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Intracellular Signal Cascade in CD4$^+$ T-Lymphocyte Migration Stimulated by Interferon-γ-Inducible Protein-10

N. B. Kukhtina*, T. I. Arefieva, and T. L. Krasnikova

Group of Neurobiology of Cardiovascular Diseases, Institute of Experimental Cardiology, Russian Cardiology Research Center, 3-ya Cherepkovskaya ul. 15a, 121552 Moscow, Russia; fax: (7-095) 415-2962; E-mail: kukhtina@cardio.ru

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Abstract—The intracellular signal cascades involved in chemokine-stimulated migration of *in vitro* activated human peripheral blood CD4$^+$ T-lymphocytes were investigated. IP-10-mediated chemotactic response of lymphocytes was decreased in the presence of selective inhibitors of Src-kinases (by 40-45%), PI3-kinases (35-40%), and MAP-kinases ERK1/2 (35-40%) and p38 (20%). Combined addition of specific inhibitors of Src-kinases and PI3-kinases and inhibitors of Src-kinases and ERK1/2 MAP-kinases did not result in the further increase of the inhibitory effect, while the combined addition of specific inhibitors of PI3-kinases and ERK1/2 MAP-kinases decreased migration of CD4$^+$ T-lymphocytes more effectively (by 55-60%) than any individual inhibitor. Immunoblotting analysis of activation of MAP-kinases ERK1/2 and p38 revealed increased level of phosphorylation of ERK1/2 and p38 MAP-kinases in the presence IP-10. Selective inhibitors of Src-kinases and PI3-kinases significantly inhibited phosphorylation of p38 but did not influence phosphorylation of ERK1/2 MAP-kinases. Our results suggest that Src-kinases, PI3-kinases, and ERK1/2 MAP-kinases are involved in intracellular signal cascade activated during IP-10-stimulated migration of T-lymphocytes, whereas p38 MAP-kinases do not participate in the migration process, although its activation induced by IP-10 depends on Src-kinases and PI3-kinases.

Key words: CD4$^+$ T-lymphocytes, chemokines, migration, MAP-kinases, PI3-kinases, Src-kinases

Activation of T-lymphocytes and their involvement in local inflammation are the ultimate stages in the development of immune response. Chemokines are a family of small chemoattractant cytokines (8-10 kD) that play a decisive role in lymphocyte migration. According to the accepted classification, chemokines are subdivided into four classes—CC, C, CXC, and CX_3C—depending on the position of the first two of four conservative cysteine residues in the N-terminal domain [1]. Chemokines CXCL9, 10, and 11 (known earlier as Mig, IP-10, and I-TAC, respectively) are high affinity ligands of the chemokine receptor CXCR3. This receptor is highly expressed on activated T-lymphocytes (preferentially on T-helpers type 1, Th1) [2, 3], natural killers (NK) and eosinophils [4], B-lymphocytes [5], and plasmocytoid dendrite cells [6]. During migration of T-lymphocytes *in vitro* and *in vivo* to inflammation sites, IP-10 is the most effective chemokine among ligands of the CXCR3 receptor [7]. CXCR3-mediated migration of CD4$^+$ T-lymphocytes is involved in development of the chronic inflammation process [2, 8], which probably represents a basis underlying the pathogenesis of atherosclerosis [9], multiple sclerosis [10], rheumatoid arthritis [2], sarcoidosis [11], psoriasis [12], etc.

Chemokine receptors belong to a large class of G-protein coupled seven domain transmembrane receptors. Chemokine binding to receptors causes dissociation of βγ-subunits of G_i protein from the complex with α-subunit and receptor. This results in fast activation of phospholipase C (PLC), activation of protein kinases A and C, formation of inositol-1,4,5-trisphosphate (PtdIns(1,4,5)P_3) and increase in intracellular calcium ions [13]. Besides the phospholipase pathway, chemokines may activate intracellular signaling cascades, employing PI3-kinases [14, 15], MAP-kinases [16, 17], and Src family non-receptor tyrosine kinases [18, 19]. We demonstrated earlier that intracellular signaling pathways of monocytes and endothelial cells activated by chemokines and leading to development of chemotaxis significantly differ [20]. Information on signaling cascades involved in chemokine stimulation of activated CD4$^+$ T-lymphocytes is rather fragmental and contradictive. According to Poggi et al. and Bonacchi et al. [21, 22], CXCR3 binding to ligands is

