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Disruption of a Single Pten Allele Augments the Chemotactic Response of B Lymphocytes to Stromal Cell-Derived Factor-1

Joanne A. Fox,*† Karen Ung,* Sonia G. Tanlimco, † and Frank R. Jirik2*†

The tumor suppressor, Pten, has emerged as a critical negative regulator of phosphatidylinositol-3-kinase-dependent intracellular signaling pathways responsible for phenomena such as cellular adhesion, proliferation, and apoptosis. Herein, we present evidence that Pten regulates chemokine-dependent events in B lymphocytes. Primary B cells isolated from Pten+/− mice demonstrated increased responsiveness to stromal cell-derived factor-1-induced chemotaxis. This was accompanied by an elevated level of protein kinase B phosphorylation on Ser473. Our results suggest not only that Pten may be an important regulator of stromal cell-derived factor-1-directed chemotaxis, but also that Pten heterozygosity is associated with increased cellular sensitivity to this chemokine, likely via dysregulation of events lying downstream of phosphatidylinositol-3-kinase. These observations suggest a mechanism by which loss of a single Pten allele may confer a selective advantage on cells during multistep tumor progression. The Journal of Immunology, 2002, 169: 49–54.

Chemokines are critical to the normal development of lymphoid lineages as well as for the appropriate homing of these cells to specific anatomical sites (1). In addition to their role in lymphocyte trafficking, specific chemokines have been shown to be capable of regulating the ontogeny and maturation of secondary lymphoid tissues (2). One of the key chemokines involved in lymphocyte development and function is stromal cell-derived factor-1 (SDF-1), a CXC chemokine originally described as a B cell maturation factor (3). SDF-1 has been shown to be a powerful chemoattractant for pro- and pre-B cells, as well as for mature B and T cells (4, 5). Attesting to the importance of SDF-1, mice lacking this chemokine exhibit a severe block in B cell maturation, demonstrating abnormally low numbers of B lymphocytes as well as myeloid progenitors. In contrast, T cell development appears to proceed normally in these animals (6). SDF-1 binds to CXCR4, thereby stimulating a series of intracellular signaling events downstream of this G protein-coupled receptor (4, 5). Mice lacking CXCR4 exhibit various developmental defects equivalent to those of SDF-1-deficient mice, confirming that the SDF-1/CXCR4 signaling pathway is essential for normal hematopoietic development (7).

SDF-1-mediated activation of CXCR4 results in increased phosphorylation of focal adhesion components, activation and phosphorylation of phosphatidylinositol-3-kinase (PI3K), and increased activity of the NF-κB transcription factor (8). PI3K activation generates the membrane bound second messengers, phosphatidylinositol-3,4-bisphosphate (PI(3,4)P2) and phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3), that recruit and activate cytosolic proteins containing pleckstrin homology (PH) domains, such as members of the family of serine/threonine kinases that includes protein kinase B (PKB). Studies using either PI3K inhibitors or activated and dominant negative mutants of PI3K have indicated that the PI3K pathway is critical to SDF-1-induced chemotaxis (9–11). Furthermore, SDF-1 is able to induce sustained signaling and to promote prolonged activation of downstream effectors such as PKB and mitogen-activated protein kinase (MAPK; Ref. 11).

Given the importance of SDF-1-induced PI3K activation, it is noteworthy that the tumor suppressor gene, phosphatase and tensin homolog deleted on chromosome 10 (PTEN), has been shown to down-regulate this signaling pathway (12). In addition to sharing a catalytic signature motif with dual-specificity phosphatases, PTEN is capable of dephosphorylating the 3′ position of PI(3,4,5)P3 and PI(3,4)P2. This lipid phosphatase activity appears to account for the tumor suppressor effect of PTEN (13). For example, by modulating phosphatidylinositol levels, PTEN can negatively regulate PKB-dependent cell survival signals (14). Also, mice heterozygous for Pten spontaneously develop malignancies and show resistance to proapoptotic stimuli (15–18). In addition to cancer susceptibility, Pten+/− mice develop a nonmalignant lymphoproliferative disorder that is accompanied by autoimmune features (16, 19) similar to that of mice expressing a constitutively active form of PI3K (20). This suggests that hyperresponsiveness of the PI3K signaling pathway to external stimuli might contribute to the lymphoproliferative disorder in Pten+/− mice. In support of this hypothesis, expression of active PKB in a T cell transgenic model system disturbed both T and B cell homeostasis and resulted in an inflammatory/autoimmune phenotype that closely resembled that seen in the Pten+/− mice (21). T cell-specific loss of Pten leads to activation of PKB and secondarily to defects in T cell
homeostasis (22), suggesting that Pten, through its ability to regulate the PI3K/PKB pathway, is important in controlling lymphoid activation and development.

