Negative regulation of CXCR4-mediated chemotaxis by the lipid phosphatase activity of tumor suppressor PTEN

Ping Gao¹, Ronald L. Wange², Ning Zhang¹, Joost J. Oppenheim¹, O. M. Zack Howard¹

¹Laboratory of Molecular Immunoregulation, Center for Cancer Research, National Cancer Institute-Frederick, National Institutes of Health, Frederick, 21702, USA.

²Laboratory of Cellular and Molecular Biology, National Institute on Aging, National Institutes of Health, Baltimore, MD 21224, USA

Correspondence:  O. M. Zack Howard, National Cancer Institute-Frederick, P.O. Box B, Bldg 560, Rm 31-19, Frederick, MD 21702-1201, USA Phone: 301-846-1348, Fax: 301-846-6789, howardz@mail.ncifcrf.gov

Word Count: 4518

Scientific Category: Chemokines.
Abstract

PTEN, a multifunctional tumor suppressor, has been shown to play a regulatory role in cell migration. D. discoideum cells lacking PTEN exhibited impaired migration towards chemoattractant gradients. In the present study, we investigated the involvement of PTEN in chemotaxis of mammalian cells by examining PTEN-null Jurkat T cells. We observed that, in contrast to observations made in D. discoideum, PTEN-null Jurkat T cells exhibited potent chemotactic responses to the chemokine, SDF-1α, indicating that PTEN was not requisite for CXCR4-mediated chemotaxis of Jurkat cells. Conversely, reconstitution of PTEN in Jurkat cells by using a Tet-on inducible expression system down-regulated CXCR4-mediated chemotaxis. Furthermore, we established the lipid phosphatase activity of PTEN as essential for its inhibitory effect on chemotaxis. In addition, using PTEN-expressing T cell lines and primary T cells; we demonstrated that down-regulation of PTEN expression with vector-based siRNAs enhanced CXCR4-mediated chemotaxis. Based on these results, we conclude that PTEN expression negatively regulates chemotaxis of lymphoid mammalian cells via its lipid phosphatase activity. Our findings may account for the reported increase in metastatic activity of PTEN-null tumor cells.

Keywords: CHEMOTAXIS, PTEN, CXCR4, CHEMOKINES, SIGNAL TRANSDUCTION
Introduction

Chemotaxis is a property of many motile eukaryotic cells that results from a cascade of intracellular events initiated in cells with the capacity to detect an extracellular chemoattractant gradient. The initiation of cell chemotaxis requires the binding of a chemoattractant to its receptor, usually a seven transmembrane G protein-coupled receptor (GPCR), and subsequent activation of pertussis toxin (PTX)-sensitive G protein (Gi), which results in a chain of signaling events, from activation of the PI3K pathway and downstream small G-proteins Rac/cdc42, polymerization of F actin at the leading edge of the cells, to the formation of a pseudopod and cell polarization. Mounting evidence has established the extensive involvement of chemoattractants, chemokines in particular, in homeostatic homing of leukocytes, inflammation, development, wound healing, angiogenesis, and immune responses. Chemokines and their receptors also play a critical role in metastasis of malignant tumor cells.

PTEN, one of the most frequently mutated or deleted tumor suppressor genes in human cancers, has been shown to play an important role in regulating cell migration. Previous studies showed that PTEN expression inhibited migration of certain human tumor cell lines, although the driving forces behind such migration and its underlying mechanisms remained obscure. The involvement of PTEN in the chemotactic movement of cells was initially evidenced in D. discoideum as cells lacking PTEN exhibited impaired migration towards chemoattractants. Although, the evidence obtained from D. discoideum study was considered conclusive, whether the observations could be extrapolated to mammalian cells remained to be determined. To date, the reports directly addressing the involvement of PTEN in chemotactic movement of mammalian cells have been confusing. While Fox et al. demonstrated that PTEN+/− mouse B
lymphocytes exhibited enhanced chemotactic responses to SDF-1α. Anzelon et al. reported that the conditional deletion of PTEN from mouse B cells resulted in a general impairment in chemotaxis induced by SDF-1α and BLC. These discrepancies point to the necessity to further elucidate the role of PTEN in regulating the chemotaxis of mammalian cells.

