Regulation of CXC Chemokine Receptor 4-mediated Migration by the Tec Family Tyrosine Kinase ITK*

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Chemokines are critical in controlling lymphocyte traffic and migration. The CXC chemokine CXCL12/SDF-1α interacts with its receptor CXCR4 to induce the migration of a number of different cell types. Although an understanding of the physiological functions of this chemokine is emerging, the mechanism by which it regulates T cell migration is still unclear. We show here that the Tec family kinase ITK is activated rapidly following CXCL12/SDF-1α stimulation, and this requires Src and phosphatidylinositol 3-kinase activities. ITK regulates the ability of CXCL12/SDF-1α to induce T cell migration as overexpression of wild-type ITK-enhanced migration, and T cells lacking ITK exhibit reduced migration as well as adhesion in response to CXCL12/SDF-1α. Further analysis suggests that ITK may regulate CXCR4-mediated migration and adhesion by altering the actin cytoskeleton, as ITK null T cells were significantly defective in CXCL12/SDF-1α-mediated actin polymerization. Our data suggest that ITK may regulate the ability of CXCR4 to induce T cell migration.

Lymphocyte trafficking is critical for proper migration to areas of inflammation and for the ability of these cells to perform their immunosurveillance roles. This trafficking is controlled in part by chemokines binding to their receptors expressed on specific cells. Understanding the signaling pathways that regulate chemokine signaling will be essential for a proper understanding of the mechanisms by which chemokines coordinate lymphocyte trafficking and the inflammatory response (1). CXCL12/SDF-1α is a chemokine that serves as a potent chemotactant for monocytes, bone marrow neutrophils, early stage B cell precursors, T lymphocytes, and CD34+ human progenitor cells (2–4). The only known receptor for CXCL12/SDF1-α, CXCR4, is a seven-transmembrane G protein-coupled receptor (5, 6). Human Jurkat T cells also express this chemokine receptor and migrate in a dose-dependent manner to its ligand, CXCL12/SDF-1α. CXCR4 is most notably recognized as a coreceptor for the binding of T-tropic human immunodeficiency virus strains (6, 7).

Recent studies using the ZAP-70-deficient Jurkat T cell line P116 have identified a role for ZAP-70 in CXCR4 signaling and migration, where ZAP-70 deficiency results in decreased migration levels to SDF-1α (8). Although the signaling events downstream of ZAP-70 that regulate T cell migration are presently unclear, ZAP-70 deficiency results in the lack of tyrosine phosphorylation of the adaptor protein Slp-76 in response to CXCR4 signaling, and although a Slp-76-deficient line of Jurkat cells are defective in CXCR4-mediated migration, this was not rescued by re-expression of exogenous Slp-76 (8). CXCR4 is coupled to the small G protein, G13, which can lead to the activation of PI3K and the accumulation of D-3 phosphoinositide lipids in Jurkat cells (9, 10). PI3K signaling may be required for CXCL12/SDF-1α-induced chemotaxis by orienting the cell toward the chemokine source. Localization of these PI3K D-3 lipid products is essential for targeting the pleckstrin homology domain-containing proteins to the leading edge of the cell and thereby directing chemotaxis.

The Tec family kinase ITK lies downstream of Zap-70 in the T cell receptor signaling pathway as well as the PI3K pathway (11–13). In addition, the adaptor protein Slp-76 is a prominent substrate and interaction partner for ITK (14, 15). We have tested whether the ITK is involved in regulating CXCL12/SDF-1α signaling and migration and now report that CXCL12/SDF-1α treatment resulted in the activation of ITK. This required the activity of Src family kinases as well as PI3K, two upstream regulators of ITK. ITK also regulated CXCR4-mediated migration as overexpression of WT ITK in the Jurkat T cell line results in increased chemotaxis, and T cells from mice lacking ITK exhibit reduced migration in response to CXCL12/SDF-1α, which accompanied reduced actin polymerization. These data suggest that ITK is involved in CXCR4 signaling and regulates T cell migration probably via its role in regulating actin polymerization.

