PI3K alpha and delta promote hematopoietic stem cell activation

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Many cytokines and chemokines that are important for hematopoiesis activate the PI3K signaling pathway. Because this pathway is frequently mutated and activated in cancer, PI3K inhibitors have been developed for the treatment of several malignancies and are now being tested in the clinic in combination with chemotherapy. However, the role of PI3K in adult hematopoietic stem cells (HSCs), particularly during hematopoietic stress, is still unclear. We previously showed that the individual PI3K catalytic isoforms p110 α and p110 β have dispensable roles in HSC function, suggesting redundancy between PI3K isoforms in HSCs. We now demonstrate that simultaneous deletion of p110 α and p110 δ in double-knockout (DKO) HSCs uncovers their redundant requirement in HSC cycling after 5-fluorouracil (5-FU) chemotherapy administration. In contrast, DKO HSCs were still able to exit quiescence in response to other stress stimuli, such as LPS. We found that DKO HSCs and progenitors had impaired sensing of inflammatory signals *ex vivo*, and that levels of IL-1 β and MIG were higher in the bone marrow (BM) after LPS than after 5-FU administration. Furthermore, exogenous *in vivo* administration of IL-1 β could induce cell cycle entry of DKO HSCs. Our findings have clinical implications for the use of PI3K inhibitors in combination with chemotherapy.

Introduction

Hematopoietic stem cells (HSCs), which are at the top of the hematopoietic hierarchy, exist mostly in a quiescent state. Under stress conditions such as infection, or after exposure to cytotoxic chemotherapy, HSCs become activated and enter the cell cycle, which drives emergency myelopoiesis and rapid regeneration of the hematopoietic system (1). These stimuli lead to the secretion of proinflammatory cytokines by a variety of immune cells, including myeloid cells and T cells, stromal cells in the BM microenvironment, and HSCs themselves (2). Furthermore, recent data suggest that HSCs can also directly respond to inflammatory signals, which leads to their activation, proliferation, and differentiation along the myeloid lineage (3, 4). Several different inflammatory signals are known to activate HSCs, including IFN- α , IFN- γ , TLR ligands, LPS, IL-1 β , TNF- α , and others (1, 5, 6). Although many of these ligands activate similar downstream signaling pathways, the specific downstream effectors for these pathways in HSCs have not been delineated.

PI3K is a lipid and protein kinase that can be activated by receptor tyrosine kinases, cytokine receptors, chemokine G protein-coupled receptors, or oncogenes, including RAS. PI3K converts phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3), which leads to activation of pyruvate dehydrogenase kinase 1 (PDK1) and the serine/threonine kinase AKT (PKB) (7). AKT has many downstream effectors, which regulate the cell cycle, apoptosis, protein translation, autophagy, and metabolism. Most cytokines and RTK ligands signal through class IA PI3K.

Although several studies have also implicated the PI3K/AKT pathway downstream of IL-1 β (8), TNF- α (9), or LPS/TLR signaling (10) in other cell types, the roles of PI3K/AKT in transducing inflammatory signals in hematopoietic stem and progenitor cells (HSPCs) are unknown. Furthermore, the specific roles of PI3K in HSC activation under stress are poorly understood. This is a critical question, as many

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kinase inhibitors and antibody-based therapeutics in clinical use can inhibit the PI3K/AKT pathway, and PI3K inhibitors are also used in cancer treatment (11). Because many patients undergoing such treatments also receive cytotoxic chemotherapy and are at increased risk for infection, it is important to understand how PI3K/AKT inhibition can affect the HSC stress response.

Class IA PI3Ks are composed of a p85 regulatory subunit and a p110 catalytic subunit. Hematopoietic cells express 3 different genes encoding 3 different isoforms of the class IA p110 subunit (*Pik3ca*, *Pik3cb*, *Pik3cd*), which encode p110 α , p110 β , and p110 δ , respectively (7). Because these 3 isoforms of p110 can all bind to p85, they can substitute for one another in some cellular contexts (7). Therefore, there is likely to be a high level of redundancy between the PI3K isoforms in HSCs. Using a conditional floxed-*Pik3ca*;Mx1-Cre mouse model, we previously demonstrated that p110 α is dispensable for HSC function, even after 5-fluorouracil (5-FU) administration (12). This was surprising, given that p110 α has been shown to be required for signaling in response to multiple growth factors in fibroblasts (13). We also observed normal peripheral blood counts in a floxed-*Pik3cb*;Mx1-Cre mouse model (14). These results support the hypothesis that PI3K isoforms play redundant roles in HSCs. Therefore, to examine the redundant roles of class I PI3K isoforms in HSCs at steady state and under stress conditions, we generated double-knockout (DKO) mice with germline deletion of *Pik3cd* and conditional deletion of *Pik3ca* in the hematopoietic system. We uncovered significant redundancy between *Pik3ca* and *Pik3cd* in hematopoiesis, and also identified a role of these two PI3K isoforms in transducing inflammatory signals in HSPCs under stress conditions.

Results

p110 δ is not required for HSC repopulating function. To determine the most highly expressed class I PI3K isoforms in murine HSCs and early progenitors, we examined the expression of each of these isoforms in a previously published RNA sequencing data set (15). We found that, of the class IA isoforms, *Pik3cd* was expressed at the highest levels in HSCs and multipotential progenitors (MPPs) (Supplemental Figure 1A; supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.125832DS1>). While *Pik3cd* germline homozygous knockout mice were previously described to have normal blood counts (16), a role for p110 δ in HSCs and early progenitors has not been reported. Consistent with previously published data (16), we confirmed that young homozygous *Pik3cd* germline knockout mice had normal blood counts (Supplemental Figure 1B). To determine whether p110 δ is required for HSC function, we performed competitive repopulation assays using whole BM from *Pik3cd*^{-/-} (p110 δ ^{-/-}) mice that were backcrossed into the C57BL/6 background for more than 9 generations, using C57BL/6 WT controls. We observed no significant differences in long-term multilineage repopulating ability between p110 δ ^{-/-} and WT BM, suggesting that p110 δ is not required for engraftment or multilineage reconstitution of the hematopoietic system (Supplemental Figure 1C). This finding further supports the hypothesis of redundancy between PI3K isoforms in HSCs.

p110 α and p110 δ play redundant roles in hematopoiesis. To determine whether p110 α and p110 δ play redundant roles in hematopoiesis, we generated DKO mice with the genotype *Pik3cd*^{-/-};*Pik3ca*-lox/lox;Mx1-Cre. Mx1 is a pan-hematopoietic promoter that can be induced postnatally by injecting mice with the double-stranded RNA polyinosinic:polycytidylic acid (pIpC). After 2 i.p. injections of pIpC at 4 weeks of age to excise *Pik3ca*, we observed significant leukopenia and anemia in DKO mice, while platelet counts were unchanged (Figure 1A). We observed a significant decrease in both neutrophil and lymphocyte counts in the peripheral blood of DKO mice (Figure 1B). DKO mice also had significantly decreased BM cellularity and decreased spleen weight (Figure 1C). Consistent with this observation, histological analysis revealed a hypocellular BM (Supplemental Figure 2A). While spleen weight was significantly decreased (Figure 1C), splenic architecture appeared intact in both p110 δ ^{-/-} and DKO spleens (Supplemental Figure 2B). We observed no significant differences in the proportions of mature myeloid or B cells in the BM of DKO mice by flow cytometry, while the frequency of Thy1.2⁺ T cells was increased in the p110 δ ^{-/-} group (Supplemental Figure 2C). However, we did observe a significant decrease in the frequency of B220⁺ B cells in DKO and p110 δ ^{-/-} spleens, with a relative increase in the frequency of Mac1^{hi}Gr-1^{hi} myeloid cells and Thy1.2⁺ T cells in DKO spleens (Supplemental Figure 2D).

