Pten loss in the bone marrow leads to G-CSF–mediated HSC mobilization

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The phosphatase and tumor suppressor PTEN inhibits the phosphoinositol–3–kinase (PI3K) signaling pathway and plays a key role in cell growth, proliferation, survival, and migration. Pten conditional deletion using MxCre or Scl-CreER1 leads to splenomegaly and leukemia formation, which occurs after the relocation of normal hematopoietic stem cells (HSCs) from the bone marrow to the spleen. Unexpectedly, dormant HSCs in the bone marrow do not enter the cell cycle upon Pten loss, they do not lose self-renewal activity, and they are not exhausted. Instead, Pten deficiency causes an up-regulation of the PI3K pathway in myeloid cells, but not in HSCs. Strikingly, myeloid cells secrete high levels of G-CSF upon Pten loss, leading to the mobilization of HSCs from the bone marrow and accumulation in the spleen. After deletion of Pten in mice lacking G-CSF, the splenomegaly, myeloproliferative disease, and splenic HSC accumulation are rescued. Our data show that although PTEN has little if any role in normal HSCs, it is essential to prevent overt G-CSF production by myeloid and stromal cells which otherwise causes HSCs to relocate to the spleen followed by lethal leukemia initiation.
functions as a tumor suppressor by negatively regulating the Akt/PKB signaling pathway. It dephosphorylates the phospholipid PIP3 to produce PIP2, and thus it is a direct antagonist of PI3 kinase (PI3K). Previous studies have shown that both PI3K-AKT–dependent and –independent signaling pathways are regulated by PTEN (Vivanco et al., 2007; Gu et al., 2011; Kalaitzidis et al., 2012; Magee et al., 2012). Loss of PTEN function typically leads to an increase in PI3K signaling, causing hyperplasia and tumorigenesis such as glioblastoma, prostate cancer, or T cell leukemias (Knobbe et al., 2008; Song et al., 2012). Previous studies have proposed that the absence of PTEN activity promotes the generation of leukemic stem cells by driving unlimited self-renewal. In contrast to the leukemic situation, it was suggested that loss of Pten in the hematopoietic system leads to the apparent depletion of normal HSCs from the BM (Yilmaz et al., 2006; Zhang et al., 2006; Lee et al., 2010; Magee et al., 2012). Thus, it was proposed that PTEN plays opposite roles in normal HSCs and leukemic stem cells with respect to self-renewal, although the mechanism for this phenomenon still remains enigmatic. In this study, we use two conditional Pten loss-of-function mouse models to show that the mobilizing cytokine G-CSF is overproduced in the absence of Pten, which has widespread consequences for the balance of the hematopoietic system.

RESULTS
Conditional elimination of the Pten<sup>fl/fl</sup> allele by SCL-CreERT

Previous studies investigated the role of PTEN in HSCs using the Mxl-Cre (MxCre) model carrying the IFN-α inducible Mxl promoter, which is activated by injecting mice with polyinosine-polycytidine (pI-pC) to induce high levels of IFN-α. However, in this model, it cannot be excluded that the type I IFN signaling cascade, activated in parallel to Cre-mediated Pten deletion, might cross talk or synergize with the hematopoietic effects upon Pten loss (Essers et al., 2009). To circumvent this issue, we generated a new genetic mouse model where Pten deletion is driven by the tamoxifen (Tx)-inducible Scl-CreERT allele (Scl-Cre). In this model, the Scl-Cre allele efficiently recombines floxed alleles in hematopoietic stem/progenitors and (to a lesser degree) in endothelial cells (Fig. 1A; Göthert et al., 2005), thus allowing the assessment of PTEN function in HSCs independently of IFN-α.

Experimental (Scl-CreERT<sup>T</sup>;Pten<sup>fl/fl</sup>) and littermate control mice (Pten<sup>fl/fl</sup>) were fed with a Tx diet starting at 4–5 wk of age. As expected, the Pten gene and PTEN protein were eliminated from the BM and spleen of mice, which, after Cre induction are referred to as Pten<sup>Scl</sup> (Fig. 1B and not depicted).

We then compared our new Pten conditional KO model to the MxCre model in which MxCre:Pten<sup>fl/fl</sup> and littermate control mice (Pten<sup>fl/fl</sup>) were injected with pI-pC every other day for a total of five doses. As shown in Fig. 1 (B and C), an efficient elimination of Pten gene and PTEN protein was achieved in the BM and spleen of mice, which after

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**Figure 1.** Conditional elimination of the Pten<sup>fl/fl</sup> allele in the hematopoietic system using SCL-CreERT<sup>T</sup> and Mx-Cre. (A) Schematic diagram of the two conditional KO approaches and description about the tissue-specific recombination efficiency (right); +, >90%; O, <50%. (B) Western blot showing the efficiency of Pten elimination in BM and spleen. (C) Analysis of Pten expression in sections of bone and spleen using immunohistochemistry. Representative figures (bone, 1 cm = 250 µM; spleen, 1 cm = 500 µM) are shown. The data presented are representative of least three different independent experiments.

MxCre-mediated deletion are referred to Pten<sup>Mx</sup> (not depicted). Our results also indicate that the two conditional KO models delete Pten with a similar efficiency.