Abbreviations: ERK1/2) extracellular signal-regulated protein kinases; IP-10) interferon-γ-inducible protein-10; MAP-kinases) mitogen-activated protein kinases; Mig) monokine induced by interferon-γ; PI3-kinase) phosphoinositol 3-kinase; I-TAC) interferon-inducible T-cell α-chemoattractant.

* To whom correspondence should be addressed.

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accompanied by obligate activation of PI3-kinases and ERK1/2 MAP-kinases. Smit et al. [23] believe that PLC-dependent pathway plays the major role, whereas PI3-kinases and ERK1/2 MAP-kinases are not involved in CXCR3-mediated chemotaxis of lymphocytes.

In the present study, we have investigated the role of Src-kinases, PI3-kinases, and MAP-kinases during IP-10-stimulated migration of activated $CD4^+$ T-lymphocytes.

MATERIALS AND METHODS

Cell culture. $CD4^+$ T-lymphocytes were isolated from venous blood of healthy donors using citrate anticoagulant; they were centrifuged in a Histopaque density gradient ($\rho = 1.077$ g/ml; Sigma-Aldrich, USA) according to the method described in [24]. Mononuclear cells of peripheral blood were colleted from the interphase and were washed twice in phosphate buffer without Ca^{2+} and Mg^{2+} (pH 7.4), and $CD4^+$ T-lymphocytes were isolated on magnetic beads (CD4 MicroBeads, Miltenyi Biotec GmbH, Germany) using the supplier's recommendations. Expression of surface antigens CD3 and CD4 was analyzed using monoclonal antibodies (MAbs) to the CD3 molecule labeled with fluorescein isothiocyanate (FITC) and to the CD4 molecule labeled with phycoerythrin (PE) (BD PharMingen, USA) by flow cytometry (FACSCalibur, BD Immunocytometry Systems, USA). The resulting suspension contained at least 98% $CD3^+CD4^+$ T-lymphocytes and cell viability was not less than 96%.

$CD4^+$ T-lymphocytes ($2 \cdot 10^6$/ml) were cultivated in RPMI 1640 medium (Gibco, UK) containing 10% fetal calf serum (Gibco), 0.01 M Hepes (Sigma-Aldrich), 100 U/ml penicillin, 100 U/ml streptomycin, 20 μM mercaptoethanol at 37°C and 5% CO_2. *In vitro* activation was induced by culturing cells with recombinant interleukin-2 (IL-2, 150 U/ml; Gibco) and anti-CD3 MAbs (15 ng/ml) plus anti-CD28 MAbs (10 ng/ml) (both from BD PharMingen).

The expression of CXCR3 chemokine receptors was evaluated in lymphocytes freshly isolated from blood or after *in vitro* activation for 24, 48, or 72 h. Cells were resuspended in phosphate buffer and incubated with anti-CXCR3 MAbs or control mouse Ig (BD PharMingen) for 20 min at 4°C, washed, fixed in CellFIX™ solution (BD PharMingen), and analyzed by flow cytometry.

Determination of MAP-kinase phosphorylation. $CD4^+$ T-lymphocytes were sedimented and then resuspended in RPMI 1640 medium containing 0.5% BSA (Sigma-Aldrich). Chemokine IP-10 (200 ng/ml; R&D Systems, Inc., UK) was used as an activator. Inhibitors of Src-kinases (PP2) and PI3-kinases (LY294002) were added to final concentration 10 μM 30 min before addition of the activator. After the stimulation, cells were washed in cold phosphate buffer and lysed by adding 100 μl of electrophoresis buffer containing 4% mercaptoethanol to the cell pellet. The cell lysate was boiled for 5 min and then it was subjected to electrophoresis in 10% polyacrylamide gel in the presence of SDS. Protein was transferred from polyacrylamide gel to nitrocellulose membrane (Sigma-Aldrich) by the method of semidry electrophoretic transfer. Membranes were blocked for 1 h with 20 mM Tris-HCl, pH 7.6, containing 165 mM NaCl, 0.05% Tween-20, and 5% defatted milk. Rabbit antibodies to human MAP-kinases and their phosphorylated forms (New England Biolabs, USA) were used as primary antibodies at concentrations recommended by the manufacturers. Horseradish peroxidase-conjugated anti-rabbit antibodies were used as secondary antibodies (Amersham-Pharmacia Biotech, UK). Incubation with all antibodies was carried out in the same buffer containing 1% milk at room temperature for 1 h or at 4°C overnight. Protein bands were detected by the chemiluminescence method using Amersham-Pharmacia Biotech kits.

