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Shaina N. Porter  
*Washington University School of Medicine in St. Louis*

Andrew S. Cluster  
*Washington University School of Medicine in St. Louis*

Robert A.J. Signer  
*University of California - San Diego*

Jenna Voigtmann  
*Washington University School of Medicine in St. Louis*

Darlene A. Monlish  
*Washington University School of Medicine in St. Louis*

*See next page for additional authors*

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**Pten** Cell Autonomously Modulates the Hematopoietic Stem Cell Response to Inflammatory Cytokines

Shaina N. Porter,1 Andrew S. Cluster,1 Robert A.J. Signer,2 Jenna Voigtmann,1 Darlene A. Monlish,1 Laura G. Schuettpelz,1 and Jeffrey A. Magee1,*

1Division of Pediatric Hematology and Oncology, Department of Pediatrics, Washington University School of Medicine, 660 South Euclid Avenue, Box 8220, St. Louis, MO 63110, USA
2Division of Regenerative Medicine, Department of Medicine, Moores Cancer Center, University of California, San Diego, La Jolla, CA 92039, USA

*Correspondence: magee_j@kids.wustl.edu
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**SUMMARY**

*Pten* negatively regulates the phosphatidylinositol 3-kinase (PI3K) pathway and is required to maintain quiescent adult hematopoietic stem cells (HSCs). *Pten* has been proposed to regulate HSCs cell autonomously and non-cell autonomously, but the relative importance of each mechanism has not been directly tested. Furthermore, the cytokines that activate the PI3K pathway upstream of *Pten* are not well defined. We sought to clarify whether *Pten* cell autonomously or non-cell autonomously regulates HSC mobilization. We also tested whether *Pten* deficiency affects the HSC response to granulocyte colony-stimulating factor (G-CSF) and interferon-α (IFNα) since these cytokines induce HSC mobilization or proliferation, respectively. We show that *Pten* regulates HSC mobilization and expansion in the spleen primarily via cell-autonomous mechanisms. *Pten*-deficient HSCs do not require G-CSF to mobilize, although they are hyper-sensitized to even low doses of exogenous G-CSF. *Pten*-deficient HSCs are similarly sensitized to IFNα. *Pten* therefore modulates the HSC response to inflammatory cytokines.

**INTRODUCTION**

The balance between hematopoietic stem cell (HSC) proliferation and quiescence is tightly regulated (He et al., 2009). Adult HSCs are usually quiescent, but their proliferation rates increase upon exposure to cytokines (Baldridge et al., 2010; Essers et al., 2009; Morrison et al., 1997; Schuettelpelz et al., 2014), bacterial infections (Rodríguez et al., 2009), and mutations that hyper-activate mitogenic pathways such as the phosphatidylinositol 3-kinase (PI3K) and Ras pathways (Kharas et al., 2010; Van Meter et al., 2007; Yilmaz et al., 2006; Zhang et al., 2006). Transient proliferation maintains the HSC pool and supports hematopoiesis, but sustained proliferation impairs HSC function and reduces long-term self-renewal capacity (Baldridge et al., 2010; Essers et al., 2009; Kharas et al., 2010; Rodríguez et al., 2009; Schuettelpelz et al., 2014; Van Meter et al., 2007; Yilmaz et al., 2006; Zhang et al., 2006). Adult HSCs therefore require mechanisms to promote quiescence.