**Pten<sup>Scl</sup>** mice develop myeloproliferative disease (MPD) and T cell acute lymphoblastic leukemia/lymphoma (T-ALL)

In Pten<sup>Mx</sup> mice, lethality occurs, on average, 7.5 wk after the first pI-pC injection. In contrast, Pten<sup>Scl</sup> mice lived significantly longer with a median survival of 16.5 wk (Fig. 2A). Similar to what has been previously reported after MxCre-mediated Pten deletion, Pten<sup>Scl</sup> mice also displayed splenomegaly (Fig. 2C). Pathological analysis of the morphology of hematopoietic organs revealed extramedullary hematopoiesis and MPD in the spleen, and granulocytic hyperplasia in the bone marrow of both types of Pten mutant mice (Fig. 2D). In agreement with the MPD pathology, immature myeloid CD11b<sup>+</sup>/Gr<sup>+</sup> cells were increased in mutant spleens (Fig. 2D and E). Consistently, in these populations Pten was efficiently deleted in BM and spleen. As a consequence, increased phosphorylation...
Figure 2. Elimination of Pten using SCL-CreER\textsuperscript{T} causes premature lethality. (A) Kaplan-Meyer analysis showing lethality upon Pten deletion in Pten\textsuperscript{Mx\textsuperscript{lo}} and Pten\textsuperscript{Scl\textsuperscript{lo}} mice. P < 0.05, n = 6 mice each group. (B) Hematoxylin & Eosin (H&E)-stained histological sections of bone and spleen showing MPD in the spleen and granulocytic hyperplasia in the bone. R, red pulp; W, white pulp. Bars: (spleen) 200 µm; (bone) 20 µm. (C) Splenomegaly in Pten\textsuperscript{Mx\textsuperscript{lo}} and Pten\textsuperscript{Scl\textsuperscript{lo}} mice 4 and 7 wk after deletion, respectively. (D) Splenic CD11b\textsuperscript{+}Gr-1\textsuperscript{+} cells in control or Pten\textsuperscript{Scl\textsuperscript{lo}} mice. Three independent experiments, n = 7 controls and n = 6 mutants. ** indicates statistically significant change, P = 0.001. (E) Splenic CD11b\textsuperscript{+}Gr-1\textsuperscript{+} cells in control or Pten\textsuperscript{Mx\textsuperscript{lo}} mice. Three independent experiments, n = 8 each group. P = 0.029. (F–H) Pten, pAktTh308, pAktSer473, and pGSK3\betaSer9 levels in BM (top) and splenic (bottom) CD11b\textsuperscript{+}Gr-1\textsuperscript{+} cells.
of AKT (on Thr308 and Ser437) and GSK3β was detected in both models, consistent with an activated PI3K–AKT signaling pathway (Fig. 2, F–K; Cross et al., 1995).

Intriguingly, in contrast, to Pten<sup>Mx</sup> mice, all Pten<sup>Scl</sup> animals developed T-ALL as early as 12 wk after deletion (Fig. 3, A–D), similar to VEC-Cre–mediated Pten deletion in fetal liver HSCs (Guo et al., 2008). Transplantation of BM isolated from sick Pten<sup>Scl</sup> mice caused 100% of NOD/SCID recipients (5/5) to die of CD4<sup>+</sup> T-ALL after 3–6 wk (Fig. 3, B and C). In contrast, only 9% of the recipient mice (1/11) transplanted with the BM of sick Pten<sup>Mx</sup> mice developed T-ALL (Fig. 3 B). This indicates that Pten<sup>Mx</sup> BM also contains some premalignant or even malignant T-ALL clones, albeit at lower frequencies than in Pten<sup>Scl</sup> BM. The reduced T-ALL malignancy detected in Pten<sup>Mx</sup> mice may be related to the liver disease of Pten<sup>Mx</sup> mice early on, which likely prevents the development of a full-blown T-ALL leukemia. In summary, in addition to being an important negative regulator of myelopoesis, PTEN is predominantly a tumor suppressor for T-cell leukemia and Pten<sup>Scl</sup> animals serve as a novel highly penetrant model of T-ALL to identify interacting pathways and to design and test novel therapeutic approaches.

