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

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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; Schuettpelz et al., 2014), bacterial infections (Rodriguez 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; Rodriguez et al., 2009; Schuettpelz 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, *Itpkb* 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 (Kalaitzidis 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 (Kalaitzidis 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). *Csf3*-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, *Csf3* 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 $Pten^{f/f}$;*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 poly-inosine:poly-cytosine (pIpC) (3 doses, 10 μg/dose every other day) to delete *Pten*. We measured bone marrow and spleen $CD45.2^{+}$ and $CD45.1^{+}$ HSC ($CD150^{+}CD48^{-}$ $Lineage^{-}Sca1^{+}c\text{-}Kit^{+}$) frequencies 14 days after Cre induction. The bone marrow of recipient mice had similar numbers of donor $CD45.2^{+}$ and competitor $CD45.1^{+}$ HSCs irrespective of genotype (Figure 1B). In contrast, the spleens of $Pten^{f/f}$;*Mx1-Cre* recipients had a ~50-fold increase in donor HSCs after *Pten* deletion, relative to control recipients, while competitor spleen HSCs increased by only ~3-fold (Figure 1C). *Pten*-deficient myeloid cell ($CD11b^{+}Gr1^{+}$) frequencies increased in the spleens of recipient mice, but competitor myeloid cell frequencies did not change (Figure 1D). *Pten* therefore regulates expansion of the spleen HSC and myeloid populations primarily via cell-autonomous mechanisms, with only minor contributions from non-cell-autonomous mechanisms.

Pten has been shown to inhibit G-CSF expression in myeloid cells (Tesio et al., 2013), but it is not clear whether myeloid *Pten* expression is actually critical for suppressing HSC mobilization. To test this, we deleted *Pten* in myeloid cells with *Lysozyme M-Cre* (*Lyz-Cre*), and we measured bone marrow and spleen HSC frequencies at 8–10 weeks after birth. As positive controls we measured bone marrow and spleen HSCs in pIpC-treated $Pten^{f/f}$;*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). $Pten^{f/f}$;*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, $Pten^{f/f}$;*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 $Pten^{f/f}$;*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 $Pten^{f/f}$;*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) $Pten^{f/f}$;$Csf3^{+/-}$;*Mx1-Cre*, and (4) $Pten^{f/f}$;$Csf3^{-/-}$;*Mx1-Cre* littermates. After 14 days we measured bone