When we performed multiparameter flow cytometric analysis on the BM of DKO mice, p110 δ ^{-/-} littermates, and WT;Mx1-Cre controls based on an established protocol (17), we observed no significant difference in the absolute numbers of phenotypically defined HSCs (Lin^{-c}-Kit⁺Sca-1⁺Flk2-CD150⁺CD48⁻), short-term HSCs (ST-HSCs: Lin^{-c}-Kit⁺Sca-1⁺Flk2-CD150⁺CD48⁻), MMP2s

Figure 1. $p110^{\alpha}$ and $p110^{\delta}$ play redundant roles in hematopoiesis. (A) Serial peripheral blood counts of $p110^{\alpha lox/lox}; p110^{\delta -/-}; Mx1-Cre$ mice (DKO; $n = 9$) and $Cre^+ p110^{\alpha lox/lox}; p110^{\delta -/-}$ littermates ($\delta^{-/-}$; $n = 8$) after 2 plpC injections. This experiment was performed 3 times. At each time point, unpaired 2-tailed Student's t test was used to compare the groups. (B) Peripheral blood absolute neutrophil and lymphocyte counts of DKO ($n = 21$), $\delta^{-/-}$ ($n = 21$), and WT; $Mx1-Cre$ (WT, $n = 6$) mice at 4 weeks after 2 plpC injections. Combined data from multiple experiments are shown. (C) BM cellularity and spleen weights of DKO ($n = 9$), $\delta^{-/-}$ ($n = 9$), and age-matched WT C57BL/6 mice ($n = 6$) sacrificed at 4 weeks after plpC injection. (D) Representative flow cytometry plots of WT, $\delta^{-/-}$ or DKO BM cells stained with HSC and progenitor markers. The gating strategy is shown for: $Lin^lo Sca-1^{hi} c-Kit^{hi}$ (LSK), mixed myeloid progenitors (MPProg), LMPP, MPP2, MPP3, ST-HSC, and HSC.

lymphoid-primed multipotent progenitors (LMPP), HSCs, short-term HSCs (ST-HSC), and multipotent progenitors 2 and 3 (MPP2 and MPP3). Absolute numbers of (E) HSCs (Lin⁻c-Kit⁺Sca-1⁺Flk2⁻CD150⁺CD48⁺), (F) ST-HSCs (Lin⁻c-Kit⁺Sca-1⁺Flk2⁻CD150⁺CD48⁺), (G) MPP2s (Lin⁻c-Kit⁺Sca-1⁺Flk2⁻CD150⁺CD48⁺), (H) MPP3s (Lin⁻c-Kit⁺Sca-1⁺Flk2⁻CD150⁺CD48⁺), (I) LSK cells (Lin⁻c-Kit⁺Sca-1⁺), (J) LMPPs (Lin⁻c-Kit⁺Sca-1⁺Flk2⁺), (K) total myeloid progenitors (Lin⁻c-Kit⁺Sca-1⁺), and (L) CLPs (Lin⁻c-Kit^{mid}Sca-1^{mid}IL-7R⁺Flk2⁺) are shown. Combined data from 2 independent experiments are shown. This experiment was performed more than 3 times, with similar results. For the experiments shown in B–L, ANOVA with the Tukey's multiple-comparisons test was used. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. Data represent mean ± SEM.

(Lin⁻c-Kit⁺Sca-1⁺Flk2⁻CD150⁺CD48⁺), or MPP3s (Lin⁻c-Kit⁺Sca-1⁺Flk2⁻CD150⁺CD48⁺) between WT and DKO groups (Figure 1, D–H). However, we observed a significant decrease in the absolute number of LSK cells (Lin⁻Sca-1⁺c-Kit⁺; Figure 1I), which contain HSCs and MPPs, in lymphoid-primed MPPs (18) (LMPPs: Lin⁻c-Kit⁺Sca-1⁺Flk2⁺; Figure 1J) and in total myeloid progenitors (Lin⁻c-Kit⁺Sca-1⁺; Figure 1K) between the WT and DKO groups. We also observed a decrease in common lymphoid progenitors (CLP) in DKO BM, but only when compared with p110δ^{-/-} BM (CLP: Lin⁻c-Kit^{mid}Sca-1^{mid}IL-7R⁺Flk2⁺; Figure 1L and Supplemental Figure 3A). To determine which stage of B cell differentiation in the BM is most affected in DKO mice, we performed Hardy fraction analysis using flow cytometry (19, 20) (Supplemental Figure 3B). Interestingly, we observed a significant decrease in the absolute numbers of multiple B cell progenitor populations in the BM, including the pre-pro B cell population (Supplemental Figure 3B). These results suggest that p110α and p110δ play redundant roles at the earliest stages of B cell specification. In contrast, we did not observe any significant differences in the absolute number of common myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs), or megakaryocyte-erythroid progenitors (MEPs) in the BM (Supplemental Figure 4A).

However, methylcellulose plating of whole BM revealed a significant decrease in the numbers of total myeloid colonies produced by either p110δ^{-/-} or DKO BM cells (Supplemental Figure 4B), granulocyte-macrophage colonies (Supplemental Figure 4C), and monocyte colonies (Supplemental Figure 4D), with a trend toward a decrease in granulocyte colonies (Supplemental Figure 4E), while the number of granulocyte erythroid myelomonocytic (GEMM) colonies was unchanged in DKO BM from the WT control (Supplemental Figure 4F). Together, these data suggest that p110α and p110δ play redundant roles in hematopoiesis, particularly in specification of the B cell lineage. In addition, p110δ may also be important for the proliferation and/or differentiation of HSCs and progenitors.

p110α and p110δ are required for multilineage reconstitution. To examine the effects of p110α and p110δ deletion on HSC function, and to determine whether the effects of p110α and p110δ deletion are cell autonomous, we performed transplantation of BM from CD45.2⁺ DKO, p110δ^{-/-}, or WT;Mx1-Cre controls into lethally irradiated CD45.1⁺ B6.SJL recipient mice. We induced excision of p110α at 4 weeks after transplantation only in recipient mice with a single pIpC injection. We confirmed excision of *Pik3ca* in the transplant recipients after one pIpC injection by excision PCR (Supplemental Figure 4G). We observed significant leukopenia in the transplant recipients in the DKO group, suggesting that this phenotype is cell autonomous. However, the transplant recipients did not develop anemia (Figure 2A). All transplant recipients survived for the entire 16-week observation period, suggesting that p110α and p110δ are not required for HSC and progenitor maintenance in the noncompetitive setting.