The PI3K pathway regulates cell metabolism, survival, and proliferation (Luo et al., 2003), and it arbitrates the balance between adult HSC quiescence and proliferation (Lee et al., 2010; Magee et al., 2012; Yilmaz et al., 2006; Zhang et al., 2006). PI3K signal transduction increases in mouse HSCs after conditional *Pten* deletion (Lee et al., 2010; Magee et al., 2012; Yilmaz et al., 2006; Zhang et al., 2006). *Pten*-deficient HSCs proliferate at increased rates and mobilize to extramedullary organs such as the spleen. They are functionally impaired and only transiently reconstitute irradiated mice (Lee et al., 2010; Magee et al., 2012; Yilmaz et al., 2006; Zhang et al., 2006). Similar phenotypes have been observed with other PI3K-pathway-activating mutations, including *Tsc1* deletion, *Hpkb* deletion, or constitutive AKT expression (Gan et al., 2008; Kharas et al., 2010; Siegemund et al., 2015). The effects of *Pten* deletion on HSC proliferation and function are mediated by the kinase mammalian target of rapamycin (mTOR) via two complexes, mTORC1 and mTORC2 (Kalaizidis et al., 2012; Lee et al., 2010; Magee et al., 2012; Yilmaz et al., 2006), mTOR impairs self-renewal by inducing p16 and p53 expression (Lee et al., 2010) and by inducing aberrantly high rates of protein synthesis (Signer et al., 2014). Together, these findings show that *Pten* is crucial for inhibiting the PI3K/mTOR pathway in HSCs, and sustained activation of the pathway compromises HSC function.

*Pten* has been proposed to regulate HSCs cell autonomously and non-cell autonomously (Tesio et al., 2013; Yilmaz et al., 2006). Several studies have noted that *Pten*-deficient HSCs have elevated PI3K pathway activity, consistent with a cell-autonomous function (Kalaizidis et al., 2012; Lee et al., 2010; Magee et al., 2012; Signer et al., 2014). However, *Pten*-deficient myeloid cells have recently been shown to express high levels of granulocyte colony-stimulating factor (G-CSF) and other pro-inflammatory cytokines that can mobilize HSCs and deplete the bone marrow HSC pool (Tesio et al., 2013). *Csf3r*-deficient mice (null for G-CSF) had reduced HSC mobilization following conditional *Pten* deletion, suggesting that *Pten* expression in myeloid cells non-cell autonomously regulates HSCs by...
suppressing G-CSF production (Tesio et al., 2013). These cell-autonomous and non-cell-autonomous mechanisms are not mutually exclusive, but their relative importance to HSC regulation has not been directly tested. Furthermore, a primary conclusion by Tesio et al. was that Pten lacks any HSC-autonomous function, in contrast to prior conclusions (Yilmaz et al., 2006; Zhang et al., 2006).

Since Pten regulates many cellular processes that are thought to be relevant for HSC self-renewal—including PI3K signal transduction, metabolism, protein synthesis, and proliferation—we sought to clarify whether it functions primarily via cell-autonomous or non-cell-autonomous mechanisms. Furthermore, we tested whether Pten interacts with two cytokines that are known to induce HSC proliferation and mobilization: G-CSF and interferon-α (IFNα). We found that Pten regulates HSC mobilization and expansion in the spleen primarily via cell-autonomous mechanisms, although non-cell-autonomous effects were also observed. In our hands, Csβ deletion did not impair Pten-deficient HSC mobilization, in contrast to prior findings (Tesio et al., 2013). However, Pten-deficient HSCs were hyper-sensitized to G-CSF such that even low doses of the cytokine caused a marked increase in spleen HSCs. Higher G-CSF doses almost completely depleted Pten-deficient HSCs from the bone marrow without further expanding the spleen HSC pool. Pten-deficient HSCs were also sensitized to mobilizing effects of IFNα. Both G-CSF and IFNα hyper-activated the PI3K pathway in Pten-deficient HSCs. Together, our data show that Pten regulates HSCs by acting downstream rather than upstream of inflammatory cytokines, and it modulates the HSC response to G-CSF and IFNα.

RESULTS

Pten Cell Autonomously Suppresses Expansion of Spleen HSCs

To test whether Pten cell autonomously or non-cell autonomously regulates HSC mobilization and expansion, we transplanted 300,000 Cre− control or Ptenf/f;Mx1-Cre bone marrow cells (CD45.2) and 300,000 wild-type competitor cells (CD45.1) into irradiated CD45.1 recipient mice (Figure 1A). Six weeks after the transplants we administered pIpC to 6-week-old control and Ptenf/f;Mx1-Cre mice (which lack Pten in all hematopoietic cells) and G-CSF-treated wild-type mice (3.8 μg/mouse twice daily for 7 days). Ptenf/f;Lyz-Cre mice had only modest increases in spleen weights, spleen HSC frequencies, and spleen HSC numbers relative to controls. They had no change in bone marrow HSC frequencies despite efficient, myeloid-specific Pten deletion (Figures 1E–1G and S1). In contrast, Ptenf/f;Mx1-Cre and G-CSF-treated mice had much larger increases in spleen HSC numbers and a concomitant reduction in bone marrow HSCs (Figures 1F and 1G). These findings again show that the non-cell-autonomous effects of Pten deletion on HSCs are small when compared with the cell-autonomous effects.