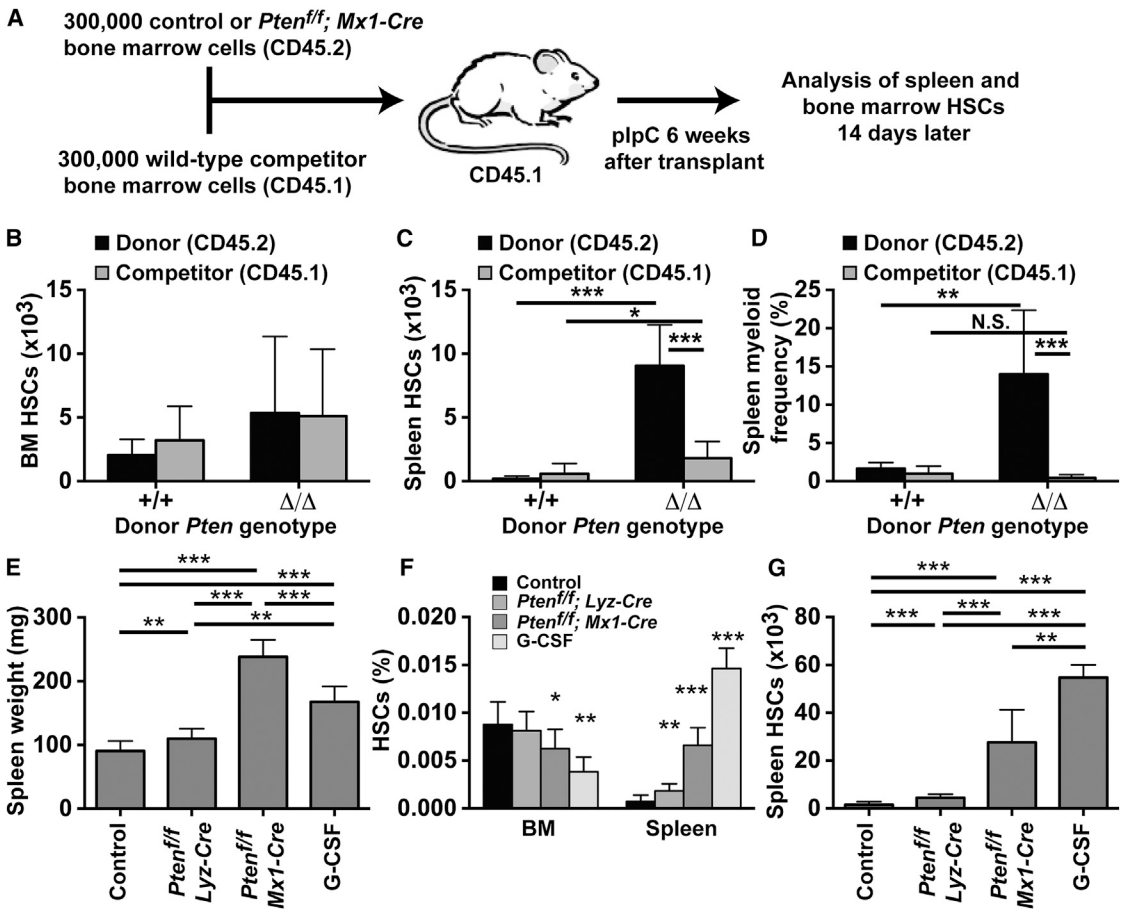

Figure 1. *Pten* Cell Autonomously Regulates HSC Mobilization to the Spleen

(A) Schematic overview of transplantation assay.

(B–D) Chimeric recipient mice had similar numbers of donor (CD45.2) and recipient (CD45.1) HSCs (B). *Pten* deletion expanded the donor spleen HSC ($CD150^+CD48^-LSK$) population to a greater extent than the recipient HSC population (C). Donor spleen $Cd11b^+Gr1^+$ myeloid cell frequencies were also disproportionately expanded following *Pten* deletion (D); n = 9 control, 11 *Pten* Δ/Δ.

(E–G) $Pten^{f/f}$;*Lyz-Cre* mice had significantly lower spleen weights (E), spleen HSC frequency (F), and spleen HSC numbers (G) when compared with $Pten^{f/f}$;*Mx1-Cre* and G-CSF-treated mice; n = 5–11 mice per genotype or treatment.

For all panels, error bars reflect SD and p values were calculated by two-tailed Student's t test; *p < 0.05, **p < 0.01, ***p < 0.001.

marrow and spleen myeloid cell frequencies and HSC numbers. As expected, $Pten^{f/f}$;$Csf3^{-/-}$;*Mx1-Cre* (G-CSF null) mice had significantly lower bone marrow and spleen myeloid cell frequencies as compared with $Pten^{f/f}$;$Csf3^{+/-}$;*Mx1-Cre* (G-CSF heterozygous) mice (Figures 2B and S2A), consistent with the established role for G-CSF in myelopoiesis. In contrast, $Pten^{f/f}$;$Csf3^{+/-}$;*Mx1-Cre* and $Pten^{f/f}$;$Csf3^{-/-}$;*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.