EXPERIMENTAL PROCEDURES

Animals and Miscellaneous Reagents—ITK−/− mice (16) were a kind gift of Dr. D. Littman (New York University School of Medicine) and were backcrossed onto the C57Bl/6 background for >10 generations. Wild-type (WT) mice were from The Jackson Laboratory (Bar Harbor, ME). 6–8-week-old mice were used for these experiments. Animal experiments were approved by the Institutional Animal Care and Use Committee of Pennsylvania State University. Antibodies against ITK have been published previously (17) against phosphotyrosine (RC20) from Transduction Laboratories (Lexington, KY) and against actin from

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The abbreviations used are: CXCL, CXC chemokine ligand; SDF-1α, stromal cell-derived factor 1α; CXCR, CXC chemokine receptor; WT, wild-type; ITK, inducible T cell kinase; PI3K, phosphatidylinositol 3-kinase; ZAP-70, ζ-chain-associated protein of 70 kDa; TcR, T cell receptor.
ITK Regulates CXCR4 Migration

FIG. 1. CXCL12/SDF-1α activates ITK. Jurkat T cells were stimulated with 50 ng/ml CXCL12/SDF-1α for the indicated times. ITK was immunoprecipitated, and phosphotyrosine levels were determined.

FIG. 2. CXCL12/SDF-1α activates ITK via an Src and PI3K pathway. Jurkat T cells were pretreated with 10 μM PP1 or 40 μM LY294002 and then stimulated with 50 ng/ml CXCL12/SDF-1α for 5 min. ITK was immunoprecipitated, and phosphotyrosine levels were determined.

FIG. 3. ITK regulates CXCL12/SDF-1α-mediated migration. A, overexpression of WT ITK in Jurkat T cells. Parental Jurkat cells (lane 1) or cells overexpressing hemagglutinin-tagged ITK (lane 2) were probed with anti-ITK antibody (top panel) or actin antibody (bottom panel). B, Jurkat (open bars) or Jurkat/ITK (filled bars) T cells were stimulated with 50 ng/ml CXCL12/SDF-1α in a migration assay as described under “Experimental Procedures.” ITK-overexpressing cells exhibited increased basal migration compared with parental Jurkat cells. * p < 0.01.

RESULTS AND DISCUSSION

Activation of ITK by CXCR4 via an Src and PI3K Pathway—To determine whether ITK is involved in CXCL12/SDF-1α stimulation, Jurkat E6-1 cells were serum-starved and stimulated with CXCL12/SDF-1α for the indicated times (Fig. 1). Cell lysates were immunoprecipitated with a specific anti-

Sigma. PP1 and LY294002 were from Calbiochem. The vector pIREShyg was purchased from Clontech (Palo Alto, CA).

Cell Culture and Transfections—The human leukemic Jurkat T cell line, clone E6-1, was maintained in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum at 37 °C in 5% CO2. For stable transfections, 7.5 × 105 cells were electroporated with 15 μg of plasmid DNA (either pIREShyg alone or containing the hemagglutinin-tagged WT ITK) (18) using a T820 square wave electroporator (BTX, San Diego, CA), stable lines were derived by selection in 1.0 mg/ml hygromycin B (Invitrogen), and expression was confirmed by Western blot analysis.

Cell Stimulation, Lysis, Immunoprecipitation, and Immunoblots—Jurkat cells were serum-starved overnight and then stimulated as indicated in the figure legends. Aliquots of 20 × 106 cells were stimulated with 50 ng/ml CXCL12/SDF-1α or carrier in phosphate-buffered saline. The cells were then lysed at the indicated times by the addition of lysis buffer, and the proteins were immunoprecipitated as described by Hao and August (18). The proteins were separated on SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Pall Gelman), and probed with anti-ITK or anti-hemagglutinin antibodies.

Chemotaxis Assay—Migration assays were performed using blind well chemotaxis chambers (Neuro Probe, Inc., Gaithersburg, MD). 1.5 × 105 Jurkat T cells and derivatives were added to the upper chamber and incubated for 2 h or 4 × 106 primary murine thymocytes for 4 h at 37 °C, 5% CO2. The cells were allowed to migrate through 0.2-μm polyvinylpyrrolidone-free filters (Osmometrics) into a lower chamber containing migration buffer with or without 50 ng/ml CXCL12/SDF-1α (100 ng/ml for primary cells), and the cells in the lower chamber were collected, and the percentage of migration was determined from the original cell input.

Adhesion Assay—Adhesion assays were performed as described previously (19). In brief, 2 × 106 primary murine thymocytes were stimulated with 50 ng/ml CXCL12/SDF-1α for 10 min at 37 °C, 5% CO2 in plates coated with 0.3 μg/ml fibronectin. Nonadherent cells were then removed by washing, adherent cells were collected and counted, and the percentage of input cells was determined.