Several lines of evidence have suggested a role for PTEN in the control of cell movement. For example, while PTEN overexpression inhibited fibroblast motility and directional movement through effects on focal adhesion kinase (FAK; Refs. 23–25), murine embryonic fibroblasts lacking Pten exhibited increased cell motility, an effect attributed to Rac1 and Cdc42 dysregulation (26). Consistent with the importance of PI3K and lipid second messengers in directed migration, increased chemotaxis was seen in both thymic and splenic hematopoietic cells obtained from mice lacking Src homology 2 domain containing inositol phosphatase (SHIP), a lipid phosphatase that specifically targets the 5’ position of PI(3,4,5)P3 (27).

We hypothesized that Pten levels might regulate chemokine-dependent cell migration. To examine this possibility, we studied the migratory response of Pten<sup>+/−</sup> cells to SDF-1 in vitro. Purified B cells from Pten heterozygous mice demonstrated an augmented sensitivity to this chemokine that was accompanied by an increase in SDF-1-dependent chemotaxis. Thus, PTEN protein levels may be an important factor in the regulation of chemokine-dependent events in lymphocytes.

**Materials and Methods**

*Ab and chemokine reagents*

SDF-1 (residues 1–67) was a generous gift from I. Clark-Lewis (University of British Columbia, Vancouver, Canada). Abs with the following specificities were used: PTEN (6H2.1; A. G. Scientific, San Diego, CA), phospho-specific MAPK, MAPK, phospho-PKB (Ser<sup>473</sup>), and PKB (from Cell Signaling Technology, Ontario, Canada).

*Cell preparations and migration assays*

Spleens were isolated from 8-wk-old Pten<sup>+/−</sup> mice and sex-matched littermate controls. All animal work was approved by the Canadian Council of Animal Care. To isolate B cells, single-cell suspensions of splenocytes were first incubated with RBC lysis buffer and then washed twice in cold HBSS. T cells were depleted from the cell suspension by two separate incubations with monclonal anti-Thy-1.2 (HO13.4), anti-CD4 (2B6), and anti-CD8 (3.155) Abs together with low endotoxin rabbit complement (Cedarlane, Hornby, Canada) for 45 min at 37 °C. The remaining B cells were then isolated by Percoll density centrifugation as previously described (28). This cell population consisted of 80–94% CD19<sup>+</sup> cells as determined by flow cytometry. For chemotaxis experiments, purified B lymphocytes were resuspended in RPMI (without serum) at 1 × 10<sup>7</sup> cells/ml. Migration assays were conducted using Transwell polycarbonate membranes (Corning–Costar, Cambridge, MA) as previously described (29).

For assessment of chemotaxis in the presence of PI3K inhibitors, 100 nM wortmannin was added to both the upper and lower chambers and the migration response compared with that seen without the inhibitor. For assessment of chemokinesis vs chemotaxis, migration was tested in both the presence and absence of an SDF-1 gradient. To create a situation with no SDF-1 gradient, cells placed in the upper chamber were resuspended in the same SDF-1 solution used for the lower chamber and then migration was allowed to proceed for 3 h.

*Immunoblotting and densitometry*

Primary B lymphocytes were resuspended in RPMI (without serum) at 1 × 10<sup>6</sup> cells/ml and stimulated with 50 μg/ml SDF-1. Immunoblotting was conducted as described (29) with 25–50 μg of total protein loaded per lane. Densitometry was conducted on between two to five immunoblots derived from independent experiments. The values obtained for the phospho-specific blots were expressed as a percentage of the density of the protein loading control. Protein loading controls were done in parallel on each of the samples using Abs specific for the nonphosphorylated forms of the proteins. For each experiment, values were normalized and results represented relative values of phosphorylation.