A longstanding challenge in tumor biology is to decipher how metastasis, the chief cause of morbidity and mortality in most cancers, occurs in human malignancy. Although accumulating evidence suggested organ-specific metastasis is governed, in part, by interactions between chemokine receptors on cancer cells and chemokine ligands in target organs, the underlying regulatory mechanisms have remained largely unknown. Thus, we reasoned that it was of significance to address directly the role of PTEN, a multifunctional tumor suppressor, in modulating chemotaxis of mammalian cells including human cancer cells. To this end, we used Jurkat cells, a naturally occurring PTEN-null and chemokine receptor CXCR4-positive human leukemia T cell line, to investigate the role of PTEN in chemotaxis. We found that PTEN-null Jurkat cells and siRNA transfected primary T-cells exhibited potent chemotactic responses to SDF-1α, indicating that PTEN was not indispensable for chemotactic movement of lymphoid cells, a sharp contrast to early observations made in D. discoideum. By using a Tet-on inducible system, we then demonstrated that reconstitution of PTEN expression inhibited CXCR4-mediated chemotaxis. Importantly, we further established that the lipid phosphatase activity of PTEN was essential for its role as a suppressor of chemotaxis. Our results may serve to invite more extensive and straightforward approaches to investigate the anti-metastatic function of PTEN in human malignancy.
Materials and Methods

Reagents

Recombinant human chemokines (SDF-1α, IP-10, RANTES, MDC, LARC, I-309, MIP-3β, Eotaxin3) and cytokines (human IL-2 and mouse IL-12) were purchased from Pepro Tech (Rocky hill, NJ). Recombinant human IGF-1 was obtained from R&D (Minneapolis, MN). Anti-human PTEN (clone 6H2.1) was from Cascade Bioscience (Winchester, MA). Anti-phospho-Akt (Ser473) and anti-ERK1/ERK2 were purchased from Cell Signaling Technology, Inc. (Beverly, MD). PE-conjugated anti-human CXCR4 were from R&D (Minneapolis, MN). PE-conjugated mouse isotype-matched IgG2a κ was from BD Bioscience Pharmingen (Palo Alto, CA). Pertussis toxin (PTX), wortmannin and LY294002 were from Sigma Chemicals (St Louis, MO). Doxycycline was purchased from BD Bioscience (Palo Alto, CA).

Cell Lines and Culture Conditions

The Tet-on inducible Jurkat cell lines have been described previously 22. 2D6 T cells and 2B4 T cells, kind gifts from Dr. Hiromi Fujiwara of Osaka University in Japan, were also described in our previous publications 23-25. While all the cell lines were maintained in RPMI 1640 medium containing 10% (v/v) FBS (HyClone, Logan, UT), 10 mM glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin (Quality Biologicals, Gaithersburg, MD), additional 800µg/ml geneticin (GIBCO, Carlsbad CA) was added into the culture medium to maintain Tet-on Jurkat cells and 250 pg/ml of IL-12 was added to maintain 2D6 T cells23. The treatment conditions for doxycycline or other chemical reagents were specified in the legends. HEK293 and HEK293/CCR5 cells were described previously 26. Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation from leukopacks.
supplied by the Department of Transfusion Medicine under an approved human subjects protocol (Clinical Center, National Institutes of Health, Bethesda, MD).

**Purification of human CD4 T cells**

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient centrifugation from leukopacks supplied by the Department of Transfusion Medicine (Clinical Center, National Institutes of Health, Bethesda, MD) under an approved human subjects protocol IRB#99-CC-0168. Approval was obtained from the National Cancer Institute institutional review board for these studies. Informed consent was provided according to the Declaration of Helsinki. Human peripheral blood CD4 T cells were purified from PBMCs by the use of magnetic cell sorting (MACS) human CD4 T cell isolation kit (Miltenyi Biotech, Auburn, CA) following the manufacturer's recommendation. The purity of CD4 T cells (> 95%) was confirmed by flow cytometry.

**Plasmids and Transfection**

Tet-on response plasmid pTRE-HA-PTEN (PTEN/wt) has been described previously. The naturally occurring PTEN mutants G129E (lipid phosphatase-dead, protein phosphatase-live) and G129R (phosphatase-dead) were introduced into pTRE-HA-PTEN by Quickchange™ (Stratagene, La Jolla, CA) site directed mutagenesis. The cDNA plasmids were transiently transfected into Tet-on Jurkat cells by utilizing Fugene 6 Transfection Reagents (Roche Diagnostic Corp. Indianapolis, IN), which was performed according to the manufacturer’s protocol. Six hours after transfection, doxycycline (1µg/ml) was added to the culture to induce
PTEN expression. After additional 48h, cells were harvested for Western blotting and chemotaxis assay.