To examine HSC repopulating function under more stringent conditions, we performed competitive repopulation experiments, in which we transplanted BM cells from DKO or p110δ^{-/-} control CD45.2⁺ donors at a 1:1 ratio with WT competitor BM cells from CD45.2⁺/CD45.1⁺ C57BL/6;B6.SJL mice into lethally irradiated CD45.1⁺ B6.SJL recipients. After confirming engraftment, we injected the recipients with pIpC at 4 weeks after transplantation to excise *Pik3ca*. We found that deletion of both p110α and p110δ led to a significant and sustained decrease in total peripheral blood donor chimerism in the DKO group, compared with the p110δ^{-/-} group (Figure 2B). However, examination of individual peripheral blood lineages revealed the most profound decrease in donor chimerism in the B220⁺ B cell population, while the effects on the myeloid (Mac1⁺Gr1⁺) and T cell (Thy1.2⁺) lineages were less pronounced (Figure 2B). The effects on the B cell lineage were not surprising, since the roles of p110δ in B cell receptor signaling and redundant roles of p110α and p110δ in B cell development in the BM have been described previously (16, 21). While there was a significant decrease in total BM donor chimerism in the DKO group, we did not observe a significant decrease in chimerism in the Flk2⁻CD150⁺ LSK compartment, which contains HSCs (Figure 2C). However, donor chimerism was decreased in both Flk2⁻ MPPs and Flk2⁺ LMPPs, as well as in CLPs (Figure 2C). We obtained similar results when we performed competitive repopulation experiments

Figure 2. p110 α and p110 δ play redundant roles in hematopoietic reconstitution. (A) Noncompetitive transplantation of 1×10^6 BM cells from p110 $\alpha^{\text{lox/lox-}}$; p110 $\delta^{-/-}$; Mx1-Cre (DKO; $n = 7$) donors, Cre $^{-}$ littermates ($\delta^{-/-}$; $n = 6$), or age-matched WT; Mx1-Cre controls (WT; $n = 7$) into 6- to 8-week-old female lethally irradiated B6.SJL recipients. Recipients were injected with plpC once at 4 weeks after transplantation. Serial peripheral blood counts of transplant recipients over time are shown from a representative experiment. This experiment was performed 3 times, with similar results. At each time point, ANOVA with the Tukey's multiple-comparisons test was used. (B) Competitive repopulation of DKO BM or $\delta^{-/-}$ (CD45.2 $^{+}$) BM mixed at a 1:1 ratio with CD45.2 $^{+}$ /CD45.1 $^{+}$ WT BM from C57 BL6/B6.SJL F $_1$ hybrids and transplanted into lethally irradiated B6.SJL (CD45.1 $^{+}$) recipients ($n = 10$ per group).

The percentage of CD45.2 single-positive cells indicates the percent donor chimerism in each cell population. Representative data are shown from one of 2 experiments. At each time point, unpaired 2-tailed Student's *t* test was used to compare the groups. **(C)** Donor chimerism in BM cell populations at the endpoint of the competitive repopulation experiment (25 weeks after transplantation). Representative data from one of 2 experiments are shown. **(D)** Secondary transplantation of sorted HSCs from primary BMT recipients of WT;Cre, DKO, or $\delta^{-/-}$ BM together with 250,000 CD45.1⁺CD45.2⁺ WT competitor BM cells. Serial donor chimerism analysis on peripheral blood (PB) populations is shown for WT;Cre (*n* = 8), $\delta^{-/-}$ (*n* = 8), and DKO (*n* = 8) transplant recipients. For the experiments shown in **C** and **D**, ANOVA with the Tukey's multiple-comparisons test was used at each time point. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. Data represent mean $\pm$ SEM.

by transplanting BM from DKO or *Pik3cd*^{-/-} mice that had been pretreated with pIpC (Supplemental Figure 5, A and B), suggesting that p110 α and p110 δ are dispensable for HSC engraftment. These results suggest that p110 α and p110 δ are more important for B cell differentiation and proliferation than for multilineage repopulation. This also suggests that p110 α and p110 δ do not play a critical role in HSCs under these conditions, but may play important roles in specific progenitor populations.

To determine whether p110 α and p110 δ are required for HSC self-renewal and multilineage repopulation under conditions requiring HSC activation, we performed secondary transplantation of 250 sorted Lin⁻c-Kit⁺Sca-1⁺Flk2⁻CD48⁻CD150⁺ HSCs from primary noncompetitive transplant recipient mice in combination with 250,000 WT competitor BM cells from CD45.2⁺/CD45.1⁺ C57BL/6;B6.SJL hybrids into lethally irradiated CD45.1⁺ B6.SJL recipients. Interestingly, we observed a significant decrease in the reconstitution of both myeloid and lymphoid lineages in the secondary transplant recipients (Figure 2D). Analysis of the BM of secondary transplant recipients revealed a significant decrease in total BM donor chimerism in the DKO group, and a trend toward decreased HSC donor chimerism (Supplemental Figure 5, C and D). We also observed a significant decrease in donor chimerism in the ST-HSCs, MPP3s, MPP4s, CLPs, and all myeloid progenitor populations in the DKO group (Supplemental Figure 5, E–L). These data reveal that DKO HSCs are defective in contributing to multiple mature lineages under stress, including the myeloid lineage. This suggests that p110 α and p110 δ are important for the regulation of HSC function during hematopoietic stress.