**Results**

Pten<sup>+/−</sup> B lymphocytes demonstrate increased responsiveness to SDF-1-induced chemotaxis

To assess the effect of Pten heterozygosity on cell migration, we examined the response of primary B cells to SDF-1. In addition to showing a characteristic bell-shaped response to SDF-1 stimulation (Fig. 1a), it was apparent that an increased number of Pten<sup>+/−</sup> cells had migrated to the lower chamber, at concentrations ranging between 500 and 2000 ng/ml, as compared with control cells. The peak chemotactic response occurred at 1500 ng/ml SDF-1 for the Pten<sup>+/−</sup> B cells, whereas the wild-type cells responded maximally at doses between 2000 and 3000 ng/ml (Fig. 1a). These results suggested that Pten<sup>+/−</sup> B cells had an altered response threshold to SDF-1.

To further explore the differential responsiveness of Pten<sup>+/−</sup> and control cells to SDF-1, B cells isolated from multiple mice were stimulated with 1500 ng/ml (Table 1 and Fig. 1b). At this concentration, Pten<sup>+/−</sup> B cells showed a ~2.5-fold (<i>p</i> < 0.01) greater chemotactic response. Pten<sup>+/−</sup> B cells also displayed elevated chemokinesis compared with controls, showing a statistically significant (~2-fold) increase in their ability to move to the lower chamber in the absence of SDF-1. To demonstrate that the increased migration of Pten<sup>+/−</sup> cells at 1500 ng/ml of SDF-1 was due to chemotaxis, as opposed to chemokinesis, migration in the presence or absence of an SDF-1 gradient was assessed. The contribution of chemokinesis, as assessed by migration in the absence of an SDF-1 gradient, was small as compared with the maximal migratory response (Fig. 1c). However, Pten<sup>+/−</sup> B cells did show a small but statistically significant increase in chemokinetic movement (<i>p</i> < 0.03) as well as the previously observed increase in migration due to chemotaxis. Taken together, these results suggest that Pten<sup>+/−</sup> leads to an alteration in the migratory response of B cells to SDF-1.

Reduced levels of Pten protein in Pten<sup>+/−</sup> B lymphocytes

Pten heterozygosity could lead to differences in lymphocyte migration owing to a reduction of levels of Pten, an important negative regulator of PI3K-mediated signal transduction pathways. To confirm that Pten<sup>+/−</sup> mice did in fact show a reduced amount of Pten protein in primary B lymphocytes in vivo, immunoblot analyses on purified populations of primary lymphocytes were conducted (Fig. 2 and data not shown). Lymphocytes from Pten<sup>+/−</sup> mice revealed an ~50% reduction in Pten as compared with lymphocytes from wild-type littermate controls. Protein levels of Pten have been shown to be regulated by phosphorylation and protein degradation (30, 31) as well as by promoter methylation (32). Thus, it was important to confirm that Pten levels did not change throughout the time course of SDF-1 stimulation. For all time points, Pten levels in Pten<sup>+/−</sup> cells remained at about half those of wild-type controls (data not shown).