Pten Deficiency Enhances G-CSF-Induced HSC Mobilization and Depletion

G-CSF has been proposed as a key mediator of Pten-deficient HSC mobilization, but systemic G-CSF levels have not been previously measured in Pten-deleted mice. To better understand how G-CSF levels change following Pten deletion, we gave pIpC to 6-week-old control and Ptenf/f;Mx1-Cre mice (3 doses, 10 μg/dose every other day), and we measured serum G-CSF levels 14 days later. As a positive control, we administered lipopolysaccharide (LPS) to wild-type mice as previously described (Boettcher et al., 2014). LPS has been shown to induce G-CSF expression in endothelial cells (Boettcher et al., 2014; Burberry et al., 2014). We found that Ptenf/f;Mx1-Cre mice had significantly elevated G-CSF levels relative to controls, but the levels were much lower than those observed in LPS-treated mice (Figure 2A). This raised the question of whether low G-CSF levels are necessary and/or sufficient to mobilize Pten-deficient HSCs.

To test whether G-CSF is necessary to mobilize Pten-deficient HSCs, we administered pIpC to: (1) Csf3−/−, (2) Csf3+/−, (3) Ptenf/f;Csf3−/−;Mx1-Cre, and (4) Ptenf/f;Csf3−/−;Mx1-Cre littermates. After 14 days we measured bone
marrow and spleen myeloid cell frequencies and HSC numbers. As expected, Pten\(^{fl/fl}\);Csf3\(^{+/−}\);Mx1-Cre (G-CSF null) mice had significantly lower bone marrow and spleen myeloid cell frequencies as compared with Pten\(^{fl/fl}\);Csf3\(^{+/+}\);Mx1-Cre (G-CSF heterozygous) mice (Figures 2B and S2A), consistent with the established role for G-CSF in myelopoiesis. In contrast, Pten\(^{fl/fl}\);Csf3\(^{+/−}\);Mx1-Cre and Pten\(^{fl/fl}\);Csf3\(^{−/−}\);Mx1-Cre had similar bone marrow and spleen HSC numbers following Pten deletion, and spleen HSC numbers were greatly expanded in both genotypes of mice relative to Cre\(^{−/−}\) controls (Figures 2C and S2B). Thus, G-CSF is not required to mobilize Pten-deficient HSCs to the spleen.

We next tested whether Pten-deficient HSCs are hypersensitive to the mobilizing effects of G-CSF, even though G-CSF is not absolutely required for mobilization. We deleted Pten from 6-week-old mice, and 2 weeks later we began a 7 day course of vehicle, low-dose (0.5 μg/day) or high-dose (5 μg/day) G-CSF. We then measured bone marrow and spleen HSC numbers. Low-dose G-CSF treatment caused a dramatic increase in spleen HSC numbers in Pten-deleted mice (Figure 2D). High-dose G-CSF treatment almost completely depleted the bone marrow HSC pool in Pten-deleted mice without further increasing spleen HSC numbers (Figures 2D and 2E). These effects of G-CSF were far more severe than those observed in wild-type mice (Figures 2D and 2E). Interestingly, Rictor deletion did not impair HSC mobilization at either G-CSF dose (Figures 2D and 2E). Thus, Pten modulates the HSC response to G-CSF, but the PI3K pathway (or at least mTORC2) is not required for physiologic HSC mobilization.
Figure 2. *Pten*-Deficient HSCs Do Not Require G-CSF to Mobilize to the Spleen, yet They Are Hyper-Sensitive to Its Effects

(A) Serum G-CSF concentrations in control, *Pten*<sup>fl/fl</sup>;Mx1-Cre, and LPS-treated mice; n = 4–9 per genotype or treatment.