Deletion of p110 α and p110 δ in HSCs affects gene expression programs associated with cell cycling and inflammatory signaling. To understand the molecular mechanisms of how p110 α and p110 δ regulate the HSC and progenitor stress response, we examined the effects of p110 α and p110 δ deletion on gene expression in HSCs and LMPPs. We sorted HSCs (Lin⁻c-Kit⁺Flk2⁻CD150⁺CD48⁻) and LMPPs (Lin⁻c-Kit⁺Flk2⁺CD150⁻CD48⁺) from pooled BMT recipients, and performed RNA microarray analysis in triplicate using the Affymetrix Mouse Transcriptome Assay 1.0 kit (Figure 3A). We observed significantly altered gene expression in DKO HSCs compared with WT HSCs (Figure 3, B and C, and Supplemental Table 1). Significantly altered expression of some genes (>2-fold change, *P* < 0.05) between p110 δ ^{-/-} HSCs and WT HSCs, or between p110 α ^{-/-} HSCs and WT HSCs, was also observed (Figure 3C and Supplemental Figure 6A). Interestingly, we did not observe any significant increase in expression of *Pik3ca* in p110 δ ^{-/-} HSCs or of *Pik3cd* in p110 α ^{-/-} HSCs or LMPPs in this dataset, suggesting that compensation between these isoforms at the transcriptional level is unlikely (Supplemental Tables 1 and 2). Furthermore, there were few overlapping dysregulated genes among the 3 comparisons, suggesting distinct roles for each isoform in regulating gene expression within this HSC-enriched population (Figure 3C). It is possible that these distinct gene expression patterns reflect enrichment for specific cell populations among the different genotypes, even within the purified Lin⁻c-Kit⁺Flk2⁻CD150⁺CD48⁻ bulk cell population. Consistent with this, we observed by gene set enrichment analysis (GSEA) using the Molecular Signatures Database (MSigDB; Broad Institute) positive enrichment of a previously published mouse long-term HSC-associated gene set in p110 δ ^{-/-} HSCs compared with WT HSCs (Supplemental Figure 6A) (17). Similarly, we observed positive enrichment of another HSC-associated gene set in DKO HSCs, when compared with either p110 α ^{-/-} or p110 δ ^{-/-} HSCs (Supplemental Figure 6, B and C). Furthermore, we saw negative enrichment of a lymphoid stem cell signature in DKO HSCs compared with p110 δ ^{-/-} HSCs (Supplemental Figure 6D). These results suggest that p110 δ plays important roles in regulating the stem cell-associated program, while p110 α and p110 δ play redundant roles in early B lineage specification.

GSEA also revealed that the most significantly negatively enriched gene sets in DKO HSCs compared with WT HSCs were those associated with cell cycling, such as HALLMARK_G2M_CHECKPOINT and HALLMARK_E2F_TARGETS (Figure 3D and Supplemental Table 3). As expected, we observed negative enrichment of gene sets associated with PI3K/mTOR signaling, which validates our microarray

Figure 3. Downregulation of gene sets associated with cell cycle progression and inflammatory signaling in DKO HSCs. (A) Experimental design for BM transplantation and sorting of HSCs and LMPPs for microarray analysis. BM cells of each genotype (CD45.2⁺) were transplanted from one donor into 7 WT recipients (CD45.1⁺) each, and the harvested BM from each set of 7 transplanted mice was pooled for sorting and RNA synthesis. Transplantation was performed 3 times for each donor genotype, resulting in a total of 3 replicates per genotype. (B) Heatmap of genes differentially expressed between DKO HSCs and WT HSCs. (C) Venn diagrams representing the total number of differentially upregulated or downregulated genes between HSCs of each genotype compared with WT HSCs (>2-fold change with $P < 0.05$). (D) Representative GSEA plots show some of the gene sets with the highest negative enrichment scores (NES) when compared with our DKO versus WT HSC gene set. (E) Gene ontology (GO) analysis of significantly downregulated genes (>2-fold, $P < 0.05$) in DKO versus WT HSCs.

Figure 4. Downregulation of inflammatory signaling gene sets in DKO LMPPs. (A) Venn diagrams representing the total number of differentially upregulated or downregulated genes between LMPPs of each genotype compared with WT HSCs (>2-fold change with $P < 0.05$). (B) Representative GSEA plots of the gene sets with the lowest negative enrichment score (NES) when compared with our DKO versus WT LMPP gene set. Gene ontology (GO) analysis of significantly downregulated genes (>2-fold, $P < 0.05$) in (C) p110 $\delta^{-/-}$ LMPPs versus WT LMPPs and (D) in DKO LMPPs versus WT LMPPs.

analysis results (Figure 3D and Supplemental Table 3). Interestingly, we also observed significant negative enrichment of gene sets associated with inflammatory signaling in DKO HSCs, such as HALLMARK_TNFA_SIGNALING_VIA_NFKB, compared with both WT HSCs and p110 α -KO HSCs (Figure 3D and Supplemental Table 3). In addition, we observed negative enrichment of several other signaling pathways that have been implicated in HSC self-renewal, including the TGF- β pathway, and the WNT/ β -catenin pathway (Supplemental Table 3). Gene ontology enrichment analysis (Gene Ontology Consortium) of the genes that were downregulated greater than 2-fold in DKO HSCs compared with WT HSCs with $P < 0.05$ also revealed significant enrichment of multiple gene sets associated with the cell cycle (Figure 3E). Furthermore, Ingenuity Pathway Analysis (IPA, QIAGEN) of genes that were dysregulated greater than 2-fold in DKO HSCs compared with WT HSCs with $P < 0.05$ also revealed significant downregulation of several pathways associated with the cell cycle (Supplemental Figure 6E).

Interestingly, we also observed negative enrichment of both the HALLMARK_TNFA_SIGNALING_VIA_NFKB gene set and several cell cycle-associated gene sets in p110 δ -KO HSCs compared with WT HSCs (Supplemental Table 1). However, a direct comparison of gene expression in DKO HSCs compared with p110 $\delta^{-/-}$ HSCs revealed more significant downregulation of cell cycle gene sets in DKO HSCs (Supplemental Table 3). In contrast, a comparison of DKO HSCs with p110 $\alpha^{-/-}$ HSCs revealed more pronounced downregulation of the HALLMARK_TNFA_SIGNALING_VIA_NFKB module in DKO HSCs (Supplemental Table 3). We also observed significant positive enrichment of RAS pathway gene sets in p110 $\alpha^{-/-}$, p110 $\delta^{-/-}$, and DKO HSCs compared with WT HSCs, suggesting

Figure 5. DKO HSCs have an impaired cycling response to 5-fluorouracil injection. (A) Cell cycle analysis of HSCs (Lin⁻c-Kit⁺Sca-1⁺CD150⁺CD48⁻ cells) from DKO ($n = 4$), $\delta^{-/-}$ ($n = 3$), or WT;Cre ($n = 3$) BM at 3 weeks after 2 plpC injections. Representative Hoechst/Ki-67 HSC plots are shown from one of 3 independent experiments, with quantification of the frequency of HSCs found in G₀, G₁, or S-G₂-M phase of the cell cycle on the right. (B) Experimental schematic for 5-fluorouracil (5-FU) experiments. (C) Kaplan-Meier survival curve of mice injected with plpC once, followed by a single injection of 200 mg/kg 5-FU (WT;Cre, $n = 6$; $\delta^{-/-}$, $n = 5$; DKO, $n = 8$). Log-rank analysis was used to compare the groups. (D) Serial WBC counts of the mice shown in C. Representative data from one of 3 experiments is shown. (E) Representative cell cycle analysis of HSCs in the BM of mice that were sacrificed at 7 days after 5-FU injection (WT;Cre, $n = 3$; $\delta^{-/-}$, $n = 3$; DKO, $n = 3$). Representative flow cytometry plots from one of 3 experiments are shown. Quantification of the frequency of HSCs found in G₀, G₁, or S-G₂-M phase of the cell cycle is shown on the right. For the experiments in A and E, ANOVA with Tukey's multiple-comparisons test was used. * $P < 0.05$, ** $P < 0.01$. Data represent mean $\pm$ SEM.

that the RAS/MAPK pathway may compensate for PI3K deletion in HSCs (Supplemental Table 3). Together, these data suggest some overlap between the roles of p110 α and p110 δ in HSCs, and also imply a functional redundancy between these 2 PI3K isoforms in regulating the cell cycle in HSCs.

