate wells. The data are representative of three independent experiments.

### 3.3. Effect of ROCK inhibition on chemokine-induced chemotaxis of T lymphocytes

Y-27632 markedly inhibited chemotaxis of T lymphocytes in response to increasing concentrations of the CCR7 ligands (Fig. 3A,B). The inhibition after stimulation with 300 nM ELC and SLC ranged from 70 to 75% (Fig. 3C). Under the same conditions, a similar inhibitory effect was obtained with CEM cells (Fig. 3C). The data on polarization and chemotaxis indicate that the ROCK kinases are involved in regulating cell morphology and migration toward a chemokine gradient.

### 3.4. Effect of ERK inhibition on chemokine-induced polarization and chemotaxis of T lymphocytes

Chemokine-induced activation of ERKs has been reported to be critical for cell migration [20–22]. SLC and ELC activated ERK-2 in T lymphocytes, as shown in Fig. 4A. On stimulation with the chemokines, ERK-2 is phosphorylated on a threonine and a tyrosine residue by the MAP-ERK kinase (MEK) and the double phosphorylated ERK-2 can be detected on SDS–PAGE by its retarded electrophoretic mobility (Fig. 4A, left panel). PD98059, a MEK-specific inhibitor [23], completely abrogated the chemokine-induced activation of ERK-2 (Fig. 4A, right panel). We have, however, not observed any inhibitory effect of PD98059 on the polarization (Fig. 4B) and chemotaxis (Fig. 4C) of the T lymphocytes in response to stimulation with SLC, ELC. The data suggest that these responses are not dependent on the activation of ERK-2.

## 4. Discussion

In the present study we have shown the crucial role of Rho kinases in CCR7-mediated polarization and migration of T lymphocytes. Both Rho kinase isoforms, ROCK I and ROCK II, were found to be expressed in T lymphocytes and CEM cells, and their inhibition induced a profound impairment of the chemokine-elicited cellular responses. The MAPK kinase ERK-2 is activated by chemokine stimulation in T lymphocytes, but it is not required for the regulation of polarization and migration.

Migration and recruitment of T lymphocytes are largely controlled by chemokines and their receptors, and are critical at almost every step of immune responses [1,2,4]. These processes require distinct changes and rearrangements in the cytoskeleton that are ruled by a very complex network of protein signals. Our findings show that the Rho kinases, which are activated by small GTPases of the Rho family [6], play an important and non-redundant role in the chemokine-induced signaling, since the specific inhibition of the kinases led to a loss of CCR7-mediated polarization and chemotaxis of T lymphocytes. The data are in agreement with a recent study reporting that SDF-1 induced the activation of RhoA and ROCK in lymphocytes, and that the inhibition of either of them blocked chemotaxis in response to SDF-1 stimulation [12]. It has also been shown that Rho kinase is involved in CCR3-mediated signal transduction in eosinophils [11]. Chemotaxis and reactive oxygen species production induced by eotaxin, the specific ligand for CCR3 [24], were inhibited by Y-27632.

It is clear that chemokines induce the activation of the MAPK ERK, but it is less clear whether ERK activation is required for chemokine-induced chemotaxis [7]. It has been reported that ERK-2 activation is involved in eotaxin-induced chemotaxis of eosinophils [20,21] and in IP10-induced chemotaxis of hepatic stellate cells [22]. However, it has also been reported that chemotaxis of neutrophils to IL-8 [25] and of monocytes to MCP-1 [26] is independent of ERK-2 activation. Similar to these observations, we have found no effect on ERK-2 inhibition by PD98059 on the polarization and chemotaxis of T lymphocytes in response to stimulation with ELC and SLC as well as SDF-1 (data not shown). We cannot rule out the possibility that ERK-2 is involved in a discrete step in the complex process of the recruitment and positioning of T lymphocytes. In conclusion our study delineates a crucial role for Rho kinases, but not for MAPKs, in the CCR7-mediated signaling that leads to lymphocyte polarization and migration. In addition, the findings suggest that the inhibition of Rho kinases can be a novel principle to suppress the homing and recruitment of CCR7-positive lymphocytes.

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