**HSC quiescence is not compromised in the BM of Pten<sup>Scl</sup> and Pten<sup>Mx</sup> mice**

We then analyzed the HSC compartment (defined here as lineage<sup>−</sup> Sca1<sup>+</sup> cKit<sup>+</sup> [LSK] CD34<sup>−</sup> CD135<sup>−</sup>) of both Pten<sup>Scl</sup> and Pten<sup>Mx</sup> mice. In agreement with previous reports (Chen et al., 2006; Yilmaz et al., 2006), we observed a decrease of BM HSCs over time in both Pten<sup>Scl</sup> and Pten<sup>Mx</sup> animals (Fig. 4 A). However, unexpectedly, no significant differences in the frequencies of quiescent (Ki67<sup>−</sup>) or cycling (BrdU<sup>+</sup>) and/or Ki67<sup>+</sup> HSCs were detected in either model (Fig. 4, B and E). Because these analyses only measure the immediate cell cycle status and may therefore not be sensitive enough to discriminate between infrequently dividing and dormant HSCs, we analyzed the frequency of dormant label retaining cells (LRCS; Wilson et al., 2008). After an initial 10-d labeling period, BrdU was removed and Pten<sup>Scl</sup> and control Pten<sup>fl/fl</sup> mice were switched onto a Tx diet for 21 d, followed by additional 49 d of chase. Analysis of the percentage of BrdU<sup>+</sup> LRCS cells within the HSC compartment revealed not a decrease as expected, but an increase of LRCS–HSCs in Pten<sup>Scl</sup> mice (Fig. 4 C). In summary, these data suggest that Pten deficiency does not alter the cell cycle distribution of primitive HSCs. Accordingly, the expression profile of control and Pten-deficient HSCs did not reveal significant differences in genes involved in cell cycle control (unpublished data). Finally, to further examine whether functional HSCs may show a differential proliferative behavior in the absence of Pten, mice were treated weekly with the chemotherapeutic agent 5-FU. As shown in Fig. 4 D, similar lethality kinetics in Pten<sup>Scl</sup>, Pten<sup>Mx</sup>, and control mice was observed, suggesting a similar proliferative index of HSC/P in Pten mutants and WT littermates. In conclusion, using the currently available methods to analyze the cell cycle of HSCs in vivo, we could not identify...
Figure 4. Phenotypic and functional analysis of Pten-deficient HSCs. (A) Kinetic analysis of the relative change of LSKCD34^-CD135^- cells in the BM of Pten^Mx and Pten^KO mice compared with controls (set to 100%). The asterisk indicates statistical significant change (P < 0.05). The cross indicates that the majority of mice do not survive beyond this time point. Each dot represents the mean value obtained from three to four mice. (B) FACS plots (top) and bar graph (bottom) showing the percentage of BrdU^+ cells within the LSKCD135^- population in BM and spleen after 90 min and in BM after.
any significant difference in cell cycle behavior or dormancy status between normal and Pten-deficient HSCs.

**Pten**<sup>ΔScl</sup> and **Pten**<sup>ΔMs</sup> HSCs are functional and retain basal Akt levels, but accumulate in the spleen

Our results suggest that the disappearance of Pten-deficient HSCs in the BM is not caused by increased HSC proliferation, followed by exhaustion. To search for an alternative explanation, we examined whether Pten mutant HSCs might have migrated from the BM to the spleen, which is known to massively increase in Pten mutant animals. Indeed, the total number of LSKCD135<sup>−</sup>CD135<sup>−</sup> cells in the spleen dramatically increased in both models after loss of Pten, raising the possibility that HSCs are not physically lost but rather relocated to the spleen (Fig. 4 F). To answer this question, we first assessed whether Pten-deficient phenotypic HSCs are functional in both locations. Indeed, although the reconstitution activity of Pten-deficient spleocytes was dramatically increased compared with controls, Pten-deficient BM contained reduced HSC activity (Fig. 4 G). To confirm these results, we performed limiting dilution experiments by transplanting into lethally irradiated recipients a limiting amount of control and mutant BM cells (0.4% of total BM) and splenocytes (1.6% of total splenocytes). As shown in Fig. 4 H, Pten-deficient BM contained a lower number of competitive repopulation units (CRUs) when compared with control BM. CRU numbers were, instead, increased in Pten-deficient spleen. This analysis suggests that the spatial distribution of HSCs is altered in Pten-deleted mice relative to control mice, whereas the absolute numbers of HSCs appear to be the same.

Assaying reconstitution of Pten-deficient BM beyond 9 wk is complicated by the fact that the BM already contains precancerous clones which progress after transplantation and thus are likely responsible for the observed sudden increase in chimera. To circumvent this complication, 2,500 FACS-sorted LSK-HSCs from Pten<sup>ΔMs</sup> and control mice were transplanted together with rescue BM. Interestingly, Pten-deficient HSCs showed the same multilineage reconstitution activity