In Vivo Migration Assay—WT or ITK null mice were lightly anesthetized with isoflurane, and 100 ng of CXCL12/SDF-1α was delivered intranasally in 50 μl of phosphate-buffered saline. After 1 h, the mice were sacrificed, and their lungs isolated, and mononuclear cells were isolated as described previously (20). The number of T cells were then determined by staining with anti-CD3 antibodies and flow cytometry, gating on the lymphocyte population as defined by forward and side scatter characteristics. Total lymphocyte numbers were determined using an Advia 1200 hematology system (Bayer, Norwood, MA) and used to determine the number of T cells in the lung. Migration was expressed as fold increase in these numbers.

Flow Cytometry—Cells were analyzed by flow cytometry using monoclonal antibodies against CD3 (conjugated to phycoerythrin), CD4 (biotinylated), CD8 (conjugated to fluorescein isothiocyanate), CDS (conjugated to phycoerythrin), and anti-CXCR4 (rabbit anti-CXCR4, Abcam, Cambridge, MA) and detected using goat anti-rabbit fluorescein isothiocyanate.

Actin Polymerization Assay—Primary murine thymocytes were stimulated with 100 ng/ml CXCL12/SDF-1α for 30 min at 37 °C. The cells were washed, fixed with fresh paraformaldehyde, stained with Alex Fluor-488 conjugated phallolidin (Molecular Probes, Eugene, OR) for 15 min, washed again, and then analyzed by flow cytometry.

Statistics—Results were compared using Student’s t test, with p values < 0.05 taken as significant.
LY294002, followed by stimulation with CXCL12/SDF-1α for 5 min. Fig. 2 demonstrates that both Src and PI3K activity were required for CXCL12/SDF-1α-mediated ITK activation. These data indicate that ITK plays a role in CXCR4 signaling and requires Src and PI3K activity for its activation by CXCL12/SDF-1α.

Fig. 4. ITK null T cells exhibit reduced migration and adhesion in response to CXCL12/SDF-1α. A, WT (open bars) or ITK−/− (filled bars) thymocytes were stimulated with 100 ng/ml CXCL12/SDF-1α in a migration assay as described under "Experimental Procedures." ITK−/− thymocytes exhibited decreased migration compared with WT thymocytes. *, p < 0.05. B, flow cytometric patterns of CD4 and CD8 expression on WT and ITK null thymocytes prior to (top panels) or after (bottom panels) CXCL12/SDF-1α-induced migration. C, WT and ITK null thymocytes were analyzed for adhesion to fibronectin in response to CXCL12/SDF-1α. Data are expressed as percent of input cells that adhered. D, CXCR4 expression is equivalent between WT and ITK null thymocytes. WT and ITK null thymocytes were analyzed for expression of CXCR4 by flow cytometry. Shaded areas, CXCR4 expression; open areas, control antibody staining. E, ITK null T cells exhibit reduced migration in vivo in response to CXCL12/SDF-1α stimulation. 100 ng of CXCL12/SDF-1α was delivered intranasally to WT and ITK null mice, and 1 h later the lung mononuclear cells were isolated and analyzed for the number of T cells by staining with anti-CD3 antibodies and flow cytometry.

Regulation of CXCR4-mediated Chemotaxis by ITK—Our analysis of CXCR4 signaling indicates that ITK is activated by this chemokine receptor. To find out whether ITK regulates migration induced by CXCR4, we tested the effect of overexpressing WT ITK in Jurkat cells on CXCL12/SDF-1α-induced migration. We established stable Jurkat cells overexpressing ITK null T cells exhibit reduced migration in response to CXCL12/SDF-1α stimulation.
WT ITK by transfecting Jurkat cells with an expression vector carrying hemagglutinin-tagged ITK (Fig. 3A). This exogenous ITK was also responsive to CXCL12/SDF-1α stimulation, similar to what we observed for the endogenous ITK (data not shown). We then compared the responses of these cells with the parental Jurkat cell line for their migratory response to CXCL12/SDF-1α. As reported previously (8), CXCL12/SDF-1α induced cell migration in the parental Jurkat cell line (Fig. 3B).