We also observed significant dysregulation of gene expression in DKO, p110 $\alpha^{-/-}$, and p110 $\delta^{-/-}$ LMPPs compared with WT;Mx1-Cre controls (Figure 4A and Supplemental Table 2). There was a surprisingly limited overlap between the upregulated gene sets of single-KO LMPPs and DKO LMPPs, though this could represent a difference in the progenitor subpopulations contained within the LMPP gate between the

3 genotypes (Figure 1J and Figure 4A). However, there was a high degree of overlap between the down-regulated genes in p110 δ -KO and DKO LMPPs, suggesting that many of these expression changes are caused by the deletion of p110 δ . GSEA revealed significant negative enrichment in multiple inflammatory gene sets in DKO LMPPs compared with WT LMPPs, including HALLMARK_INFLAMMATORY_RESPONSE, HALLMARK_TNFA_SIGNALING_VIA_NFKB, and HALLMARK_IL6_JAK_STAT3_SIGNALING (Figure 4B and Supplemental Table 4). We also observed negative enrichment of similar gene sets in p110 δ -KO LMPPs (Supplemental Table 4). Gene Ontology analysis of the gene set downregulated greater than 2-fold ($P < 0.05$) in p110 $\delta^{-/-}$ LMPPs or DKO LMPPs revealed enrichment for multiple inflammatory and stress response modules (Figure 4, C and D). Consistent with this finding, IPA of these downregulated gene sets confirmed the downregulation of several inflammatory signaling pathways in both p110 δ -KO and DKO LMPPs (Supplemental Figure 6F and Supplemental Tables 5 and 6). These data suggest that p110 δ could be important in the response of both HSCs and LMPPs to inflammatory signals.

p110 α and p110 δ are essential for hematopoietic recovery after 5-FU. Given our findings that p110 α and p110 δ appear to be more important in regulating HSC function under stress, we wondered whether the defect in cell cycle regulation we observed would be accentuated under stress conditions. To address this question, we first performed cell cycle analysis using Hoechst 33342 and Ki-67 staining on p110 $\delta^{-/-}$ and DKO HSPCs at 4 weeks after a single pIpC injection to excise *Pik3ca*. Although most HSCs were quiescent at steady state, we observed a modest increase in the quiescence of DKO HSCs compared with p110 δ -KO and WT HSCs (Figure 5A). To examine whether the effects of p110 α and p110 δ deletion on HSC cycling may be secondary to an impaired response to hematopoietic stress, we analyzed the hematopoietic system in p110 $\delta^{-/-}$ and DKO mice after 5-FU administration. A single injection of 5-FU initially causes pancytopenia in mice due to depletion of mature blood cells, followed by cell cycle entry of HSCs and progenitors in order to rapidly replenish the hematopoietic system (22). We treated DKO mice, p110 $\delta^{-/-}$ littermates, and WT;Mx1-Cre control mice with a single 5-FU injection (200 mg/kg) at 3 weeks following a single pIpC injection. We followed blood counts in all groups every 7 days until count recovery. Strikingly, while all of the WT and p110 $\delta^{-/-}$ mice survived 5-FU administration, and recovered normal blood counts within 21 days, 6 of 8 DKO mice died within 15 days after 5-FU injection (Figure 5, B and C). DKO mice remained pancytopenic at 15 days after 5-FU injection, while both control groups recovered their counts within this time period (Figure 5D). To understand the etiology of this pancytopenia, we performed a separate experiment in which all mice were sacrificed at 7 days after a single 5-FU injection (180 mg/kg), and the cell cycle status of BM populations was analyzed. Strikingly, while WT;Mx1-Cre and p110 $\delta^{-/-}$ HSCs were actively cycling at this time point, with only 8%–15% of the cells in G₀, we observed a significant increase in the proportion of DKO HSCs that remained quiescent after 5-FU treatment, with a significant decrease in the proportion of cycling cells (Figure 5E). Because the expression of several HSC markers, such as c-Kit and Sca-1, changes after 5-FU administration, making the gating of HSCs challenging, we also examined the cell cycle profile in other BM cell populations. We observed the same trend toward increased quiescence in myeloid progenitors, Lin⁻Sca-1⁺c-Kit⁺ cells, and total Lin⁻ cells in DKO mice (Supplemental Figure 7). This impaired ability to enter the cell cycle upon stress likely can explain the lack of hematopoietic recovery and poor survival after 5-FU administration in DKO mice.

p110 δ is required for the maximal response of HSPCs to IL-1 β and TNF- α . 5-FU has been reported to increase the levels of several inflammatory cytokines in the BM, including IL-1 α and IL-1 β , and IL-1R signaling is required for myeloid recovery after 5-FU (23). Given the downregulation of inflammatory signaling gene sets that we detected in p110 $\delta^{-/-}$ and DKO HSCs and LMPPs, and the defect in cell cycle entry after 5-FU injection in DKO HSPCs, we wondered whether the observed defect in HSC activation and cell cycle entry after 5-FU was due to the impaired ability of HSPCs to transduce inflammatory signals. One of the most significantly downregulated inflammatory signaling pathways observed in our IPA analysis in DKO LMPPs was the p38 MAPK signaling pathway (Supplemental Figure 6F). Interestingly, p38 MAPK is activated in BM cells after 5-FU treatment and has been found to be essential for promoting cell cycle progression of HSCs during stress hematopoiesis (24). The inflammatory cytokines IL-1 β and TNF- α stimulate signaling pathways through the cell surface receptors IL-1R and TNFR, respectively, which leads to the phosphorylation of p38 MAPK (reviewed in ref. 25). Furthermore, TNF- α is known to signal through the PI3K/AKT pathway in other cell types (9). Therefore, we harvested BM cells from DKO, p110 $\delta^{-/-}$, and WT;Mx1-Cre mice at 3 weeks after pIpC treatment, stimulated lineage-negative cells ex vivo with IL-1 β or TNF- α , and then examined phosphorylation of p38 MAPK in HSPCs and myeloid progenitors by intracellular phospho-flow cytometry. We observed a decreased level of p38 MAPK phosphorylation in both p110 $\delta^{-/-}$ and DKO HSPCs, both at baseline and after stimulation with either IL-1 β or

TNF- α (Figure 6, A–D). To confirm the impaired response of p110 $\delta^{-/-}$ and DKO cells to these inflammatory signals, we also performed Western blotting on c-Kit–enriched BM cells harvested from DKO, p110 $\delta^{-/-}$, or WT;Mx1-Cre mice at 3 weeks after 2 pIpC injections, following ex vivo stimulation with IL-1 β or TNF- α . As expected, we observed a significant decrease in the phosphorylation of AKT at Ser473 in DKO cells, but not in p110 $\delta^{-/-}$ cells, with IL-1 β or TNF- α stimulation (Figure 6E and Supplemental Figure 8A). This confirmed that p110 α and p110 δ play redundant roles in the activation of AKT in hematopoietic progenitors. We also observed a trend toward a decrease in phosphorylation of p38 MAPK in DKO cells in response to IL-1 β or TNF- α , though not to as great an extent as the decrease in AKT phosphorylation in these cells (Figure 6E and Supplemental Figure 8B). Overall, these data suggest that p110 α and p110 δ are both required for optimal activation of signaling downstream of IL-1 β or TNF- α in hematopoietic stem and progenitor cells in the BM.