Sustained SDF-1-induced PKB phosphorylation in Pten<sup>+/−</sup> B lymphocytes

To investigate a potential mechanism by which Pten heterozygosity leads to enhanced chemotaxis in B lymphocytes, we examined two signaling events lying downstream of SDF-1, PKB, and MAPK phosphorylation. Chemotaxis was abrogated when 1500 ng/ml SDF-1 was added to B cells in the presence of 100 nM wortmannin, indicating that the chemotactic response of both Pten<sup>+/−</sup> and control B cells was PI3K-dependent (Fig. 1b). Hypothesizing that differences in the activation of the PI3K pathway might account for the differential response of the Pten<sup>+/−</sup> and
control B lymphocytes to SDF-1, we examined PKB phosphorylation, an event reflective of PI3K activation. It has been demonstrated that PKB phosphorylation at Ser473 correlates with the kinase activity in vitro (14, 33, 34). We observed an increased level of phosphorylation of PKB on Ser473 upon stimulation of B cells with 500 ng/ml SDF-1, compared with controls (Fig. 3a). This SDF-1 concentration was selected so that differences in intracellular signaling events occurring within minutes of SDF-1 stimulation could be readily evaluated. An alteration in the kinetics of PKB phosphorylation was also evident in Pten+/− B cells, which showed a prolonged phosphorylation response to SDF-1 as compared with wild-type cells. To quantitate the differential response of Pten+/− and control cells to SDF-1, densitometric analysis of multiple independent experiments was conducted (Fig. 3b).

FIGURE 1. Pten+/− B cells show increased sensitivity to SDF-1. a, Dose response of Pten+/− B cells to SDF-1. Purified splenic B cells (1 × 10⁶ per well) were evaluated for their ability to migrate from the upper chamber toward SDF-1 at indicated concentrations in the lower chamber. After 3 h, cells in the lower chamber were counted by flow cytometry. Results represent pooled data from four independent splenic B cell isolations and transmigration experiments. b, Purified splenic B cells were examined for their ability to transmigrate from the upper chamber toward media with or without addition of 1500 ng/ml SDF-1 in the lower chamber. Results represent pooled data obtained from five independent splenic B cell isolations with each transmigration experiment performed in duplicate as a minimum. For the inhibitor study, 100 nM wortmannin was added to both the lower and upper chambers and results shown are triplicates from one splenic B cell isolation. c, Purified splenic B cells were examined for their ability to transmigrate from the upper chamber in the presence or absence of an SDF-1 gradient. Cells were placed in the top chamber in the absence of SDF-1 and allowed to migrate toward media with (1500 bottom) or without SDF-1 (0). For the assessment of chemotaxis in the absence of an SDF-1 gradient, cells were resuspended in media containing 1500 ng/ml SDF-1 and placed in the top chamber and allowed to migrate toward media containing 1500 ng/ml SDF-1 (1500 top/bottom). Results represent pooled data obtained from at least three independent splenic B cell isolations with each transmigration experiment performed in triplicate.

Table I. Chemotaxis of primary B cells from Pten+/− mice

<table>
<thead>
<tr>
<th>Gradient</th>
<th>+/+</th>
<th>SEM</th>
<th>n/a</th>
<th>+/-</th>
<th>SEM</th>
<th>n/a</th>
<th>t Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>118</td>
<td>24</td>
<td>19</td>
<td>361</td>
<td>65</td>
<td>22</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>SDFb</td>
<td>688</td>
<td>211</td>
<td>20</td>
<td>1871</td>
<td>329</td>
<td>22</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

a Pooled data from five independent B cell isolations.
b 1500 ng/mL SDF.
As with PKB, MAPK also becomes phosphorylated following SDF-1 stimulation, subsequent to the activation of receptor-associated G proteins (8). Therefore, we assessed the phosphorylation status of p44/42 MAPK in B lymphocytes using phospho-specific Abs. SDF-1 stimulation resulted in MAPK phosphorylation in Pten−/− B lymphocytes; however, when compared with wild-type cells, no reproducible difference in either the level or kinetics of phosphorylation was evident. Representative immunoblots are shown in Fig. 4a. Densitometric analyses from multiple experiments revealed that Pten−/− cells did not exhibit any significant differences in phosphorylation of MAPK as compared with wild-type cells (Fig. 4b). These results support the idea that the differential response of Pten−/− B lymphocytes to SDF-1 is linked to dysregulation of PI3K-dependent events, including PKB phosphorylation.

**Discussion**

We present evidence of a role for Pten in the regulation of SDF-1-induced lymphocyte chemotaxis. The enhanced response of Pten−/− B lymphocytes to SDF-1 was associated with increased and sustained phosphorylation of PKB, one of the key molecules downstream of the products of PI3K activity. Our results suggest that Pten may be an important negative regulator of SDF-1-stimulated cell migration.