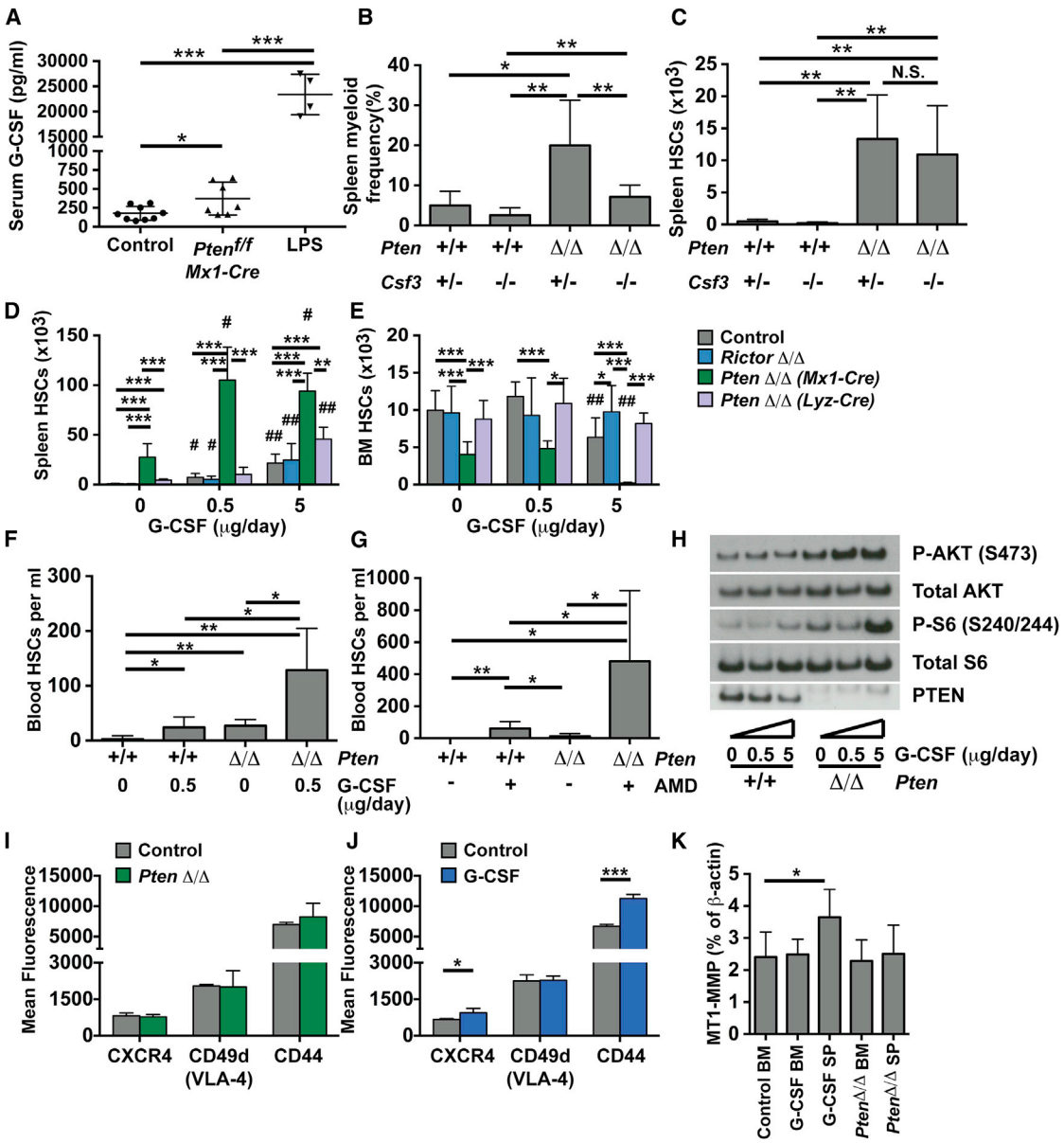

Figure 2. *Pten*-Deficient HSCs Do Not Require G-CSF to Mobilize to the Spleen, yet They Are Hyper-Sensitive to Its Effects

(A) Serum G-CSF concentrations in control, *Pten*$^{f/f}$;*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*$^{f/f}$*;Lyz-Cre* adult mice for 7 days, and we measured bone marrow and spleen HSC numbers. HSCs in *Pten*$^{f/f}$*;Lyz-Cre* mice were only modestly sensitized to G-CSF as evidenced by a small increase in spleen HSC numbers after high-dose treatment, relative to wild-type controls (Figure 2D). This change contrasted with the much greater effects of low- and high-dose G-CSF on *Pten*$^{f/f}$*;Mx1-Cre* HSCs (Figures 2D and 2E). These findings suggest that: (1) low concentrations of G-CSF can stimulate HSC mobilization and extramedullary expansion, (2) these effects are normally suppressed by *Pten*, and (3) the mechanisms are independent of PI3K pathway regulation in myeloid cells.