18 h of BrdU labeling. P = 0.99, P = 0.19, and P = 0.75, respectively. (C) Percentage of BrdU LRCs within the LSKCD34<sup>−</sup>CD135<sup>−</sup> population. Mice were initially labeled for 10 d with BrdU, followed by simultaneous induction of SCL-CreERT with Tx and 70 d of a BrdU free chase period. P = 0.02. Each dot represents a single mouse. Horizontal bars indicate mean values. (D) Kaplan-Meyer curve showing the survival of Pten<sup>ΔMs</sup> and Pten<sup>ΔScl</sup> mice (15 d [Mx] or 21 d [Scl] after deletion) during weekly 5-FU administration. n = 7 Pten<sup>ΔMs</sup>mice, n = 4 Pten<sup>ΔScl</sup>mice, n = 4 of each control group. (E) Analysis of intracellular Ki67 expression in LSKCD34<sup>−</sup> cells from BM (top, P = 0.43) and spleen (bottom, P = 0.81) of pten<sup>ΔMs</sup> mice. (F) Kinetic analysis of the relative change of LSKCD34<sup>−</sup>CD135<sup>−</sup> cells in the spleen of Pten<sup>ΔMs</sup> and Pten<sup>ΔScl</sup> mice compared with controls (set to 100%). The asterisk indicates statistically significant change (P < 0.05). The cross indicates that the majority of mice do not survive beyond this time point. Each dot represents the mean value obtained from three to four mice. (G) Bar graph showing CD45.2 donor contribution 5, 7, and 9 wk after transplantation of 0.4% of total BM and 1.6% of total splenocytes of control and Pten<sup>ΔMs</sup> mice into CD45.1 hosts. n = 4 each group. (H) Limiting dilution analysis of BM cells (top) and splenocytes (bottom) of pten<sup>ΔMs</sup> (KO) and control (wt) mice. Values represent reconstituted mice (chimerism > 0.5%) versus total mice transplanted. CRUs were calculated using the method of maximum likelihood. The number of mice per group is indicated. (I) Percentage of CD45.2 donor contribution 12 wk after transplantation of 2500 LSK cells of control and Pten<sup>ΔMs</sup> mice into CD45.1 hosts. (J and K) Multilineage reconstitution after transplantation of 2500 BM (J) or splenic (K) LSK cells from pten<sup>ΔMs</sup> mice. n = 2 controls, n = 3 mutants. (L) PCR analysis of DNA from FACS-sorted donor (CD45.2) splenocytes 12 wk after transplantation showing the absence of the Pten<sup>ΔMs</sup> but presence of the Pten<sup>ΔD</sup> allele. (M–O) Pten, pAktThr308, pAktSer473, and pGSK3βSer9 levels in BM LSKCD135<sup>−</sup>CD34<sup>−</sup> (top) or splenic LSK cells (bottom) of Pten<sup>ΔMs</sup> and controls (p-i.p. treated). A representative FACS plot (M) and the fold change in the expression levels (N and O) are shown. Three independent experiments, n = 6 for each group. BM: P = 0.13 Pten, P > 0.05 pAktThr308, pAktSer473, and pGSK3βSer9. Spleen: P = 0.04 Pten, P > 0.05 pAktThr308, pAktSer473, and pGSK3βSer9. Unless otherwise indicated, the data presented in this figure refer to at least three independent experiments using at least three mice per group. Data are provided as mean ± SE.
(with the notable exception of B cells) after 3 mo as their normal counterparts (Fig. 4, I–L), demonstrating that Pten-deficient BM and splenic HSCs have myelo/lymphoid reconstitution potential. In agreement with their normal function, BM LSKCD34+ and splenic LSK cells of PtenAlox and PtenΔlox mice did not show an increase in the levels of P-AKTthr308, P- AKTser473, or P-GSK3βser9, despite Pten being deleted (Fig. 4, M–O; and not depicted). To exclude that these results could be due to a poor sensitivity of the detergent–based assay used to detect phospho-antigens, we further evaluated AKT levels by using an alternative paraformaldehyde/acetone method (Krutzik et al., 2011). However, with this method, AKT activation was also exclusively found in Pten-deficient myeloid cells and not in HSCs (unpublished data). Surprisingly, these data indicate that PI3K-Akt signaling is not significantly up-regulated in Pten-deficient HSCs. Collectively, these results suggest that Pten-deficient HSCs are functional and retain basal levels, but not significantly increased P-AKT levels, and are predominantly localized in the spleen rather than in the BM. To further address whether Pten deletion in the stromal compartment contributes to HSCs relocalization to the spleen, we performed reverse chimeras experiments. Wild-type cells were transplanted into lethally irradiated PtenB6+ or MxCre;PtenB6+ hosts, and after hematopoietic reconstitution, Pten deletion was induced by pl-pC (Fig. 5 A). Although Pten was efficiently deleted in BM stroma (Fig. 5 B), PtenAlox and control recipients presented a similar number of donor-derived LSK cells in their spleens (Fig. 5 C). Moreover, no changes in the distribution of donor-derived mature splenic cells were observed in PtenAlox recipients (Fig. 5 D). These data show that Pten loss–induced relocalization of HSCs to the spleen is due to a hematopoietic cell autonomous mechanism.

Pten deletion increases expression of mobilizing cytokines

Accumulation of normal HSCs in the spleen is observed after BM injury and establishment of extramedullary hematopoiesis, or in response to high levels of mobilizing cytokines such as G-CSF. Therefore, we examined whether the level of this cytokine was increased after Pten deletion. As shown in Fig. 6 (A and B), Pten mutant CD11b+Gr1+ BM cells produced significantly higher G-CSF levels as compared with their control counterparts (Fig. 6, A and B; and not depicted). To examine whether other cytokines were increased in Pten-deficient serum, a cytokine antibody array screen was used to simultaneously study the expression of 62 cytokines. This global analysis revealed that, in addition to G-CSF levels of both CXCL12, and the mouse analogue of human IL-8, known as keratinocyte-derived chemokine (KC), were elevated (Fig. 6 C). The results obtained in this screen were then validated by flow cytometry. As shown in Fig. 6 D, MCP-5 was significantly increased in mutant splenic Gr-1highCD115− and CD4+CD8− cells. In addition, a threefold up-regulation of IL-9 occurred in mutant BM myeloid cells and lymphoid cells (Fig. 6 D) and Pten-deficient osteoblasts and endothelial cells produced higher levels of CXCL12 (Fig. 6 E). In summary,