The DNA vectors for small interfering RNA (siRNA) constructs, pRNA-T-U6.1/Hygro/GFP, were from GenScript Corp. (Piscataway, NJ). Three siRNA insert sequences for PTEN targeting, which were selected by using the software siRNA Construct Builder (GenScript Corp.), were as the follows:

GGATCCCGTTCGCCACTGAACATTGGAATTGATATCCGTTCCAATGTTTCAGTGGCGGAATTTTTTCCAAAAGCTT (siPTEN construct #1),
GGATCCCGCACGCTCTATACTGCAAATGTTGATATCCGCAATTTGCGATATAGAGCGTGCCTTTTTTCCAAAAGCTT (siPTEN construct #2)
GGATCCCGTATAGGTCAAGTCTAAGTCGATTGATATCCGTCGACTTAGACTTGACCTATATTTTTTCCAAAAGCTT (siPTEN construct #3). As a negative control, a mock vector was also designed; whose inserted sequence was

GGATCCCGCGAGTTAATTACACGCGATCCTTGATATCCGGGATCGCGTGTAATTAACGCGTTTTTTCCAAAAGCTT (scrambled). The three siRNA constructs were combined into one pool and transfected into 2D6 T cells or 2B4 T cells using Fugene 6 Tranfection Reagents according to the manufacturer’s protocol (Roche Diagnostic Corp. Indianapolis, IN). Stable cell clones were obtained with hygromycin selection, based on GFP expression. PTEN expression following the transfection was measured by Western blotting.

The transfection of primary CD4 T cells with vector-based siRNA constructs were carried out by using Nucleofect™ II Device and Human T cell Nucleofector™ Kit Amaxa Biosystems (Amaxa
GmbH, Nattermannallee, Cologne, Germany) according to the manufacturer’s protocol for unstimulated human T cells. We used 5x10^6 purified unstimulated CD4 T cells, 3 µg DNA and Program U-014 for each transfection because our pilot studies revealed that these conditions yielded a higher proportion of surviving transfected cells than when we used PHA stimulated T-cells. The cells were cultured in RPMI-1640 supplemented with 10% (v/v) FBS for 4 hours after transfection, then human IL-2 (20U/ml) was added to the culture. After additional 20 hours of culture, we pooled multiple wells of cells transfected with the same vector and purified the viable and positive cells by cell sorting using the DakoCytomation/MoFlo System (Fort Collins, Colorado, USA), based on GFP expression. The purified cells were 93% positive for GFP and 98% of them excluded trypan blue. PTEN expression was measure by Western blotting.

Chemotaxis

Chemotaxis assays were performed using 48-well microchemotaxis chambers. Jurkat cells, other T-cell lines or peripheral blood T-cells were resuspended in binding medium (RPMI 1640 medium containing 1% bovine serum albumin (Sigma A4503, 25mM HEPES, pH8.0) at 2.0x10^6. In some experiments, cells were pretreated with chemical inhibitors under conditions specified in the legends. Chemokines, diluted in binding medium, were placed in the lower wells of the micro-chemotaxis chambers (Neuroprobe, Rockville, MD). Five-micrometer polyvinyl-free polycarbonate membranes (Neuroprobe) were treated overnight at 4°C in RPMI containing 10µg/ml of fibronectin (Sigma, Saint Louis, MI), air-dried and placed over the chemoattractants to separate the lower and upper wells of the chamber. After the micro-chemotaxis chamber was assembled, 50 µl of cells were added in the upper wells. After incubation at 37°C for 3 h in humidified air with 5% CO₂, the membranes were removed, scraped and stained. The cells
migrated across the membranes were counted with the use of a Bioquant semiautomatic counting system, which objectively determines the average number of cells in six defined microscopic fields of each condition on the filter membrane. The results were reported as the mean number of cells per high-powered field (No./HPF).

Flow Cytometry

The surface expression of chemokine receptor CXCR4 on Jurkat cells was monitored by fluorescence-activated cell sorter analysis. Jurkat cells (1x10^6, with/without doxycycline treatment) were washed with FACS buffer (1xDPBS w/o calcium and magnesium, 1% FCS and 0.02% sodium azide), and stained with PE-conjugated mouse anti human CXCR4 mAb (20µl/sample), or PE-conjugated mouse isotype-matched IgG2a. Stained cells were washed and analyzed on a FACScan (BD, Biosciences, Mountain View, CA) using CellQuest software. Cell sorting of GFP positive T cells after siRNA transfection was carried out using the DakoCytomation /MoFlo System (Fort Collins, Colorado, USA)