To determine whether AKT or p38 MAPK signaling is essential for myelopoiesis after 5-FU administration, we injected WT and DKO mice with 200 mg/kg 5-FU, and then euthanized the mice after 24 hours, harvested the BM, and performed colony-forming assays in methylcellulose media with myeloid growth factors (M3434, STEMCELL Technologies). We added either the AKT inhibitor MK2206 or the p38 MAPK inhibitor SB203580 to the WT BM cultures. As expected, DKO BM cells formed significantly fewer myeloid colonies than WT;Cre⁺ control BM cells after 5-FU injection (Figure 6F). Interestingly, treatment of WT BM with the AKT inhibitor, but not with the p38 MAPK inhibitor, could phenocopy the results with DKO cells, suggesting that activation of AKT, but not of p38 MAPK, is critical for myeloid differentiation after 5-FU administration (Figure 6F).

p110 α and p110 δ are not essential for HSC cell cycle entry in response to LPS administration. To determine whether p110 α and p110 δ are also required for the transduction of inflammatory cytokines in HSPCs under conditions simulating bacterial infection, we examined the response of p110 $\delta^{-/-}$, DKO, and WT;Mx1-Cre HSPCs to LPS, a bacterial cell wall product (26). LPS is the best-known ligand for TLR signaling and can directly stimulate HSCs in vivo to induce HSC cycling (3). In HSPCs, this process is mediated via the TLR4/TRIF/ROS/p38 pathway and not through MyD88 signaling (3). Upon a single injection of 5 μ g LPS, HSPCs enter the cell cycle within 24 hours (27). To examine the response of DKO HSPCs to LPS, we treated DKO mice, p110 $\delta^{-/-}$ littermates, and WT;Mx1-Cre controls with a single pIpC injection and then administered a single LPS injection after 4 weeks. Mice were sacrificed after 24 hours to examine the cell cycle profile of HSPCs using Hoechst 33342 and Ki-67 staining. Surprisingly, we found that DKO and p110 $\delta^{-/-}$ HSPCs had cell cycle profiles similar to those of WT;Mx1-Cre HSPCs at 24 hours after a single LPS injection (Figure 7A). Consistent with this, we observed phosphorylation of both p38 MAPK and AKT in both WT and DKO HSPCs in response to in vivo LPS administration (Supplemental Figure 8C). Therefore, we conclude that p110 α and p110 δ are not required for LPS to activate HSPCs.

Higher levels of inflammatory cytokines and chemokines were detected in the BM after LPS injection than after 5-FU injection. In addition to the direct action of LPS on HSCs via TLR, LPS can also activate HSCs indirectly, by stimulating the secretion of inflammatory cytokines and chemokines by hematopoietic cells and by the BM microenvironment (28). To determine whether differences in cytokine or chemokine levels in the BM could account for some of the differences in HSPC activation that we observed between 5-FU and LPS, we measured the expression of inflammatory cytokines and chemokines in the BM serum at baseline and after injection of 5-FU or LPS using a multiplex Luminex assay. Interestingly, we observed significantly elevated levels of IL-1 β , IL-1 α , and MIG (CXCL9) after exposure to LPS, but not to 5-FU, in WT BM under these conditions (Figure 7B and Supplemental Figure 9A). We also observed significantly increased expression of IL-1 β and MIG in p110 $\delta^{-/-}$ or DKO BM after LPS exposure (Figure 7B). In contrast, 5-FU only increased the expression of MIG in DKO BM, but did not increase the levels of IL-1 β in p110 $\delta^{-/-}$ or DKO BM (Figure 7B). This suggests that LPS but not 5-FU induces increased secretion of some inflammatory cytokines and chemokines in the BM.

DKO HSCs can be activated by in vivo administration of IL-1 β . It is known that IL-1 β can have multiple effects in vivo, both cell-autonomous and -nonautonomous. To test the hypothesis that administration of a high dose of IL-1 β in vivo can overcome the impaired sensing of IL-1 β by DKO HSCs, we injected DKO, p110 $\delta^{-/-}$, or WT;Mx1-Cre mice with 0.5 μ g IL-1 β , a dose that we found can induce cycling of WT HSCs in vivo (29) (Supplemental Figure 9B). We then euthanized the mice at 24 hours after injection, harvested the BM, and performed cell cycle analysis with Hoechst and Ki-67. We found that more than 50% of HSCs from all 3 groups of mice entered the cell cycle in response to IL-1 β , though there was a trend toward more quiescence

Figure 6. Optimal signal transduction of IL-1 β or TNF- α in HSPCs requires p110 α and p110 δ . (A) Phospho-flow cytometry analysis of p-p38 MAPK with IL-1 β stimulation in HSPCs (Lin^c-Kit⁺-Sca-1⁺ BM cells). Representative histograms are shown. (B) Quantification of p-p38 median fluorescence intensity (MFI) for the experiment shown in A (WT;Cre, $n = 3$; $\delta^{-/-}$, $n = 3$; DKO, $n = 3$). Data are representative of 3 independent experiments. (C) Phospho-flow cytometry analysis of p-p38 MAPK with TNF- α stimulation in HSPCs. Representative histograms are shown. (D) Quantification of p-p38 MFI for the experiment shown in C (WT;Cre, $n = 3$; $\delta^{-/-}$, $n = 3$; DKO, $n = 3$). Data are representative of 3 independent experiments. (E) Western blot analysis of c-Kit-enriched BM cells, which were serum starved and then stimulated with IL-1 β or TNF- α . Representative data from one of 4 independent experiments are shown. Quantification of Western blot data from all experiments is shown in Supplemental Figure 8. (F) BM colony forming assays in methylcellulose media using BM harvested from WT ($n = 6$) or DKO ($n = 6$) mice at 24 hours after 5-FU injection. The AKT inhibitor MK2206 (AKTi; 1 μ M) or p-38 MAPK inhibitor SB 203580 (p38MAPKi; 10 μ M) was added to the methylcellulose where indicated. For the experiments in A–D and F, ANOVA with the Tukey's multiple-comparison test was used. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$. Data represent mean $\pm$ SEM.

in the DKO group (Figure 7C). Furthermore, we found that in vivo administration of IL-1 β can also induce phosphorylation of both p38 MAPK and AKT (Ser473) in HSPCs in p110 $\delta^{-/-}$ and DKO mice (Supplemental Figure 9C). This supports the hypothesis that the inefficient transduction of IL-1 β signals in DKO HSCs can be at least partially overcome in vivo by high levels of exogenous IL-1 β .