Pten-deficient HSCs proliferate in the spleen more than G-CSF-mobilized, wild-type HSCs (Figures S2C and S2D). The marked expansion of *Pten*-deficient spleen HSCs that we observed after G-CSF treatment could reflect increased extramedullary proliferation as opposed to increased mobilization. To better assess mobilization without the confounding effects of extramedullary proliferation, we splenectomized 5-week-old wild-type and *Pten*$^{f/f}$*;Mx1-Cre* mice. We deleted *Pten* 1 week later and administered vehicle or low-dose G-CSF for 5 days beginning at 8 weeks after birth (2 weeks after pIpC). We then measured peripheral blood HSC numbers by flow cytometry. *Pten* deletion and G-CSF treatment both independently and synergistically increased peripheral blood HSC numbers (Figure 2F). *Pten* deletion also synergistically enhanced HSC mobilization after treatment with the CXCR4 inhibitor AMD3100 (Figure 2G). Thus, *Pten* suppresses HSC mobilization in response to both low-dose G-CSF treatment and CXCR4 inhibition.

To better understand why *Pten*-deficient HSCs are hypersensitive to G-CSF, we analyzed PI3K pathway activity in wild-type and *Pten*-deficient HSCs and multipotent progenitors (HSC/MPPs; CD48^{-}LSK) after 2 days of treatment with vehicle, low-, or high-dose G-CSF. We assessed phosphorylation of AKT (an mTORC2 target) and S6 (an mTORC1 target) by western blotting. In wild-type HSC/MPPs, G-CSF had no effect on AKT or S6 phosphorylation at either dose (Figure 2H). In *Pten*-deficient HSC/MPPs, low-dose G-CSF increased AKT phosphorylation relative to untreated *Pten*-deficient HSC/MPPs, and high-dose G-CSF markedly increased S6 phosphorylation (Figure 2H). Thus, G-CSF hyper-activates the PI3K/mTOR pathway in *Pten*-deficient HSCs but not wild-type HSCs.

We tested whether *Pten* modulates the expression of surface proteins that are known to regulate HSC homing including CXCR4, VLA-4, and CD44 (Avigdor et al., 2004; Rettig et al., 2012). We also characterized expression of *MT1-MMP*, a matrix metalloproteinase that is expressed in G-CSF-stimulated HSCs in an mTOR-dependent manner and that promotes mobilization (Shirvaikar et al., 2010; Vagima et al., 2009). Whereas G-CSF treatment caused a modest increase in CXCR4 and CD44 expression as determined by flow cytometry (Figure 2J), *Pten* deletion had no effect on CXCR4, VLA-4, or CD44 expression (Figure 2I). Likewise, *MT1-MMP* expression was elevated in G-CSF-mobilized HSCs, consistent with prior studies (Shirvaikar et al., 2010; Vagima et al., 2009), but it was not changed in *Pten*-deficient HSCs (Figure 2K).

Our data suggest that G-CSF mobilizes and expands *Pten*-deficient spleen HSCs through mechanisms that are distinct from its normal physiologic activity. HSCs likely require *Pten* to keep low levels of G-CSF and other inflammatory cytokines from hyper-activating the PI3K/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 IFNα. This hypothesis was based on prior data showing that IFNα 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*$^{f/f}$*;Mx1-Cre* mice (which require pIpC-induced IFNα signaling to delete the *Pten* allele) have been shown to have more severe HSC phenotypes than tamoxifen-inducible *Pten*$^{f/f}$*;Scl-CreER* mice (Tesio et al., 2013). This

(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.
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.