Western Blotting

The cultured cells (1x10^6) were harvested and lysed by adding 100 µl of 1xSDS sample buffer (62.5 mM Tris-HCl, pH 6.8 at 25°C, 2%w/v SDS, 10% glycerol, 50 mM dithiothretol, 0.01% bromophenol blue). The samples were then sonicated for 10 seconds on ice, boiled for 5 min, cooled on ice, and centrifuged at 14,000 rpm for 5 min. The protein concentration of the extracts was estimated with a BCA Protein Assay kit (PIERCE, Rockford, IL) and 25 µg of proteins were loaded and separated on a 4-12% NuPAGE™ Bis-Tris Gel (Invitrogen, Carsbad, CA). The proteins in the gel were then electrotransferred onto Immobilon™ membrane (Millipore,
Bedford, MA) with an Xcell™ blot Module (Invitrogen, Carsbad, CA) using 1x NuPAGE™
transfer buffer. The PVDF membrane with the transferred proteins was sequentially washed with
1xTBS/T (1x TBS, 0.1% Tween20) for 5 min, incubated for 1h in blocking buffer (1 x TBS,
0.1% Tween20, 5% w/v nonfat milk), and incubated at 4°C overnight in blocking buffer
containing 1:1000 dilution of the primary antibodies. The membrane was then washed three
times for 5 min each with TBS/T and incubated with 10 ml of 1:2000 dilution of HPR-
conjugated second antibodies (Cell Signaling, Beverly, MD) for 1h. After washing three times
with TBS/T, the membrane was incubated with working solution of ECLplus Detection Reagents
(Amersham, Piscataway, NJ) for 5 min and exposed to an X-ray film, which was then developed
using an automatic processor (Kodak X-OMAT 2000A). The same membrane was incubated in
stripping solution (62.5 mM Tris-HCl, pH6.7, 2% SDS, 100mM 2-ME) at 50°C for 30 min and
then re-probed as before.
Results

Several previous studies have reported that Jurkat cells were PTEN deficient\textsuperscript{22,28-30}, however, there were also reports showing that Jurkat cells expressed wild type PTEN\textsuperscript{31}. Thus, we started our experiments by examining PTEN protein expression in Jurkat cells. Several Jurkat cell lines, obtained from different laboratories, were tested by Western blotting and none was found PTEN-positive (Figure 1A and data not shown).

Jurkat cells are human acute leukemia T cell lines\textsuperscript{32}. As expected, FACS analysis confirmed surface expression of CXC chemokine receptor CXCR4 in these cells (Fig 1B and data not shown). Thus, PTEN-null Jurkat cells represented a useful model to study the role of PTEN in chemotaxis of mammalian cells.

When stimulated with the CXCR4 ligand, SDF-1\(\alpha\) (CXCL12), Jurkat cells exhibited potent migratory responses in chemotaxis assays. Since earlier reports showed that D. discoideum lacking PTEN were defective in directional movements towards chemoattractant sources (chemotaxis), but exhibited enhanced random movements (chemokinesis)\textsuperscript{17}, we further characterized SDF-1\(\alpha\)-stimulated Jurkat cell migration in chemotaxis assays to determine whether it was due to chemotaxis or chemokinesis. As shown in Figure 1, SDF-1\(\alpha\) stimulated typical bell-shaped dose-dependent chemotactic responses (Fig. 1C) that were inhibited by pertussis toxin (PTX) (Fig.1D). When the chemokine concentration gradient was eliminated by adding equal concentrations of SDF-1\(\alpha\) to the upper wells of chemotaxis chambers, Jurkat cell migration was abolished (Data not shown). Furthermore, stimulation with various other chemokines did not elicit chemotactic response in Jurkat cells (Fig 5A). Taken together, our
results indicated that, in contrast to previous findings in D.discoideum, PTEN-null Jurkat cells exhibited potent chemotactic responses to SDF-1α.

To more directly address the role of PTEN in chemotaxis, PTEN expression was reconstituted in Jurkat cells by using a tetracycline-inducible expression system as previously described. PTEN expression upon doxycycline (dox) treatment was confirmed in a PIJ (PTEN-Inducible Jurkat)-17 clone but not in a non-PTEN-expressing control clone, Con18, by Western blotting (Figure 2A). PTEN expression in PIJ-17 Jurkat cells was detectable as early as 3h after dox induction, reaching maximum expression between 24 and 48h, and was persistent at the maximal level 120 h after induction (Fig 2C). PTEN expression inversely correlated with phosphorylation of Akt, indicating that the expressed PTEN was functional (Figure 2A & C).

Chemotaxis assays showed that expression of PTEN substantially inhibited SDF-1α-stimulated chemotaxis of PIJ-17 cells (Figure 2B). The effect on chemotaxis, which was detectable 6h after dox induction and persistent for at least 5d, correlated with the level of PTEN expression and phosphorylation of Akt (Figure 2C and D). However, expression of PTEN did not affect surface expression of CXCR4 on Jurkat cells (Figure 2E), suggesting that PTEN negatively modulated SDF-1α-stimulated chemotaxis, presumably by inhibiting chemotactic signaling pathway downstream of CXCR4. In agreement with the previous report, in the timeframe of our experiments, dox treatment did not affect the viability of PIJ-17 Jurkat cells (Data not shown).