SDF-1 is known to activate multiple signal transduction pathways including receptor-associated trimeric G proteins, phospholipase C and protein kinase C, PI3K and PKB, small G proteins, and specific protein tyrosine kinase pathways (8–10, 35). SDF-1-induced chemotaxis is a complex and coordinated phenomenon that involves polarized localization and activation of signaling molecules such as PKB (36). These events in turn lead to increased intracellular Ca2+ levels, cytoskeletal reorganization, and ultimately, the chemotactic response of the cell. Tyrosine phosphorylation of multiple focal adhesion proteins such as RAFTK/Pyk2, p130Cas, Paxillin, FAK, CrkL, and Crk is also seen following SDF-1 stimulation (37). Human PTEN has been implicated in the dephosphorylation of some of these intermediates, such as FAK.

**FIGURE 2.** Pten−/− B lymphocytes have decreased levels of Pten protein. Splenic B cells from three separate litters (A–C) were isolated and immunoblot analyses with a mAb against PTEN were conducted. Littermate sex-matched controls for each genotype were analyzed. The numbers below the blots represent relative densitometry units for each band.

**FIGURE 3.** Pten−/− B cells show increased phosphorylation of PKB in response to SDF-1 stimulation. Purified splenic B cells from Pten−/− and sex-matched littermate controls were stimulated with 50 μg/ml SDF-1 for the indicated times. a, Representative blots from parallel immunoblot analyses using either a phospho-specific Ab for PKB (Ser473) or an Ab against PKB. b, Densitometric analysis of autoradiographs derived from four independent experiments. In each case, splenic B cell isolations consisted of two mice pooled per experiment. Results have been normalized for maximal PKB phosphorylation, as determined by percentage of PKB phosphorylated at 2 min in Pten−/− cells, and represent the percentage of phosphorylated PKB relative to parallel loading controls. Bars represent mean and SEM percentage of maximal PKB phosphorylation upon SDF-1 stimulation (p < 0.002).

**FIGURE 4.** Pten−/− B cells show no significant difference in MAPK phosphorylation following SDF-1 stimulation. Representative blots from parallel immunoblot analyses using either a phospho-specific Ab for p42/p44 MAPK or an Ab against MAPK. a, Splenic B cells were pooled from two mice per group for each isolation, and four independent experiments were conducted. b, Densitometric analysis derived from three independent experiments. Results were normalized relative to the amount of MAPK phosphorylated at 2 min in Pten−/− cells, and represent the percentage of phosphorylated MAPK relative to parallel loading controls. Bars represent mean and SEM percentage of maximal MAPK phosphorylation upon SDF-1 stimulation.
and p130Cas, suggesting a role for the protein tyrosine phosphatase domain of PTEN in the regulation of integrin-mediated cell adhesion (24, 25). In addition to the activation of protein kinase signaling pathways, SDF-1 also causes an accumulation of P(l3,4,5)P3 in the membrane and SDF-1-induced chemotaxis is fully dependent on PI3K activation (38). Because Pten has been implicated as an important negative regulator of the PI3K pathway, the link between SDF-1 and Pten could be through dysregulation of signaling pathways dependent on P(l3,4,5)P3 and P(l3,4)P2 in Pten−/− cells.

Using specific inhibitors, it has been shown that the PI3K pathway, but not the MAPK pathway, is required for both SDF-1-induced phosphorylation of focal adhesion proteins and SDF-1-induced migration (37). Also, consistent with the importance of induced phosphorylation of focal adhesion proteins and SDF-1-way, but not the MAPK pathway, is required for both SDF-1-fl CD4+/H11032 CD4+ lymphocytes lacking the 5′ induced migration (37). Also, consistent with the importance of induced phosphorylation of focal adhesion proteins and SDF-1-way, but not the MAPK pathway, is required for both SDF-1-fl CD4+/H11032 CD4+ lymphocytes lacking the 5′ induced migration (37). Also, consistent with the importance of induced phosphorylation of focal adhesion proteins and SDF-1-way, but not the MAPK pathway, is required for both SDF-1-fl CD4+/H11032 CD4+ lymphocytes lacking the 5′ induced migration (37).