**Figure 6. Increased level of mobilizing cytokines in ptenAlox and ptenΔlox mice.** (A and B) FACS plot (A) and histogram showing GCSF expression in BM CD11b+Gr-1+ cells of control and PtenAlox mice. Two independent experiments were done for controls (n = 7) and mutants (n = 8; ** indicates statistically significant change, P = 0.0008). (C) Cytokine array screening serum levels of indicated cytokines 15 d (PtenAlox) or 28 d (PtenΔlox) after deletion. This screen has been performed once with n = 2 per group. (D) MCP-5 and IL-9 levels in splenic Gr-1highCD115− and CD4+CD8− cells or BM CD11b+Gr-1+ and CD4+CD8− cells of PtenAlox mice and control counterparts. Data are shown as fold change as compared with control levels. P = 0.01, MCP-5 in Gr-1highCD115− cells; P = 0.007, MCP-5 in CD4+CD8− cells; P = 0.03, IL-9 in BM CD4+CD8− cells; P = 0.04, IL-9 in BM CD11b+Gr-1+ cells. Three independent experiments were performed, n = 11 controls and n = 6 mutants. (E) CXCL-12 levels observed in osteoblasts (CD45−CD31+Ter119−CD51+Sca-1−) and endothelial cells (CD45−Ter119−CD31+) of PtenAlox mice and controls. Data are shown as fold change as compared with control levels. Two independent experiments (5 controls and 10 mutants) were performed. P < 0.01, osteoblasts, P < 0.05 endothelial cells. Data are provided as mean ± SE. Unless otherwise indicated, * indicates statistically significant change with P < 0.05.
the up-regulation of cytokine production, including G-CSF, provides a mechanistic explanation for the observed mobilization of Pten-deficient HSCs from the BM to the spleen.

**Genetic elimination of G-CSF rescues Pten loss--induced phenotypes**

Next, we examined whether the genetic lack of G-CSF could rescue the phenotype observed in Pten\(^{Abx}\) and Pten\(^{Ax}\) mice. Pten\(^{Abx}\);G-CSF\(^{-/-}\) double-mutant mice did not develop splenomegaly (Fig. 7, A and B), nor did they massively increase the number of splenic CD11b\(^+\)Gr-1\(^+\) cells (Fig. 7, C and D). Importantly, Pten\(^{Abx}\);G-CSF\(^{-/-}\) mice did not accumulate LSKCD34\(^+\)CD135\(^+\) cells in their spleens (Fig. 7 E). Similarly, Pten\(^{Ax}\);G-CSF\(^{-/-}\) mice showed no significant increase in spleen size when compared with control animals (Fig. 7, F and G). Moreover, Pten\(^{Ax}\);G-CSF\(^{-/-}\) animals did...
not accumulate CD11b+Gr-1+ cells in their spleen as observed in Pten+/−;G-CSF−/− mice (Fig. 7 J). As expected, Pten−/−;G-CSF−/− mice only demonstrated a modest nonsignificant decrease in the number of splenic immature myeloid cells (Fig. 7, H and I). Additionally, the significant accumulation of LSKCD34−CD135− cells observed in the spleen of Pten+/+;G-CSF−/− mice did not occur in the absence of G-CSF (Fig. 7 J). Consistent with these observations, Pten+/+;G-CSF−/− mice showed a moderate increase in the numbers of BM LSKCD34−CD135− cells when compared with Pten+/+;G-CSF+/− mice (Fig. 7 K). Importantly, Pten+/+;G-CSF−/− mice did not significantly differ in the numbers of splenic or BM HSCs compared with controls (Fig. 7, J and K).

In summary, our data suggest that loss of Pten promotes HSC mobilization to the spleen, which is caused by the production of G-CSF by mutant BM myeloid cells. Thus, HSC mobilization in mutants is not a cell-autonomous effect caused by the lack of Pten in HSCs but rather a consequence to the loss of Pten in myeloid and stromal cells. This, in turn, induces the secretion of cytokines that feeds back to HSCs in their BM niche leading to their mobilization to the spleen.

**DISCUSSION**

In this study, we generated a new conditional ScICre-mediated Pten KO model, which enabled us to provide new mechanistic insights into the role of Pten in the hematopoietic stem and progenitor cells. Particularly, we show that Pten deletion induces production of G-CSF in myeloid cells, which subsequently leads to HSC mobilization to the spleen, followed by development of MPD and leukemia. Although some of our data are consistent with previous studies showing that Pten deletion promotes MPD, leukemia development, and loss of HSCs in the BM, our data challenge the suggested mechanism in which Pten is put forward as an important regulator of HSC proliferation and self-renewal. (Chen et al., 2006; Yilmaz et al., 2006; Lee et al., 2010). Instead, our results suggest an indirect role for Pten in HSCs mobilization. Pten mutant mice in our hands have a normal number of quiescent and dormant HSCs, and a normal stress response after 5-FU induced myeloid ablation. Consistently, the expression profiles of control and Pten-deficient HSCs have revealed a strikingly overlapping pattern with only very few differentially expressed genes after Pten deletion (unpublished data). In agreement with these data, transplantation studies showed that Pten−/−deficient HSCs can generate all hematopoietic lineages (except mature B cells) and are fully functional. Strikingly, no activation of AKT signaling was observed on BM LSKCD34− cells after Pten loss. Collectively, our ex vivo and in vivo studies revealed neither a significant role for PTEN in the control of the balance between HSC quiescence and self-renewal nor a critical role for HSC function, as was previously suggested (Yilmaz et al., 2006).