PTEN is a dual-functional protein and lipid phosphatase. The major known physiologically relevant substrate of PTEN is the lipid second messenger PIP3. However, both lipid and
protein phosphatase activities have been suggested to be involved in regulating cellular migration 16,34. To investigate which of the phosphatase activities of PTEN, lipid or protein, were responsible for the inhibitory effect of PTEN on chemotaxis, Tet-on inducible vectors expressing PTEN/wt, PTEN/G129E and PTEN/G129R were transiently transfected to Tet-on-Jurkat cells. G129E and G129R are two naturally occurring PTEN mutants found in human malignancy. G129E is a mutant lacking lipid phosphatase activity while retaining protein phosphatase activity, whereas G129R lacks both lipid and protein phosphatase activities15. After 6h following transfection, dox (1µg/ml) was added to the medium to induce PTEN expression. After an additional 48h, PTEN expression was detected in PTEN-vector-transfected cells but not in the mock transfection group. Phosphorylation of Akt was decreased in the PTEN/wt group, but not in mutant groups (PTEN/G129R or PTEN/G129E), indicating that the expressed mutant PTEN indeed lacked lipid phosphatase activity (Fig 3A). Chemotaxis assays were performed 48h after transfection. As shown in figure 3B, the effect of PTEN on chemotaxis was abolished by a single point mutation, which inactivates PTEN’s lipid phosphatase activity. Of note, PTEN/G129E, a mutant lacking lipid phosphatase activity but retaining protein phosphatase activity, was also defective in modulating chemotaxis, indicating that the lipid, but not protein, phosphatase activity of PTEN was essential.

Since the lipid phosphatase activity was essential for the effect of PTEN on chemotaxis, we postulated that, by antagonizing the PI3K-PIP3 pathway, PTEN negatively modulated CXCR4-mediated chemotaxis in Jurkat T cells. Although PI3K-PIP3 have been proposed as central players in chemotaxis in a number of mammalian cells, its effect varies, depending on the cell types and chemokine receptors involved 35,36. PI3K-independent pathways have also been
proposed\textsuperscript{35,36}. Therefore, we examined whether blocking the PI3K-PIP3 pathway impaired CXCR4-mediated chemotaxis. PIJ-17 Jurkat cells, with or without dox treatment, were pre-incubated with two chemical inhibitors of PI3K; wortmannin or LY294002, and chemotaxis assays were performed. As shown in Figure 4, both wortmannin and LY294002 inhibited SDF-1\(\alpha\)-stimulated chemotaxis in a dose-dependent manner. Interestingly, although chemotaxis was markedly inhibited with PTEN expression, it was further decreased by the pretreatment with PI3K inhibitors. However, chemotaxis was never decreased to basal levels, even at higher doses of chemical inhibitors (Figure 4 and data not shown), suggesting that pathways independent of PI3K may also regulate SDF-1\(\alpha\)-stimulated chemotaxis of Jurkat cells.

Next, we addressed whether the effect of PTEN was restricted to CXCR4-mediated chemotaxis. We tested Jurkat cell responses to other chemokines whose receptors are reportedly expressed on different subsets of T lymphocytes. Of all the chemokines tested, Jurkat cells responded only to SDF-1\(\alpha\) (Figure 5A). However, Jurkat cells demonstrated substantial chemotactic responses to IGF1, whose receptor IGFR, a receptor tyrosine kinase, was reportedly expressed on Jurkat cells\textsuperscript{37}. Interestingly, expression of PTEN in PIJ-17 Jurkat cells substantially inhibited IGF1-stimulated chemotaxis as well (Figure 5B).

To address whether our observation that PTEN expression inhibited Jurkat T cell migration could be extrapolated to other mammalian cells, we utilized vector-based small interfering RNAs (siRNAs) to inhibit PTEN expression in two additional T cell lines, 2D6 T cells and 2B4 T cells. 2D6 cells were established from a terminally differentiated Th 1 cell\textsuperscript{38} clone that is IL-12-responsive. Our previous observations demonstrated that 2D6 T cells expressed chemokine
receptors including CXCR4 and showed chemotactic responses to chemokine ligands (and our unpublished observations). The chemotactic ability of 2B4/CXCR4 transfectant T cells were described in our previous publication. Following transfection with vector-based PTEN-targeting siRNAs, stable clones were obtained with hygromycin selection, based on GFP expression (Data not shown). Western blotting confirmed a substantial reduction in PTEN expression in siRNA/PTEN-transfected cells, but not in mock vector-transfected cells (Figure 6A). Consistent with results in Jurkat T cells, inhibition of PTEN expression significantly enhanced SDF-1α-induced chemotaxis of 2D6 T cells (Figure 6B) and 2B4 T cells (Figure 6C), indicating that PTEN negatively regulated CXCR4-mediated chemotaxis in two additional T cell lines, 2D6 and 2B4 T cells.