Cell migration. Intracellular signaling cascades involved in IP-10-stimulated migration of $CD4^+$ T-lymphocytes were studied by inhibitory analysis of cell migration *in vitro*. Lymphocyte chemotaxis was measured in a modified Boyden chamber [20, 25, 26]. To initiate chemotaxis, chemokine IP-10 was diluted in RPMI 1640 (200 ng/ml) and placed into lower wells, whereas upper wells were filled with 50 μl of cell suspension. Upper and lower wells were separated with porous membrane (pore size of 3-5 μm; Osmonics Inc., USA) covered with fibronectin (50 ng/ml; Imtek, Russia). The chamber was placed into a CO_2-incubator for 2 h. Non-migrated cells were removed and cells remaining on the filter were fixed for 5 min and stained with Giemsa stain (Sigma-Aldrich). The membrane with stained cells was scanned using an HP ScanJet 5300C (Hewlett Packard, USA); results were analyzed using SigmaGel software (Jandel Scientific, USA). Results were expressed in relative units representing the ratio of cell migration under experimental conditions to control value (obtained during evaluation of migration of cells penetrated through the porous membrane in the absence of the chemoattractant). Kinase inhibitors were added to cell suspension right before experiment and also to the lower cell of the Boyden chamber into a chemokine solution. PD98059 inhibitor was used at 50 μM concentration; SB202190, LY294002, PP2 (Calbiochem, USA) and genistein (Sigma-Aldrich) were used at 10 μM concentration.

Statistical analysis. Data representing mean ± SD were statistically treated using Student's *t*-test.

RESULTS AND DISCUSSION

Stimulation of $CD4^+$ T-lymphocytes increases expression of chemokine receptor CXCR3. Non-activated $CD4^+$ T-lymphocytes freshly isolated from peripheral

blood do not exhibit chemokine-mediated migration. For cell activation, we used treatments that mimic stimulation of T-lymphocytes by antigen [27, 28]. $CD4^+$ T-lymphocytes were incubated with MAbs to CD3 and CD28 molecules in the presence of recombinant IL-2. Cell activation was accompanied by reduction in the number of cells expressing CXCR3 receptor. Maximal expression of CXCR3 receptor was noted on the third day of incubation (Fig. 1).

IP-10 activates MAP-kinases. The putative role of IP-10 as an activator of MAP-kinase cascades in $CD4^+$ T-lymphocytes was investigated using specific antibodies recognizing phosphorylated forms of these kinases.