As an alternative mechanism for the observed loss of Pten-deficient BM HSCs, our data provide strong evidence that upon Pten deletion, functional HSCs are mobilized from the BM to the spleen. This is mediated by expression of several cytokines by mutant myeloid cells, which are known to mobilize HSCs. These include KC, the mouse homologue of the CXC chemokine IL-8, a chemoattractant and activator of neutrophils previously shown to rapidly induce mobilization of HSCs in mice and primates (Laterveer et al., 1995). In addition, after Pten deletion, CXCL12, a well characterized chemoattractant for HSCs and a key mediator of HSC trafficking (Lapidot and Petit, 2002; Nervi et al., 2006; Tesio et al., 2011), is up-regulated. Finally, and most importantly, mutant myeloid cells significantly up-regulate G-CSF, the most common mobilizing cytokine routinely used for harvesting human HSC from donors in clinical settings (Nervi et al., 2006). The exact molecular mechanisms that up-regulate G-CSF expression in myeloid cells after Pten loss remains to be explored. However, it may be mediated by the KC/IL-8 receptor Cxcr2, which has been previously implicated in G-CSF regulation (Mei et al., 2012).

The role of G-CSF is critical, as Pten mutant cells lacking the G-CSF gene fail to significantly mobilize HSCs to the spleen. Nevertheless, G-CSF may have other functions that contribute to the phenotype. Moreover, the additional mobilizing cytokines induced by mutant BM deficient HSCs and does not appear to be a cell-intrinsic phenomenon of HSCs. Indeed, despite Pten being efficiently deleted in HSCs, no activation of the PI3K–AKT pathway could be detected. In contrast, mutant CD11b+Gr-1+ cells displayed activated Akt signaling in response to Pten loss. Thus, our results indicate that loss of Pten in BM myeloid cells is the driving force leading to G-CSF production and HSC mobilization to the spleen. In agreement with these data, recent studies revealed the crucial role of BM myeloid cells such as monocytes and macrophages in driving HSC egress from their niches (Winkler et al., 2010; Chow et al., 2011). Interestingly G-CSF signaling in BM monocytes was shown to be sufficient to induce HSC mobilization (Christopher et al., 2011); however, the molecular mechanisms underlying these processes remain mostly uncertain.

Because AKT signaling is not altered in Pten-deficient HSCs, other phosphatases might be operative in HSCs. Another negative regulator of the PI3K pathway, SHIP, is a potential candidate, as it was shown to cooperate with other negative regulator of the PI3K pathway, SHIP, is a potential candidate, as it was shown to cooperate with Pten in B cell lymphoma (Miletic et al., 2010). Additionally the serine/threonine phosphatase PP2A negatively regulates P-AKT in human CD34+ progenitor cells, contributing to their motility (Basu et al., 2007). Interestingly, our results demonstrate that HSCs and myeloid cells show a different degree of AKT activation in response to Pten deletion. Mammalian cells express three isoforms, AKT1, AKT2, and AKT3, with the first two being expressed in hematopoietic cells. Functional differences between these two isoforms have been shown, and AKT1 and AKT2 deletion in Pten heterozygous mice have different effects on tumorigenesis (Chen et al., 2006; Xu et al., 2012). Importantly, the amplitude of AKT inactivation by PHLPP phosphatases largely depends on the isoform type (Brognard et al., 2007). Thus, a differential expression and/or activity of the two
AKT isoforms in myeloid and HSCs might explain why the two cell types show a different response to *Pten* deletion. Several lines of evidence indicate that splenomegaly and MPD development are, at least partially, a secondary effect of *Pten* deficiency in BM myeloid cells. First, the production of G-CSF by mutant BM CD11b<sup>Gr-1<sup>+<sup></sup></sup> cells is crucial for the development of splenomegaly and MPD, as both phenomena were abolished in mice lacking G-CSF. Second, *Pten*-deficient myeloid cells do show overactive AKT signaling, which is crucial in driving MPD development (Kharas et al., 2010). The role of AKT in HSC self-renewal is controversial, as its constitutive activation was shown to deplete HSCs (Kharas et al., 2010), whereas fetal liver HSCs lacking AKT were unable to reconstitute long-term hematopoiesis (Juntilla et al., 2010). Our data, showing that HSC self-renewal is maintained in the presence of a physiological level of active AKT, indicate that a fine-tuned modulation of AKT activation guarantees HSC maintenance, whereas too low or too high levels are detrimental for HSC function. In any case, PTEN does not seem to be a major regulator for AKT activity in HSCs in vivo.

The discrepancies observed between this and previous studies, with respect to HSC proliferation and AKT activation, might be due to the different genetic background used. Previous studies used mice on a clean C57BL/6 inbred background; in contrast, our data are obtained with mice on a mixed genetic background. These data may indicate that the observed effects on a C57BL/6 background are strain specific and our model might therefore better reflect the genetic complexity observed in clinical settings. This difference might be crucial, as several aspects of HSC function show genetically determined variation and HSC cycling activity, as well as HSC pool size being controlled at a genetic level (Van Zant et al., 1983; de Haan and Van Zant, 1997). Similarly, strain-dependent variations affect responsiveness of primitive progenitor cells to cytokines such as SCF, Flt3 ligand, and TGF-β (Henckaerts et al., 2002, 2004; Avagyan et al., 2008). Intriguingly, the mobilization response to G-CSF is also affected by genetic variation (Hasegawa et al., 2000). Moreover, tumor development in *Pten*-deficient mice has been demonstrated to be highly dependent on genetic background (Freeman et al., 2006; Svensson et al., 2011).