Finally, we tested the effect of PTEN down-regulation on chemotaxis of primary T cells. CD4+ T cells were purified from human peripheral blood, with a purity of more than 95%, followed by transfection with vector-based siRNAs using the Amaxa System. After 24 hours of culture, we purified GFP-positive, viable cells by cell sorting. As shown in Figure 6A, transfection of vector-based siRNAs substantially inhibited PTEN expression in primary CD4 T cells. Consistent with results in 2B4 and 2D6 T cell lines, inhibition of PTEN expression markedly enhanced SDF-1α-stimulated chemotaxis of human primary T cells (Figure 6D).
Discussion

In this report, we investigated the naturally occurring PTEN-null human Jurkat T cell line to characterize the involvement of PTEN in chemotaxis of mammalian cells. Our findings are as follows: 1) in contrast to early observations made in D. discoideum, PTEN-null Jurkat cells exhibited potent CXCR4-mediated chemotaxis, indicating that PTEN was not indispensable for proper chemotactic movement of Jurkat cells; 2) utilizing a Tet-on inducible system, we found that reconstitution of PTEN expression in Jurkat cells down-regulated CXCR4-mediated chemotaxis; 3) using two naturally occurring PTEN mutant, G129E and G129R, we further identified the lipid phosphatase activity as essential for PTEN’s role as a negative modulator of CXCR4-mediated chemotaxis. This was further supported by the observations that PI3K inhibitors mimicked PTEN’s effect of negatively regulating CXCR4-mediated chemotaxis. The inhibitory effect of PTEN on chemotaxis was not restricted to CXCR4-mediated chemotaxis of Jurkat cells since restoration of PTEN expression down-regulated IGF1-stimulated chemotaxis as well. Furthermore, suppression of PTEN expression by siRNA also enhanced CXCR4-mediated chemotaxis by PTEN-expressing T cell lines and primary T cells. These findings indicate that PTEN expressed by mammalian cells performs a critical inhibitory function in the signal cascade that leads to cell migration.

Considering that the single-cell D. discoideum that lacked PTEN showed defective directional movement towards chemoattractant sources but enhanced random movement\textsuperscript{17-19}, our contrasting observations in PTEN-null Jurkat cells are of interest. Although single-cell organisms such as D. discoideum share many mechanisms in common with leukocytes of mammalian species for sensing and responding to chemoattractant gradients, it is highly probable that the
complexity of the signaling pathways elicited by chemoattractants may vary with the species of migrating cells or types of the chemoattractants. Interestingly, in D. discoideum, results from both Devreotes’ and Firtel’s laboratories elegantly demonstrated that, following stimulation by chemoattractants, PI3K was relocated to the leading edge while PTEN moved to the lateral sides and the posterior of the chemotaxing cells, thus increasing the intracellular PIP3 gradient and sharpening the subsequent localization of PH domain-containing proteins to the leading edge of the cells. However, Bourne’s group did not observe PTEN exclusion from the leading edge of the HL-60 myeloid cells following chemoattractants’ stimulation. These earlier results together with the present study suggest that the observations made in single-cell organisms such as D. discoideum are not recapitulated in certain mammalian cell lines. At present, we have no specific answers to the fundamental question as to how lymphoid cells, in the absence of PTEN, can generate the intracellular molecular gradient that is essential for cellular polarization and chemotaxis. Presumably, prolonged phosphorylation of components in this pathway, which occurs in the absence of PTEN, favors chemotaxis and the actual chemotactic mechanisms in mammalian cells are distinct from those of prokaryotic cells.

There have been few earlier studies of the role of PTEN in mammalian cell chemotaxis. Two groups have directly observed chemotactic behaviors of PTEN deficient B lymphocytes, but the results were divergent. Fox et al. demonstrated that PTEN+/- mouse B lymphocytes exhibited enhanced chemotactic responses to SDF-1α stimulation, however, Anzelon et al. reported just the opposite: the conditional deletion of PTEN in mouse B cells resulted in an overall impairment in chemotaxis. The reason for this discrepancy remains unknown. In PTEN+/- cells used by Fox et al., the expression level of PTEN never went below 50% of the
wild-type cells, whereas the PTEN<sup>loxp/loxP</sup> B cells utilized by Anzelon et al. were PTEN deficient. Since the level of PTEN has been proposed as a decisive factor in determining its multiple functions<sup>40</sup>, it is possible that cells might behave differently, or in the opposite manner, as PTEN level is further reduced. However, our study does not support such a possibility. In PTEN-null Jurkat cells, enhanced, instead of impaired, chemotaxis was observed. Restoration of PTEN expression substantially down-regulated CXCR4-mediated chemotaxis, which was correlated with PTEN expression levels (Figure 2 C and D). On the other hand, although Anzelon et al. reported impaired chemotaxis of the PTEN<sup>loxp/loxP</sup> B cells in the in vitro assays, it is interesting to notice that, in those conditional knockout mice, as indicated by the preferential generation of marginal zone B cells, B1 cells and germinal center formation<sup>21</sup>, the general homeostatic homing of the PTEN deficient B cells to specific secondary lymphoid compartments was not impaired, as has been observed in the knockout phenotypes of certain chemokine receptors, such as CCR7 or CXCR5<sup>41-43</sup>. The reported increase in B cell cellularity in specific second lymphoid compartments suggest that PTEN deficiency did not actually impair the specific homing of lymphocytes to secondary lymphoid organs in vivo, a process in which chemotaxis plays an indispensable role<sup>44</sup>.