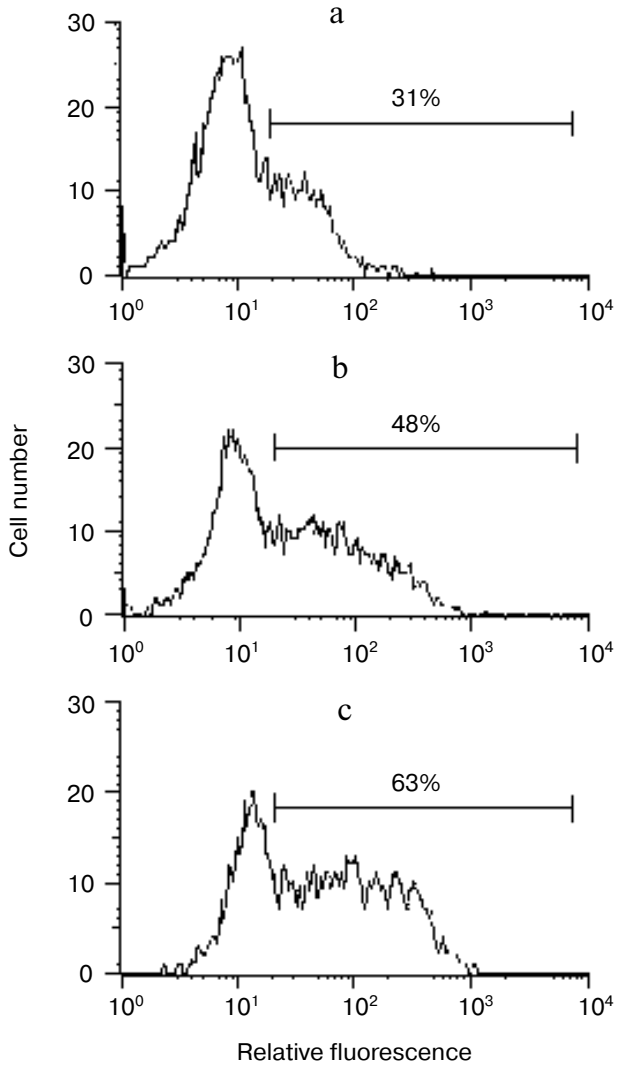

Fig. 1. Cytofluorimetric analysis of chemokine receptor CXCR3 expression on the surface of $CD4^+$ T-lymphocytes. Expression of CXCR3 was determined using PE-labeled MAbs right after isolation of lymphocytes (a) and after their *in vitro* activation for 24 (b) and 72 h (c). Results are expressed as % of CXCR3-expressing cells. Results of one of three independent experiments are shown.

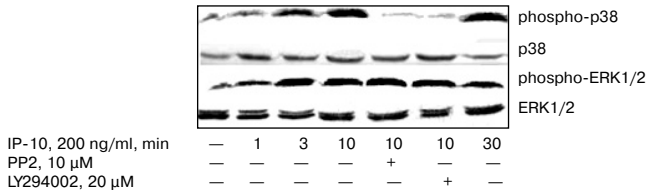

Fig. 2. Evaluation of phosphorylation of p38 and ERK1/2 MAP-kinases (1-30 min) by immunoblotting (see "Materials and Methods" section for details). Typical results of one of three experiments are shown.

Addition of IP-10 into a cell suspension caused transient activation of both ERK1/2 MAP-kinases and p38 MAP-kinase; the maximal response was observed within 3-10 min after chemokine addition (Fig. 2). Specific inhibitors of Src- and PI3-kinases, PP2 and LY294002, respectively, did not influence phosphorylation of ERK1/2 MAP-kinase, whereas phosphorylation of p38 MAP-kinase significantly decreased in the presence of both PP2 and LY294002.

Inhibitory analysis of intracellular signaling pathways involved in IP-10-stimulated migration of activated $CD4^+$ T-lymphocytes *in vitro*. For investigation of intracellular signaling pathways realizing signal transduction from IP-10 and influencing cellular mobility, we studied IP-10-induced cell migration in the presence of protein kinase inhibitors. Concentrations of inhibitors did not influence cell viability evaluated by incorporation of 0.1% Trypan blue dye. (Cell viability was not less than 95%.) Since all stock solutions of inhibitors were prepared using dimethyl sulfoxide, corresponding amounts of this solvent was also added to the control cells.

IP-10 caused twofold stimulation of $CD4^+$ T-lymphocyte migration compared with spontaneous migration (data not shown).

Maximal inhibition of IP-10-stimulated migration of $CD4^+$ T-lymphocytes (by 40-45%) was observed in the presence of selective Src-kinase inhibitor PP2. Significant decrease in migration activity of cells was also observed after addition of inhibitors of PI3-kinase (~35-40%) and ERK1/2 MAP-kinase (~35-40%). Specific p38 MAP-kinase inhibitor (SB202190) had an insignificant influence on migration of $CD4^+$ T-lymphocytes (Fig. 3). Combined addition of inhibitors of Src- and PI3-kinases or inhibitors of Src-kinases and ERK1/2 MAP-kinases did not potentiate the inhibitory effect, whereas combined addition of inhibitors of PI3-kinases and ERK1/2 MAP-kinases decreased migration of T-lymphocytes more effectively (55-60%).