Figure 7. LPS signaling in HSPCs does not require p110 α and p110 δ . (A) Representative cell cycle flow cytometry plots of HSCs at 24 hours after a single injection of LPS (5 μ g). Quantification of the cell cycle analysis is shown on the right. (B) Luminex cytokine assays in BM serum from mice treated with plpC only ($n = 6$ per group), plpC followed by LPS (5 μ g; $n = 6$ per group), or plpC followed by 5-FU (180 mg/kg; $n = 6$ per group). (C) Representative cell cycle flow cytometry plots of HSCs at 24 hours after a single i.p. injection of IL-1 β (0.5 μ g). Quantification of the cell cycle analysis is shown on the right. (D) Model figure summarizing the proposed roles of p110 α and p110 δ in HSPCs in inflammatory signaling. In the setting of chemotherapy treatment, some inflammatory cytokines and chemokines cannot be efficiently transduced by DKO HSCs, leading to impaired HSC activation and impaired multilineage differentiation. However, increased levels of some inflammatory cytokines or chemokines in the BM after LPS administration can compensate for impaired AKT signal transduction in DKO HSCs and can lead to HSC activation. For the experiments in A–C, ANOVA with the Tukey's multiple-comparisons test was used. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Data represent mean $\pm$ SEM.

Discussion

It has been reported that $p110\delta^{-/-}$ mice have normal blood counts, but the HSC phenotype of these mice has not been previously described (16). Surprisingly, we discovered that while HSCs and progenitors were largely unaffected in $p110\delta^{-/-}$ BM, and $p110\delta^{-/-}$ HSC function was normal in competitive repopulation experiments, gene expression was altered in $p110\delta^{-/-}$ HSCs and LMPPs. The most pronounced changes we observed in our microarray analysis in $p110\delta^{-/-}$ HSCs were negative regulation of multiple inflammatory signaling pathways and positive enrichment in HSC-associated genes compared with WT HSCs. In contrast, we observed fewer changes in gene expression in $p110\alpha^{-/-}$ HSCs, with the predominant changes reflecting a downregulation in cell cycle-associated gene sets. Only upon deletion of both $p110\alpha$ and $p110\delta$ did we observe a decrease in AKT activation in HSPCs upon IL-1 β or TNF- α stimulation and a significant defect in HSC function under stress. Therefore, our data suggest that $p110\delta$ may play an important role in HSPCs in transducing some inflammatory signals, though $p110\alpha$ can functionally compensate in $p110\delta^{-/-}$ HSCs.

Our studies in $p110\alpha/\delta$ -DKO mice suggest that $p110\alpha$ and $p110\delta$ are not critical for HSC function at steady state. The decrease we observed in the numbers of LMPPs and myeloid progenitors in DKO mice, and the competitive repopulation defect that primarily affects the B cell lineage, suggest defects at the level of HSC differentiation or proliferation, as well as in B lineage specification. These findings are consistent with the reported phenotype of compound deletion of the PI3K regulatory isoforms $p85\alpha$ and $p85\beta$ in fetal liver HSCs, which were found to be defective in proliferation and in competitive repopulation, particularly in lymphoid lineages (30). However, direct comparisons of adult and fetal HSCs are difficult, given the more proliferative status of fetal HSCs. Using CD2-Cre, which only deletes in B cells, Ramadani et al. have shown that in the absence of activity of both $p110\alpha$ and $p110\delta$, pre-B cell receptor signaling failed to promote developmental progression of B cell progenitors in the BM (21). In our Mx1-Cre system, which allows for deletion of $p110\alpha$ and $p110\delta$ in HSCs and early progenitors, we have uncovered evidence of an earlier defect in B lineage specification. More investigation will be necessary to understand the mechanisms by which $p110\alpha$ and $p110\delta$ promote early fate decisions toward the B cell lineage.

Our results also suggest that additional compensatory mechanisms exist in HSCs that allow for multilineage hematopoietic differentiation and self-renewal at steady state. One possibility is that another PI3K isoform, such as $p110\beta$, could compensate for $p110\alpha$ and $p110\delta$ in HSC self-renewal. Alternatively, a parallel signaling pathway could substitute for PI3K in HSCs. For example, our gene expression analyses suggest the upregulation of the RAS/MAPK signaling pathway in PI3K-KO HSCs, which could be a potential compensatory pathway. This would be consistent with a recent report suggesting that both the MEK/ERK and AKT/mTORC1 pathways are activated in HSCs, and that there is feedback regulation between these two pathways in controlling HSC quiescence (31).

In the secondary transplantation recipients, we observed a multilineage reconstitution defect, more severe than the defect that was observed after primary transplantation. Therefore, we conclude that while DKO HSPCs are capable of reconstituting the myeloid lineage at steady state, they are impaired during emergency myelopoiesis. This suggests that while other PI3K isoforms, or parallel signaling pathways, may functionally compensate for $p110\alpha$ and $p110\delta$ in HSCs at steady state, they are critical for HSC self-renewal and differentiation under stress conditions.

Our findings that cell cycle-associated gene sets are significantly downregulated in DKO HSCs are consistent with the known role of the PI3K/AKT pathway in direct regulation of cell cycle intermediates, such as CDK4 (32, 33). Surprisingly, we observed significantly decreased survival of DKO mice after a single 5-FU injection, associated with inefficient HSC cell cycle entry. Interestingly, in our previous studies we did not observe any significant defects in hematopoietic recovery in $p110\alpha$ -lox-lox;Mx1-Cre conditional knockout mice after 5-FU administration (12). In addition, we did not observe any significant defect in the survival of $p110\delta^{-/-}$ mice after 5-FU administration, or in the ability of $p110\delta^{-/-}$ HSPCs to enter the cell cycle. Therefore, we conclude that $p110\alpha$ and $p110\delta$ play critical but redundant roles in promoting HSPC cell cycle entry under chemotherapy-induced stress.

A recent report has implicated phosphatase and tensin homolog (PTEN) in the inflammatory response in hematopoietic cells (34). Since PTEN antagonizes PI3K signaling, leading to AKT activation, our results are consistent with those of Porter et al., who showed that *Pten* deletion sensitizes HSCs to growth factors and inflammatory signals. We and others have previously reported on the importance of the PI3K/AKT

pathway in maintaining the balance between quiescence and proliferation of adult HSCs in studies of AKT pathway activation (35–38). Given the increased cycling of HSCs that was observed in these studies, our observation of increased quiescence in PI3K-KO HSCs is consistent with an important role for PI3K/AKT in controlling HSC quiescence under conditions of hematopoietic stress.