In the unstimulated state, the ITK overexpressing Jurkat cells exhibited increased migration, and stimulating these cells with CXCL12/SDF-1α under the same conditions led to increased migration, although the fold increase in adhesion in response to CXCL12/SDF-1α was actually lower than that observed in the parental cell line perhaps because of the increased basal migration in these cells (Fig. 3B). Both cell lines had equivalent levels of CXCR4 expression as analyzed by flow cytometry (data not shown). These data suggest that ITK can control the ability of CXCR4 to induce migration. To determine whether the absence of ITK would affect the ability of cells to migrate, we took advantage of mice lacking ITK (16). We compared the ability of CXCL12/SDF-1α to induce migration of WT or ITK null thymocytes (on a C57Bl/6 background) and found that the absence of ITK resulted in a significant inhibition of migration in response to CXCL12/SDF-1α (Fig. 4A). Since the lack of ITK alters T cell development such that the ratio of CD4⁺:CD8⁺ thymocytes is reduced, we examined the migrating cells to determine whether the migration of particular populations of ITK null thymocytes was preferentially altered. The CD4 and CD8 patterns of expression of the WT and ITK null thymocytes were examined prior to and after CXCL12/SDF-1α-induced migration. We found that in both populations, there seemed to be preferential migration of double positive thymocytes and less migration of double negative cells after CXCL12/SDF-1α-induced migration (Fig. 4B; note that we recovered fewer cells from the ITK null populations). We have shown previously that ITK is involved in regulating TcR-induced adhesion (19). To determine whether ITK is also involved in the ability of thymocytes to adhere to fibronectin in response to CXCL12/SDF-1α, we performed adhesion assays, stimulating cells with CXCL12/SDF-1α and examining adhesion to dishes coated with fibronectin. We found that, similar to the results observed with migration, ITK also affected the ability of these thymocytes to adhere to fibronectin in response to CXCL12/SDF-1α stimulation (Fig. 4C). It was also possible that ITK null thymocytes expressed lower levels of CXCR4, the receptor for CXCL12/SDF-1α. However, analysis of CXCR4 expression indicated that both WT and ITK null populations expressed equivalent levels of CXCR4 (Fig. 4D). Finally, we determined whether ITK could affect the ability of T cells to migrate in response to CXCL12/SDF-1α stimulation in vivo. For these experiments, we delivered CXCL12/SDF-1α intranasally and determined the ability of T cells to migrate to the lung in response to this stimulus, which requires adhesion and migratory properties. These experiments confirmed the in vitro experiments, showing that although the number of WT T cells increased in the lung following CXCL12/SDF-1α stimulation, the number of ITK null T cells did not and actually decreased instead (Fig. 4E). This was similar to what we have observed previously in a murine model of allergic asthma, in that ITK null T cells were defective in getting into the lung following allergic challenge (20). These data indicate that ITK regulates the ability of T cells and thymocytes to adhere and migrate in response to CXCL12/SDF-1α.

**Regulation of CXCR4-mediated Actin Polymerization by ITK**—Shimizu and colleagues (19) as well as Tsoukas and colleagues (22) and Schwartzberg and colleagues (27) have recently reported that ITK can regulate the ability of the TcR to induce actin polymerization. As chemokine-induced migration is dependent on signal-induced changes in the actin superstructure (23), we asked whether actin polymerization in response to CXCL12/SDF-1α was affected in ITK null thymocytes. We stimulated WT or ITK null thymocytes with CXCL12/SDF-1α and measured the level of actin polymerization using Alexa Fluor-468-labeled phalloidin and flow cytometry. We found that, although CXCL12/SDF-1α-induced WT thymocytes to increase F-actin levels ~2-fold, the absence of ITK resulted in a significant reduction in the ability of CXCL12/SDF-1α to induce actin polymerization (Fig. 5). These data suggest that ITK may regulate the ability of CXCL12/SDF-1α to induce actin polymerization and therefore the ability of cells to migrate in response to CXCL12/SDF-1α.

The requirements for Zap-70, Slp-76, and PI3K in regulating CXCR4 signaling suggest that signaling between these molecules is important for this pathway (8, 24). In the TcR pathway, Zap-70 is required for ITK activation, and ITK requires PI3K for efficient activation (11–13). In addition, Slp-76 is a binding partner for ITK, and ITK can phosphorylate Slp-76, which may lead to the assembly of critical components of this pathway leading to migration, including the molecule ADAP/Fyb/Slap-130 (15, 25). Thus ITK may play a critical role in the proper assembly of these components, leading to altered actin rearrangement and migration. Indeed, ITK is involved in regulating the actin cytoskeleton downstream of the TcR (22, 26, 27), and our data suggest that ITK may play a larger role in regulating actin cytoskeletal changes downstream of CXCR4 as well. Interestingly, CXCR4 is involved in regulating T cell
migration to lymph nodes, and although we have not observed any difference in the percentages of T cells in the lymph nodes of ITK null mice under steady state conditions (other than an overall decrease in total T cell numbers, reported previously (16)), we have observed altered recruitment of ITK null T cells to the lung during the induction of allergic asthma in ITK−/− mice (20). It will be of considerable interest to determine whether T cells from ITK null mice exhibit altered homing of the lymph node under stimulated conditions and whether ITK or other Tec family kinases also regulate other chemokine responses.

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