(B and C) *Csf3* deletion significantly reduced spleen myeloid cell frequencies after *Pten* deletion (B), but it did not affect spleen HSC numbers (C); n = 5–9 per genotype.

(D) *Pten* deletion with Mx1-Cre and low-dose G-CSF synergistically expanded the spleen HSC population. *Pten* deletion with Lyz-Cre had only minimal effects on spleen HSC numbers. *Rictor* deletion did not impede G-CSF mediated HSC mobilization.

(E) *Pten* deletion with Mx1-Cre in conjunction with G-CSF treatment (5 μg/day for 7 days) almost completely depleted the bone marrow HSC pool. *Pten* deletion with Lyz-Cre had no effect on bone marrow HSCs. *Rictor* deletion prevented G-CSF-mediated depletion of the bone marrow HSC pool.

(F) *Pten* deletion enhanced HSC mobilization to the peripheral blood following treatment with low-dose G-CSF (0.5 μg/day for 5 days); n = 4–6.

(G) *Pten* deletion enhanced mobilization of AMD3100-treated HSCs; n = 4–8.

(H) Western blot of 30,000 sorted HSC/MPPs. G-CSF treatment caused increased phosphorylation of AKT and S6, but only in *Pten*-deficient HSCs/MPPs.

(legend continued on next page)
G-CSF induces physiologic HSC mobilization by binding the G-CSF receptor on monocyte-macrophage lineage cells that, in turn, modulate stromal levels of CXCL12 (Christopher et al., 2011; Day et al., 2015; Liu et al., 2000). We therefore tested whether Pten-deficient myeloid cells could indirectly hyper-sensitize HSCs to G-CSF. We administered vehicle, low-, and high-dose G-CSF to Pten



 after birth (2 weeks after pIpC) We then measured periph-



vehicle or low-dose G-CSF for 5 days beginning at 8 weeks



(D and E) #p < 0.05 for 0.5 g/day and 0 µg/day G-CSF for the indicated genotypes; ##p < 0.05 for 5 mg/day G-CSF versus 0.5 µg/day and 0 µg/day G-CSF for the indicated genotypes; n = 4–16.



mTOR pathway. This observation is consistent with prior data showing that mobilization and extramedullary expansion of Pten-deficient HSCs requires mTORC2 (Magee et al., 2012), while physiologic mobilization does not (Figure 2D).



Pten Deficiency Enhances pIpC-Induced HSC Mobilization

In light of the observed interaction between Pten and G-CSF, we tested whether Pten also modulates the HSC response to IFNz. This hypothesis was based on prior data showing that IFNz induces AKT phosphorylation in less-pure progenitor populations (Essers et al., 2009) and recognition of the fact that interferon signaling, like Pten deletion, drives HSCs into cycle and depletes the HSC pool (Baldridge et al., 2010; Walter et al., 2015). Furthermore, Pten



expression; n = 4–6.

(K) G-CSF, but not Pten deletion, stimulated MT1-MMP expression; n = 4–5.

For all panels, error bars reflect SDs and p values were calculated by two-tailed Student’s t test; *p < 0.05, **p < 0.01, ***p < 0.001; (D and E) #p < 0.05 for 0.5 µg/day G-CSF versus 0 µg/day G-CSF for the indicated genotypes; ##p < 0.05 for 5 mg/day G-CSF versus 0.5 µg/day and 0 µg/day G-CSF for the indicated genotypes; n = 4–16.

(I and J) Pten deletion did not cause differences in CXCR4, CD49d, or CD44 expression in HSCs (I), while G-CSF treatment caused increased CXCR4 and CD44 expression in mobilized spleen HSCs (J); n = 3–6.

(K) G-CSF, but not Pten deletion, stimulated MT1-MMP expression; n = 4–5.
raised the question of whether plpC treatment contributes to the phenotypes observed in Ptenf/f;Mx1-Cre mice.