The present study demonstrated that Jurkat cells with complete loss of PTEN exhibited enhanced directional movement towards SDF-1α as well as IGF1 and restoration of PTEN expression inhibited such migration. Although SDF-1α and IGF1 use receptors of different families, their intracellular signaling pathways converge at PI3K-PIP3 level<sup>37</sup>. Indeed, we established that the lipid phosphatase activity was essential for the role of PTEN as a negative regulator of chemotaxis (Figure 3 and 4), suggesting that, by antagonizing the PI3K-PIP3 pathway, PTEN
inhibited chemotaxis. Of note, we never observed complete abolition of chemotaxis by PTEN expression in Jurkat cells, indicating a delicate balance existing between PTEN and PI3K, and perhaps other pathways, that favor cell migration. Although the lipid substrates of PTEN and its implications in tumorigenesis have been intensively studied, the present results reveal a previously unappreciated aspect of the multifunctional nature of PTEN in tumor biology. Since chemokines, and perhaps other chemoattractants of the growth factor family, have been proposed as promoters of cancer cell metastasis to specific target organs, the present results prompted us to hypothesize that tumor cells with “loss of function” of PTEN may develop enhanced chemotactic ability, resulting in increased metastatic spread. Indeed, accumulating evidence demonstrated that “loss of function” of PTEN has been associated with late stage, more aggressive and metastatic cancers. Experiments utilizing vector-based siRNA constructs to knockdown PTEN or Tet-inducible system to restore PTEN expression in several human tumor cell models are currently in progress and their in vitro and in vivo behaviors in directional migration will be examined.
Acknowledgements

The authors are grateful to Drs. Paul Hu, Gareth Davies and Xin Chen for advise. We thank Drs. Ji-Ming Wang, William L. Farrar and Xia Zhang for critical review of this manuscript. We thank Dr. Helene Tonoli for kindly providing us with IGF-1. Dr. Esta Sterneck for sharing Amaxa Nucleofector device, and Dr. Hiromi Fujiwara for 2D6 and 2B4 T cells.
PTEN inhibits CXCR4-mediated chemotaxis

References

Legends

Figure 1. PTEN-null Jurkat T cells exhibited potent chemotactic response to SDF-1α.

A. Jurkat cells were PTEN-deficient. Lanes 1-6 contained lysates of wild-type Jurkat cells obtained from the laboratories of Drs. Ji-Ming Wang, William L. Farrar, Daniel W. McVicar and our NCI-Frederick lab, respectively, and the Tet-on and Tet-off Jurkat T cell lines purchased from Clontech. Lanes 7-9 showed the lysates of HEK-293 cells and PBMC, used as positive controls. Cultured cells were lysed and electrophoresed on a SDS-PAGE gel, transferred to nitrocellulose membranes, and probed with anti-PTEN, or anti-ERK1/2 as a loading control. B. Jurkat cells express CXCR4. The surface expression of chemokine receptor CXCR4 in Jurkat cells was measured by flow cytometry. Cells were stained with anti-hCXCR4 (solid line) or isotype-matched human IgG2a as a negative control (dotted line). C. Jurkat cells exhibit dose-dependent chemotactic responses to SDF-1α. Chemotaxis was performed using 48-well chemotaxis chambers as described in Material and Methods. Different concentrations (0-1000 ng/ml) of SDF-1α were used for chemotaxis. D. SDF-1α-induced chemotaxis of Jurkat cells was PTX-sensitive. Jurkat cells were pretreated with different doses of PTX for 30 min at RT and chemotaxis assay was carried out as described. All the results in this figure were representative of at least three independent experiments.

Figure 2. Reconstitution of PTEN in Jurkat cells down-regulated CXCR4-mediated chemotaxis. A. Doxycycline (dox) induced PTEN expression in PIJ-17 but not in Col-18 or Tet-on Jurkat cells. PIJ-17, Con-18 and Tet-on Jurkat cells were treated with 1µg/ml dox for 48h and the expression of PTEN (top panel) and Ser473-phosphorylated AKT (middle panel) was measured by Western blotting. The samples were also blotted with anti-ERK1/2 as loading
control (bottom panel). **B.** Expression of PTEN down-regulated SDF-1α-mediated chemotaxis. PIJ-17 and Con-18 Jurkat cells were cultured with/without the presence of dox (1µg/ml) for 48h and chemotaxis was carried out as in Fig.1. The results were representative of at least five independent experiments. **C & D.** Time course of PTEN expression under DOX induction and its effects on chemotaxis. PIJ-17 Jurkat cells were treated with 1µg/ml dox for the indicated periods of time, and the levels of PTEN and Ser473-phosphorylated AKT were detected by western blotting (C). The same cells were also assayed for chemotactic responses (D). The results were representative of three independent experiments. **E.** PTEN expression had no effects on CXCR4 surface expression. PIJ-17 Jurkat T cells were cultured with/without dox (1µg/ml) for 48h and CXCR4 expression were detected by flow cytometry. The results were representative of two independent experiments.