Interestingly, although in the absence of G-CSF *Pten* mutant mice do not develop splenomegaly or accumulate HSCs in their spleens, G-CSF absence did not rescue T-ALL development (unpublished data). Additional mechanisms, such as activation of Notch signaling, might be involved in this phenomenon. Mutations in NOTCH1 are the most recurrent genetic lesions in T-ALL, and a cross talk between PTEN and NOTCH1 signaling in regulating T-ALL development has been reported (Palomero et al., 2007). Furthermore, a recent study has suggested that PTEN down-regulation contributes to the activation of the oncogene c-Myc, providing another mechanism for the promotion of T-ALL in the absence of PTEN (Bonnet et al., 2011).

Collectively, our data provide a novel and important mechanism for HSCs mobilization and MPD initiation, indicating that PTEN is dispensable for HSCs but critical for proliferation and cytokine production of myeloid progenitors. In conclusion, our data show that in response to *Pten* loss, BM Gr-1<sup>CD11b<sup>+</sup></sup> cells hyper-activate AKT signaling and up-regulate G-CSF, which promotes HSC accumulation in the spleen, splenomegaly, and MPD development. The increased G-CSF levels are further supported by elevated CXCL-12 production by mutant stromal and endothelial cells to promote mobilization of HSCs from the BM to the spleen. Importantly, mobilized *Pten*-deficient splenic HSCs are functional, as they maintain their self-renewal activity and do not show increased AKT activation. Inhibition of AKT signaling by one of the various inhibitors in leukemia patients would therefore lead to the return of mobilized HSCs to the BM with little if any direct functional effects on the HSCs themselves.

**MATERIALS AND METHODS**

**Mice.** All mice were maintained in the ISREC and in the DKFZ animal facility under specific pathogen-free (SPF) conditions and housed in individually ventilated cages (HVIC). Animal procedures were performed according to protocols approved by the Swiss Bundesamt für Veterinärwesen no. 1728 and by the German authorities no. G-22/09. *Pten<sup>fl<sup/>flox</sup></sup>* (control) mice (The Jackson Laboratory) were purchased as in Grozzer et al. (2001) and crossed with the MsCre transgenic mice (Kühn et al., 1995) to obtain MsCre:*Pten<sup>fl<sup/>flox<sup>/+</sup></sup>* (mutant) mice on a mixed genetic background (≈50% C57BL/6, rest 129SV, FVB/N). IFN-α-induced deletion was induced by five i.p. injections of 10 mg/kg poly-I:polyC (poly-IC; InvivoGen) every 2 d as previously described (Wilson et al., 2004). For the LRC assay, mice were B6U labeled for 10 d using 0.8 mg/ml BrdU water (glucose), followed by a 70-d chase. 5-FU studies were performed using weekly i.p. injections of 150 mg/kg 5-Fluorouracil (Sigma-Aldrich). Unless otherwise stated, all control mice were littermates of analyzed mutant mice and were all treated with p-c. *Pten<sup>fl<sup/>flox<sup>/+</sup></sup>* (control) mice were crossed with the SCL-CreER<sup>2</sup> transgenic mice (Göthert et al., 2005) to obtain SCL-CreER<sup>2</sup>*Pten<sup>fl<sup/>flox<sup>/+</sup></sup>* (mutant) mice on a mixed genetic background (63% C57BL/6; 47% 129SV and CBA/J). Recombination was achieved by Tx diet (1 g/kg food; Sigma-Aldrich) for 20 d. G-CSF−/− mice were provided by G.J. Lieschke (Australian Regenerative Medicine Institute, Victoria, Australia).

**Generation and analysis of chimeras.** B6.SJL-Ptpnca-Pep3b/Boj donor mice (*Cd45.1; The Jackson Laboratory*) were purchased and maintained in the ISREC animal facility. To generate HSC chimeras, transplantation was performed using 2,500 FACs-sorted LSK-HSCs from each donor mouse, along with 2 × 10<sup>6</sup> CD45.1 rescue BM cells, which were i.v. transferred into two CD45.1− lethally irradiated (2 × 550 rad) recipient mice, which were then pretreated (48 h before) with anti-NK1.1 mAbs. For limiting dilution transplants, the indicated dilutions of BM and SP CD45.2 cells from experimental mice were transferred i.v., along with 1.5 × 10<sup>6</sup> Sca1-depleted CD45.1 BM into lethally irradiated CD45.1 recipients. To address the leukemic potential of *Pten*-deficient cells, control and mutant cells were transplanted into sublethally irradiated NOG/SCID recipients. All chimeric mice were maintained on antibiotics containing water (Bactrim; Roche) for 20 d. G-CSF−/− mice were provided by G.J. Lieschke (Australian Regenerative Medicine Institute, Victoria, Australia).
(EMD Millipore) and antibodies, followed by anti–mouse–HRP (Promega) and the ECL WB detection kit (GE Healthcare), were used for blot development.