We first tested whether plpC activates the PI3K pathway in wild-type and Pten-deficient HSCs. We gave three doses of plpC (10 μg/dose) to 6-week-old wild-type and Ptenf/f; Mx1-Cre mice to delete Pten. Two weeks later, we treated the mice with vehicle or a single additional 10 μg dose of plpC to re-induce IFNα. We isolated HSC/MPPs 24 hr later and performed western blots to assess PI3K pathway activity. HSC/MPPs from wild-type mice that received the additional plpC dose (hereafter called plpC-treated) had high AKT and S6 phosphorylation relative to controls that did not receive the additional dose (Figure 3A). S6 phosphorylation was even higher in plpC-treated, Pten-deficient HSC/MPPs (Figure 3A). STAT1, a known IFNα target, was also more highly phosphorylated in plpC-treated HSC/MPPs, consistent with prior studies (Essers et al., 2009). These data show that IFNα induces PI3K/mTOR signaling in HSCs and that its effect on mTORC1/S6K signaling is greater in Pten-deficient HSCs than in wild-type HSCs.

We next devised a strategy to delete Pten without plpC so that we could characterize the interaction between Pten and IFNα independently of Cre induction. We isolated HSCs from E16.5 Ptenf/f mice and treated half of the cells with Tat-Cre for 30 min ex vivo to delete Pten. Two weeks later, we treated the mice with vehicle or a single additional 10 μg dose of plpC to re-induce IFNα. We isolated HSC/MPPs 24 hr later and performed western blots to assess PI3K pathway activity. HSC/MPPs from wild-type mice that received the additional plpC dose (hereafter called plpC-treated) had high AKT and S6 phosphorylation relative to controls that did not receive the additional dose (Figure 3A). S6 phosphorylation was even higher in plpC-treated, Pten-deficient HSC/MPPs (Figure 3A). STAT1, a known IFNα target, was also more highly phosphorylated in plpC-treated HSC/MPPs, consistent with prior studies (Essers et al., 2009). These data show that IFNα induces PI3K/mTOR signaling in HSCs and that its effect on mTORC1/S6K signaling is greater in Pten-deficient HSCs than in wild-type HSCs.

We next devised a strategy to delete Pten without plpC so that we could characterize the interaction between Pten and IFNα independently of Cre induction. We isolated HSCs from E16.5 Ptenf/f mice and treated half of the cells with Tat-Cre for 30 min ex vivo to delete Pten (Figure 3B). We transplanted control or Tat-Cre-treated HSCs into irradiated recipients (350 HSCs and 300,000 competitor bone marrow cells per recipient). Unlike adult HSCs, Pten-deficient fetal HSCs were able to reconstitute, consistent with previously published findings (Magee et al., 2012). Six weeks after the transplants we administered plpC to half of the mice (three doses, 10 μg/dose every other day). Two weeks later we measured spleen cellularity and HSC frequencies. plpC caused a significant increase in spleen cellularity and spleen HSC frequency in recipients of Tat-Cre-treated HSCs, but bone marrow HSC frequencies did not differ between the treatment groups (Figures 3C and 3D). Spleen HSC numbers were significantly increased in recipients of Tat-Cre-treated HSCs, and this value further increased after plpC treatment (Figure 3E). Thus, Pten modulates the...
HSC response to IFNα, much like it modulates the response to G-CSF.

**DISCUSSION**

We have conducted experiments to clarify whether Pten regulates HSCs cell autonomously or non-cell autonomously. Our data confirm that Pten regulates HSCs via both cell-autonomous and non-cell-autonomous mechanisms (Figure 1). The data also show that the cell-autonomous effects of Pten deletion are significantly greater than the non-cell-autonomous effects (Figure 1). While our analyses of Pten$^{−/−}$/Csf3$^{−/−}$;Mx1-Cre mice do not support an obligate role for G-CSF in HSC mobilization (Figure 2C), we have found that Pten-deficient HSCs are hyper-sensitive to the mobilizing and depleting effects of G-CSF (Figures 2D and 2E). This hyper-sensitivity does not appear to reflect enhancement of normal physiologic mobilization mechanisms. Rather, G-CSF enhances PI3K/mTOR pathway activation in HSCs that lack Pten (Figure 2H). This could occur through direct engagement of the G-CSF receptor or through indirect activation of other cytokines in the microenvironment. Pten-deficient HSCs also hyper-activate mTORC1/56K and mobilize in response to IFNα (Figure 3). Together, these data suggest that Pten modulates the HSC response to inflammatory cytokines, and pIpC contributes to the HSC proliferation, mobilization, and self-renewal phenotypes that have been widely described using Pten$^{−/−}$/Mx1-Cre mice (Kalaitzidis et al., 2012; Lee et al., 2010; Magee et al., 2012; Signer et al., 2014; Yilmaz et al., 2006; Zhang et al., 2006).