The chemotaxis of Pten−/− B lymphocytes that we observed indicated that reduced Pten levels were associated with an increased responsiveness to SDF-1, implicating Pten in the control of lymphocyte migration to this chemokine. In Pten−/− B cells, Pten levels were about half those of wild-type controls. We hypothesized that the reduced levels of Pten were accompanied by a decrease in Pten activity. Other studies support the hypothesis that Pten heterozygosity leads to haploinsufficiency (39).

We found that phosphorylation of MAPK remained unchanged. Although there is some debate as to whether Pten is able to regulate MAPK phosphorylation (40–44), we observed no reproducible difference in MAPK phosphorylation in Pten−/− B lymphocytes exposed to SDF-1. Taken together, our results suggest that the differential response of Pten−/− B lymphocytes to SDF-1 is linked to dysregulation of PI3K dependent events, including PKB phosphorylation.

The PKB PH domain rapidly and transiently translocates to the plasma membrane upon chemoattractant stimulation and is found at the leading edge in migrating cells (36). It has been proposed that activation of PI3K at the leading edge leads to the formation of P(l3,4,5)P3- and P(l3,4)P2-enriched lipid domains that function as docking sites for diverse PH domain containing proteins. This results in a clustering of these signaling proteins, which leads to the formation of a new pseudopod and directed cellular movement (45, 46). In addition to this pivotal role at the leading edge of PKB, PKB has also been implicated in the regulation of chemotaxis through its ability to cause the phosphorylation of G protein-coupled receptors (47). Although the increased phosphorylation of PKB in Pten−/− cells suggests a plausible mechanism for the enhanced response of these cells to SDF-1, it is possible that other PH domain containing proteins are also involved.

Pten heterozygosity does not appear to initially compromise normal lymphoid development, as reflected in the normal ratios of CD4−, CD8−, and B220− lymphocytes in the lymph nodes of young Pten−/− mice (16). However, over time a progressive lymphoproliferative disorder develops in these mice (16). If Pten−/− lymphoid cells were abnormally sensitive to chemokines in vivo, it is plausible that the aberrant recruitment and accumulation of lymphoid cells would contribute to the lymphoproliferative disorder of Pten−/− mice.

It has been well-established that activation of PKB-dependent signaling pathways promote cell growth and survival (48). A complete lack of functional Pten, due to biallelic gene mutations that are frequently seen in tumors and tumor cell lines, and the resulting constitutive activation of PKB is thought to be a major contributing factor in tumorigenesis. In contrast, our observations suggest that even a reduction in Pten protein levels accompanying the loss of a single Pten allele might be able to confer a selective advantage on cells during multistep tumorigenesis. In keeping with the importance of this, chemokines have been implicated in tumor cell growth, angiogenesis, and metastasis (49); and recently, the SDF-1:CXCR4 axis has been shown to be involved in breast cancer metastasis (50). This latter study and others have found that CXCR4 is overexpressed in specific cancer cell lines and tumor samples as compared with normal tissues (50–52). Also, SDF-1 expression is high in tissues that are often targets for metastatic cells such as lung, liver, and lymph nodes (50). Pten−/− mice have been shown to develop malignant lymphomas that produce metastatic infiltrates of solid organs including the lung and the liver (16, 18, 19). It has also been shown that neutralization of CXCR4 in vivo inhibits tumor cell metastasis, suggesting that SDF-1 acts as an important attractive force in metastasis (50). Thus, our finding that Pten−/− cells have enhanced responsiveness to SDF-1 suggests a mechanism by which loss of a single Pten allele would be under selective pressure. For example, Pten−/− cells might have an increased ability for directed migration toward favorable microenvironments, thus promoting tumor progression.

Acknowledgments
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References
is essential but not sufficient for PKB activation; phosphatidylinositol3,4,5-P3 is required for PKB phosphorylation at Ser-473. Studies using cells from SHIP−/− knockout mice. J. Biol. Chem. 277:9027.


54. PTEN REGULATES SDF-1-INDUCED SIGNALING AND CHEMOTAXIS