Isolation of BM, spleen, and stromal cells. To collect BM cells, mouse legs were dissected and flesh removed, bones were crushed using a mortar and pestle, and cell suspensions were filtered before further use. Mouse spleens were isolated and crushed using the bottom of a syringe punch and filtered to obtain cell suspensions. Blood cells were collected from tail vein bleedings into tubes containing 10,0000 U/ml heparin (Sigma-Aldrich) and peripheral blood lymphocyte were isolated using a histopaque gradient (1,900 rpm, 8 mm, no brake; Sigma-Aldrich). To isolate HSCs, lineage magnetic depletion was performed to enrich for lineage-negative cells. Therefore, BM cells were incubated with lineage antibodies (CD4, CD8, CD11b, Gr1, B220, and Ter119) and lineage-positive cells were removed using sheep antimouse IgG-coated M450 Dynabeads (Invitrogen). Lineage-negative cells were stained for hematopoietic subsets, and LSK-HSCs were sorted using a FACS-Aria (BD). Stromal cells were obtained by three subsequent digestions of the long bones. In brief, the bones were first digested with a solution containing Trypsin 0.1% Collagenase P and DNase (20 min at 37°C). Bones were then washed and crushed. The remaining bone fragments were then incubated with a second solution containing trypsin 0.1% and DNase (20 min at 37°C). After being washed, bone fragments underwent a third digestion containing collagenase P, dispase, Heps, and CaCl2 (1 h at 37°C). The cells present in the supernatant were isolated using a histopaque gradient.

Flow cytometry and cell cycle analysis. Cell cycle analysis on HSCs was performed using a cell surface staining in combination with BrdU or Ki67 antibodies. For BrdU analysis, mice were injected i.p. with 7.2 mg/kg BrdU (Sigma-Aldrich) before analysis. Mice were sacrificed and BM and SP cells were isolated. Lineage bead depletion was performed to enrich for lineage-negative cells. Lineage-negative cells were stained for hematopoietic subsets, and LSK-HSCs were sorted using a FACS-Aria (BD). Stromal cells were obtained by three subsequent digestions of the long bones. In brief, the bones were first digested with a solution containing Trypsin 0.1% Collagenase P and DNase (20 min at 37°C). Bones were then washed and crushed. The remaining bone fragments were then incubated with a second solution containing trypsin 0.1% and DNase (20 min at 37°C). After being washed, bone fragments underwent a third digestion containing collagenase P, dispase, Heps, and CaCl2 (1 h at 37°C). The cells present in the supernatant were isolated using a histopaque gradient.

Immunohistochemistry. All histological samples were collected and fixed in 10% neutral buffered formalin solution (HT50 1–2; Sigma-Aldrich) for 2–4 h in a cold room on a rotor. For bones, an additional decalcification step in 0.4 M EDTA, pH 7.2, for 4–6 d at 4°C was performed on a rotor. For immunohistochemistry, slides were pretreated with peroxidase blocking buffer (120 mM Na2HPO4, 43 mM citric acid, 30 mM Na2MoO4, and 0.2% H2O2; pH 5.8) prior to antigen retrieval (20 min at 70°C, 10 mM citrate buffer, pH 6.0) and then sections were incubated with a rabbit polyclonal Peroxidase antibody (Neomarkers, 1:200; LABVISION), followed by an anti–rabbit HRP (Dako). DAB (Sigma-Aldrich) was used to reveal staining, and Mayer's Hematoxylin (Sigma-Aldrich) was used to counterstain the nucleus. Light microscopic analysis was performed using an Axio Scope equipped with a Progress C10 camera (Carl Zeiss).

ELISA and cytokine array. Cytokine levels in the serum were quantified using the RayBio Mouse Cytokine Array III kit (Hoelzel Biotech) according to the manufacturer's protocol. The cytokine levels were calculated by measuring the normalized intensity of each spot, using AIDA Image Analyser (v.4.06) software.

Antibodies. Gr-1 (Ly-6G, RB6-8C5)-FITC, -biotin, and -Alexa Fluor 647; Ter-119-FITC and -biotin; B220 (RA3-6B2)-FITC and -biotin; CD11b-FITC and -biotin; CD4 (clone GK1.5)-FITC and -biotin; CD8a (53.6.7)-FITC and -biotin; TCR-β (H57)-FITC; CD161 (NK1.1, PRK3)-FITC and -biotin; CD45.1 (A20.1)-FITC, -biotin, -PE, or -Alexa Fluor 647; CD45.2 (ALI-4A2)-FITC, -biotin, -PE, or -Alexa Fluor 647; and TCR-γδ (GL3)-FITC and -Alexa Fluor 647 were purified and conjugated in this laboratory according to standard protocols. CD34 (RAM34)-FITC; CD135 (A2F10)-PE; CD117 (2B8)-PE and -PE-Cy5; Sc1 (D7)-APC and -biotin; CD43 (57)-FITC, -BPI (FG5.4)-biotin, and -IgM (11/4)-PE were all purchased from ebioscience. CD44 (MWReg30)-FITC was purchased from BD. Primary antibody anti–G-CSF was from Santa Cruz Biotechnology, Inc., and anti–pAKTThr308, -pAKTser473, and -pGSK3ser9 were from Cell Signaling Technology. Anti–CXCL12 antibodies were purchased from R&D Systems, anti–MCP-5-PE from Bioss, and IL-9–PE from BioLegend.

Statistical analysis. Significance levels of data were determined by Student's t test for the differences in mean values.

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