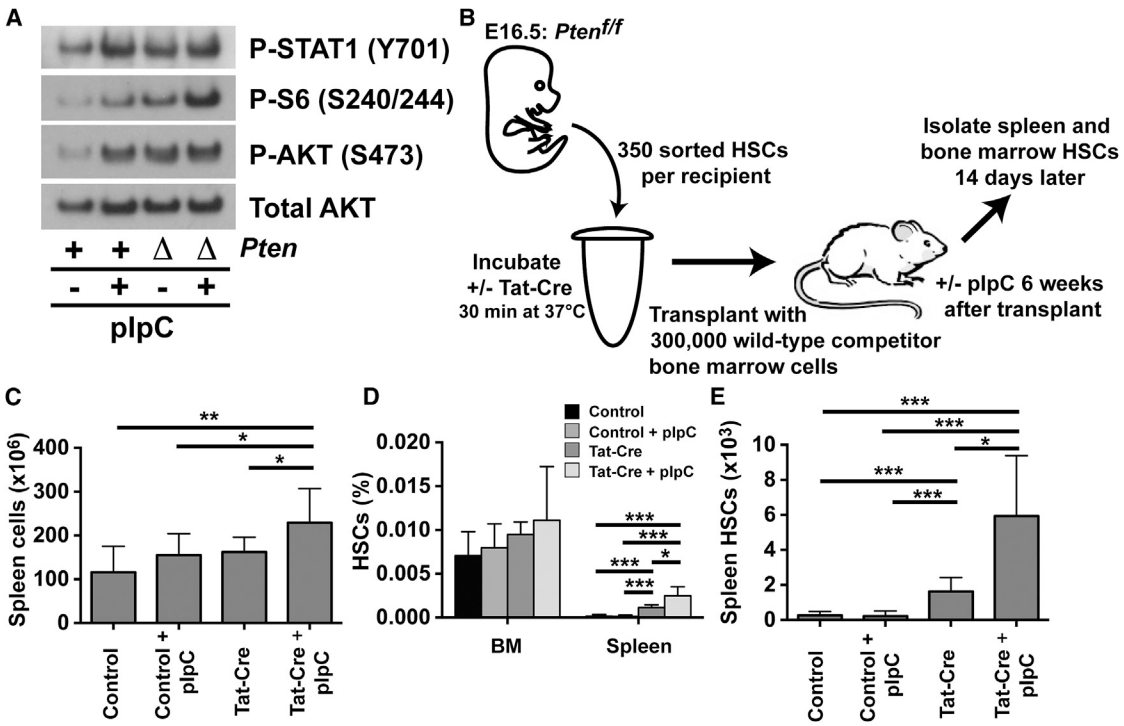

Figure 3. *Pten*-Deficient HSCs Are Mobilized by pIpC Treatment

(A) Western blot of 30,000 HSC/MPPs after pIpC treatment to delete *Pten* followed by a single vehicle or pIpC dose 2 weeks later to induce IFNα.

(B) Schematic overview for Tat-Cre-mediated deletion of *Pten* followed by pIpC treatment.

(C–E) pIpC treatment resulted in a significant increase in the spleen cellularity (C), spleen HSC frequency (D), and spleen HSC numbers (E) in recipients of Tat-Cre-treated $Pten^{f/f}$ HSCs. (C–E) n = 4–7 for each genotype and treatment.

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.

raised the question of whether pIpC treatment contributes to the phenotypes observed in $Pten^{f/f}$;*Mx1-Cre* mice.

We first tested whether pIpC activates the PI3K pathway in wild-type and *Pten*-deficient HSCs. We gave three doses of pIpC (10 μg/dose) to 6-week-old wild-type and $Pten^{f/f}$;*Mx1-Cre* mice to delete *Pten*. Two weeks later, we treated the mice with vehicle or a single additional 10 μg dose of pIpC 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 pIpC dose (hereafter called pIpC-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 pIpC-treated, *Pten*-deficient HSC/MPPs (Figure 3A). STAT1, a known IFNα target, was also more highly phosphorylated in pIpC-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 pIpC so that we could characterize the interaction between *Pten* and IFNα independently of Cre induction. We isolated HSCs from E16.5 $Pten^{f/f}$ 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 pIpC to half of the mice (three doses, 10 μg/dose every other day). Two weeks later we measured spleen cellularity and HSC frequencies. pIpC 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 pIpC 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*$^{f/f}$;*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 hyperactivate mTORC1/S6K 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*$^{f/f}$;*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 (Baldridge 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*$^{f/f}$, *Rictor*$^{f/f}$, *Mx1-Cre*, and *Lyz-Cre* strains have been described previously and are available from The Jackson Laboratory (Clausen et al., 1999; Groszer et al., 2006; Kuhn et al., 1995; Magee et al., 2012). Expression of *Mx1-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*$^{f/f}$ 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

J.A.M. designed experiments and interpreted the data. J.A.M. directed the study and wrote the manuscript.

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