Our data potentially link two major modes of HSC regulation—the PI3K pathway and pro-inflammatory cytokines—as common causes of HSC depletion during illness or aging. The consequences of PI3K pathway activation in HSCs have been extensively studied (Gan et al., 2008; Kharas et al., 2010; Lee et al., 2010; Magee et al., 2012; Siegemund et al., 2015; Signer et al., 2014), but the signals that activate the pathway—either in native or stressed conditions—have not been well characterized. Likewise, inflammatory signals have drawn scrutiny for their putative role in bone marrow failure, HSC aging, and pre-leukemic clonal evolution (Baldrige et al., 2010, 2011; Essers et al., 2009; Walter et al., 2015), but the downstream mechanisms by which these signals deplete HSCs have not been fully resolved. Our data suggest a model in which inflammatory cytokines hyper-activate the PI3K pathway in HSCs leading to increased protein synthesis and tumor-suppressor expression, which ultimately depletes the HSC pool. Ongoing experiments will test whether mTORC1 or mTORC2 inactivation can preserve the function of cytokine-stimulated HSCs. If so, mTOR inhibitors may have a role in sustaining the HSC pool in patients with inflammation and otherwise tenuous HSC function (e.g., bone marrow transplant patients with complicating graft-versus-host disease or infections).

**EXPERIMENTAL PROCEDURES**

**Mouse Strains and Injections**

The Pten$^{−/−}$, Rictor$^{−/−}$, Mxl-Cre, and Lzy-Cre strains have been described previously and are available from The Jackson Laboratory (Claussen et al., 1999; Groszer et al., 2006; Kuhn et al., 1995; Magee et al., 2012). Expression of Mxl-Cre was induced by three intraperitoneal injections of pIpC (GE Life Sciences; 10 μg/dose) over 5 days beginning 6 weeks after birth. Recombinant human G-CSF (Amgen) was given subcutaneously at the doses noted in the text and figure legends. AMD3100 (Genzyme) was given subcutaneously at 5 mg/kg 2 hr before euthanizing the mice, as previously described (Devine et al., 2008). All mice were housed in the Department for Comparative Medicine at Washington University. All animal procedures were approved by the Washington University Committees on the Use and Care of Animals.

**Isolation of HSCs, Flow Cytometry, and HSC Transplantation**

HSCs were isolated, analyzed, and transplanted as described previously (Magee et al., 2012), and as described in detail in the Supplemental Experimental Procedures.

**G-CSF Measurements**

Serum G-CSF levels were measured with the mouse G-CSF Quantikine ELISA Kit (R&D Systems). For positive controls, mice were injected with 35 μg of LPS at 72 and 24 hr prior to sacrifice, as described previously (Boettcher et al., 2014).

**Western Blots**

Western blots were performed as described previously (Magee et al., 2012) and in the Supplemental Experimental Procedures.

**Tat-Cre Treatments**

Fetal Pten$^{−/−}$ HSCs were sorted into 0.1 ml of Iscove’s modified Dulbecco’s medium with 2% fetal bovine serum and incubated with 30 units of Tat-Cre (Millipore) for 30 min at 37°C. A complete description of the optimization protocol is provided in the Supplemental Experimental Procedures (Figure S3).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.04.008.

**AUTHOR CONTRIBUTIONS**

S.N.P., A.S.C., J.V., and J.A.M. performed all experiments except the analysis of serum G-CSF levels and cell-cycle assays (performed by D.A.M. and L.G.S.) and analyses of Pten/Csf3 compound mutant mice (performed by R.A.J.S.). S.N.P., A.S.C., R.A.J.S., L.G.S., and
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J.A.M. designed experiments and interpreted the data. J.A.M. directed the study and wrote the manuscript.

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