It should be noted that the data on activation and migration of $CD4^+$ T-lymphocytes significantly varied in cells isolated from various donors.

In this study, we tried to characterize intracellular signaling pathways activated in response to CXCR3 bind-

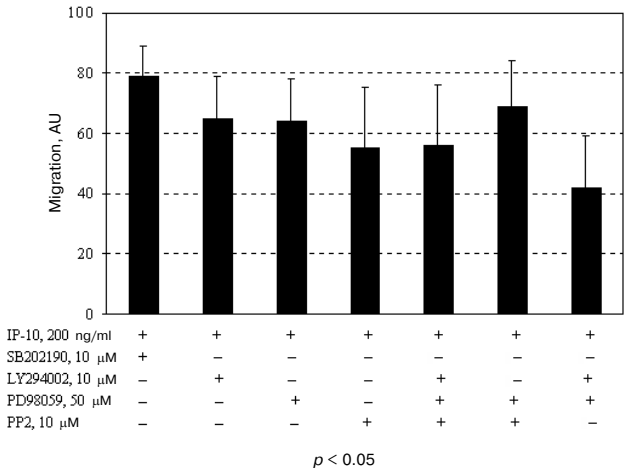

Fig. 3. Inhibitory analysis of IP-10-stimulated migration of activated CD4+ T-lymphocytes. AU, arbitrary units; maximal value of migration in the absence of inhibitor was defined as 100 AU. Results of one of three experiments are shown.

ing with IP-10 ligand in T-lymphocytes. Human CD4+ T-lymphocytes activated *in vitro* were used as a model. Stimulation of CD4+ T-lymphocytes resulted in the increase in relative amount of CXCR3+ lymphocytes. We detected IP-10-induced activation of p38 and ERK1/2 MAP-kinases. These kinases are involved in regulation of motility and other important processes, such as cell proliferation, secretory activity, etc. Many cytokines might activate this signaling pathway [29].

Activity of PI3-kinases is critical for many cell functions including chemotaxis [30]. Bonacchi et al. [22] demonstrated that IP-10 caused activation of PI3-kinases and transitory phosphorylation of downstream tyrosine PI3-kinases (e.g., Akt). Involvement of tyrosine kinases in CXCR3 signaling suggests that non-receptor tyrosine Src-kinases might also be involved in chemotaxis of CD4+ T-lymphocytes. It is known that Src-kinases are components of signaling pathways involved in signal transduction from various G-protein coupled receptors including chemokine receptors; they may be effectively activated by ERK1/2 MAP-kinases [31]. In the signaling pathway mediating CXCR3 the PI3-kinases may either activate Ras/ERK1/2 kinase pathway or represent its target [32, 33]. Nevertheless, specific inhibitors of PI3-kinase (LY294002) or Src-kinase (PP2) did not inhibit phosphorylation of ERK1/2 MAP-kinases, but it blocked activation of p38 MAP-kinase. This suggests that neither PI3-kinases nor Src-kinases are involved in activation of ERK1/2 MAP-kinases of CD4+ T-lymphocytes induced by IP-10. It is possible that ERK1/2 MAP-kinases can be activated by alternative signal cascades, whereas p38 MAP-kinase is obviously activated by Src-kinases and/or PI3-kinases but this activation does not affect chemotaxis of activated CD4+ T-lymphocytes.

CXCR3 receptor plays an important role in cell migration during inflammation and tissues damage. Blockade of CXCR3 by MAbs inhibited migration of Th1 to inflammation nidi in mice [3, 34, 35]. Data on expression of CXCR3 not only by T-lymphocytes but also by endothelial cells and vascular pericytes [22] caused by vascular damage support involvement of this receptor in wound healing and angiogenesis. Study of mechanisms of cell migration is important for understanding the pathogenesis of chronic inflammatory diseases and for development of various drugs for their amelioration.

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