**Figure 3. The lipid phosphatase activity was essential for PTEN’s role as a negative regulator of chemotaxis.** Tet-on Jurkat cells were transiently transfected with Tet-inducible vectors expressing PTEN/wt, PTEN/G129E, PTEN/G129R, or a mock vector. After 6h, dox (1µg/ml) was added to the culture medium. Cells were cultured for an additional 48h and then harvested for Western blotting and chemotaxis (B). The results were representative of three independent experiments.

**Figure 4. PI3K inhibitors reproduced the inhibitory effect of PTEN on CXCR4-mediated chemotaxis.** PIJ-17 Jurkat cells were cultured with/without dox (1µg/ml) and were then pretreated with wortmannin or LY294002 (B) for 1h at RT at the indicated concentrations. The cells were then subjected to chemotaxis assays with SDF-1α (100ng/ml). The basal migration in
the absence of SDF-1α was indicated as “medium only”. The results were representative of four independent experiments.

**Figure 5. Restoration of PTEN expression in Jurkat cells down-regulated IGF1-mediated chemotaxis.** A. The chemotactic responses of cultured PIJ-17 Jurkat cells (without dox) to different chemoattractants were tested. The concentrations used were determined previously as optimal for chemotaxis of different T lymphocyte subsets: RANTES (50 ng/ml), MDC (100 ng/ml), Eotaxin3 (100 ng/ml), LARC (1000 ng/ml), IP-10 (100 ng/ml), I-309 (10 ng/ml), MIP-3β (100 ng/ml), IGF-1 (10 ng/ml), SDF-1α (100 ng/ml). The results were representative of three independent experiments. B. PIJ-17 Jurkat cells were cultured with/without dox (1 µg/ml) for 48h and chemotaxis assays were performed as in panel A. The results were representative of three independent experiments.

**Figure 6. Inhibition of PTEN expression by vector-based siRNA enhanced SDF-1α-induced chemotaxis of T cells.** A. Western blotting confirmed down-regulation of PTEN expression in siRNA/PTEN transfected cells. The non-transfected cells and the cells transfected with siRNA/PTEN constructs or mock vectors (Stable transfection for 2D6 and 2B4 T cell lines and transient transfection for primary T cells) were lysed and PTEN protein expression detected by Western blotting as described before. The samples were also blotted with anti-ERK1/2 as loading control (bottom panel). The results were representative of three independent experiments for T cell lines and two independent experiments for primary T cells. B, C, and D. CXCR4-mediated chemotaxis of 2D6 T cells (B), 2B4 T cells (C), and primary CD4 T cells (D) was enhanced by inhibition of PTEN expression. The chemotaxis assays were performed as described...
in “Materials and Methods”. The results were representative of three independent experiments for T cell lines and two independent experiments for primary T cells.
Figure 1 (Gao et al.)

A

PTEN
ERK1/2

Jurkat cell lines
HBK93
HBK/CCI, S
PBMC

B

C

D

SDF-1α (ng/ml)
PTX (ng/ml)

Migrated Cells/HPF

Medium
SDF-1
(100ng/ml)
Figure 2 (Gao et al.)

A

B

C

D

E

DOX(−)

DOX (+)
Figure 3 (Gao et al.)

A

B

PTEN inhibits CXCR4-mediated chemotaxis
Figure 4 (Gao et al.)

A

![Graph A showing the effect of Wortmannin on migrated cells/HPF under different conditions.

B

![Graph B showing the effect of LV294002 on migrated cells/HPF under different conditions.]}
Figure 5 (Gao et al.)

A

B

IGF-1 (ng/ml)

DOX(-)
DOX (+)
Figure 6 (Gao et al.)

A

2D6 Cells 2B4 Cells Primary T Cells

PTEN

ERK1/2

Parental Mock siPTEN Parental Mock siPTEN un-transfected Mock siPTEN

B

C

D

Medium SDF-1α (100ng/ml)

Medium SDF-1α (100ng/ml)

Medium SDF-1α (100ng/ml)

Primary T cells Mock siRNA/PTEN
Negative regulation of CXCR4-mediated chemotaxis by the lipid phosphatase activity of tumor suppressor PTEN

Ping Gao, Ronald L Wang, Ning Zhang, Joost J Oppenheim and O. M. Zack Howard