Surprisingly, we observed that DKO HSPCs enter the cell cycle normally upon stimulation with LPS, suggesting that p110 α and p110 δ are not absolutely required for HSPC cell cycle entry or progression. Therefore, direct regulation of cell cycle intermediates cannot completely explain the stress hematopoiesis phenotype of DKO mice after 5-FU administration. Our data support the hypothesis that reduced sensing of inflammatory cytokines and chemokines is a potential mechanism for maintained DKO HSC quiescence in response to some forms of hematological stress, such as chemotherapy. Our results suggest that the increased secretion of some inflammatory cytokines and chemokines in the BM upon LPS exposure can overcome the inefficient transduction of such signals by DKO HSPCs, leading to sufficient activation of downstream signaling to induce HSC cycling (Figure 7D). Consistent with this hypothesis, we show that in vivo administration of high-dose exogenous IL-1 β can activate HSC cycling and downstream signal transduction in DKO HSCs. It is possible that this effect with in vivo administration of IL-1 β is mediated by both cell-autonomous and -nonautonomous functions of IL-1 β , and may reflect indirect effects of IL-1 β on other cell types in the BM. Therefore, the roles of p110 α and p110 δ in the sensing of specific cytokines or chemokines in HSCs in vivo, and their relative roles in HSC activation, are still unclear. For example, while we observed decreased activation of p38 MAPK and AKT with TNF- α stimulation in DKO HSCs and progenitors, the levels of TNF- α in the BM were not significantly different between these two conditions. Therefore, impaired sensing of TNF- α alone is unlikely to explain the impaired activation of DKO HSCs after 5-FU administration. In addition, it is also possible that direct activation of TLR in DKO HSCs by LPS could play a role in HSC activation. It is likely that proper sensing of multiple inflammatory signals via p110 α and p110 δ is required for full HSC activation in response to stress stimuli.

In conclusion, our analysis of p110 α and p110 δ deletion in the hematopoietic system has uncovered substantial redundant roles for these two PI3K isoforms in HSC activation in response to hematopoietic stress and in the sensing of some inflammatory signals in HSCs, such as IL-1 β . Our findings of the importance of the PI3K/AKT pathway in the HSC stress response also have important clinical implications. Several isoform-selective PI3K inhibitors are approved to treat lymphoid malignancies (39, 40). For instance, idelalisib, which selectively targets p110 δ , is used to treat chronic lymphocytic leukemia (CLL) and indolent non-Hodgkin's lymphoma. However, it has been recently reported that patients treated with idelalisib experience an unexpectedly high rate of neutropenia, especially when it is administered in combination with chemotherapy (41). Copanlisib, a PI3K inhibitor with activity against the p110 α and p110 δ isoforms, is approved for indolent lymphoma and is being tested in combination with chemotherapy (42). Our results in the DKO mouse model suggest that caution should be exercised when PI3K inhibitors targeting these isoforms are combined with chemotherapy. However, differences in the immune response between mice and humans should also be taken into account when applying these results to the clinic. Clinical trials using alternating sequencing of PI3K inhibitors with chemotherapy should be considered to directly examine the potential for these approaches to minimize toxicity in patients.

Methods

Mice. Mice were maintained under pathogen-free conditions in barrier facilities at the Dana-Farber Cancer Institute (DFCI) and Albert Einstein College of Medicine (AECOM). *Pik3cd* germline KO mice (16) were provided as a gift from James Ihle (St. Jude's Children's Hospital, Memphis, Tennessee, USA) as frozen embryos, re-derived by the Boston Children's Hospital Transgenic Facility, and backcrossed to the C57BL/6 strain for a total of 9 generations. To generate DKO mice, *Pik3ca*-lox-lox;Mx1-Cre mice (12) were crossed with *Pik3cd*^{-/-} mice, resulting in progeny with the genotypes *Pik3ca*-lox-lox;*Pik3cd*^{-/-};Mx1-Cre (DKO) and *Pik3ca*-lox-lox;*Pik3cd*^{-/-} (p110 δ ^{-/-}) littermate controls. pIpC (Sigma-Aldrich) was dissolved in HBSS, and 250 μ g was injected i.p. into 4- to 8-week-old mice twice on nonconsecutive days, unless otherwise indicated. Experimental mice always included both males and females. Peripheral blood was obtained under isoflurane anesthesia by retro-orbital or facial vein bleeding. Genotyping of tail genomic DNA and excision status of exon 1 of *Pik3ca* in the peripheral blood or BM DNA and deletion of *Pik3cd* were determined by PCR as described previously (13, 16). For each experiment, excision of *Pik3ca* was confirmed in the peripheral blood of DKO mice at 3–4

weeks after pIpC injection by peripheral blood analysis. Peripheral blood counts were analyzed on Hemavet (Drew Scientific), Forcyte (Oxford Science), or Genesis (Oxford Science) blood analyzers.

Colony-forming unit assays. Whole BM cells were subjected to RBC lysis (RBC lysis solution, QIAGEN) and resuspended in IMDM media containing 10% FBS and 1% penicillin-streptomycin. Cells were seeded at 10,000 cells per dish in methylcellulose semisolid medium (M3434, STEMCELL Technologies). The colonies were scored and phenotyped at 7–10 days after plating.

Colony-forming unit assays after *in vivo* 5-FU treatment. Whole BM cells were subjected to RBC lysis and resuspended in IMDM media containing 10% FBS and 1% penicillin-streptomycin and 1 μ M AKT inhibitor (MK-2206 2HCl, Selleckchem) or 10 μ M P38 MAPK inhibitor (SB 203580, Selleckchem). The colonies were counted after 7 days of plating.

Flow cytometry. For the flow cytometry analysis of HSPC populations, RBC-lysed BM cells were stained with a panel of lineage and HSC antibodies listed in Supplemental Table 7. Flow cytometry analysis was performed on the BD FACS LSR II. The absolute number of each population per femur was calculated based on the number of whole BM cells flushed from one femur.

Cell cycle analysis. BM cells were first stained with cell surface antibodies (Supplemental Table 7), followed by mild fixation and permeabilization using the FIX & PERM Cell Fixation & Permeabilization Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The fixed and permeabilized cells were incubated with Ki-67-FITC overnight. Hoechst 33342 (Thermo Fisher Scientific) was used to stain DNA at 25 μ g/ml. Flow cytometric analysis was performed on the BD FACS LSR II.

5-FU treatment. Mice were given a single pIpC injection (250 μ g) at 4–6 weeks of age. After 3 weeks, they were injected once i.p. with 200 mg/kg 5-FU (Sigma-Aldrich). Blood was collected 1 week prior to 5-FU and on days 7, 14, 21, and 28 after 5-FU administration. For cell cycle analysis after 5-FU treatment, mice were injected with 180 mg/kg i.p. 5-FU at 3 weeks after a single pIpC injection, and then sacrificed on day 7 after the 5-FU injection.

LPS/IL-1 β injection. After a single pIpC injection (250 μ g) at 4–6 weeks of age, mice were injected once i.p. with 5 μ g LPS (InvivoGen Ultrapure LPS, *E. coli* 0111:B4) or 0.5 μ g IL-1 β (PeproTech) and then sacrificed after 24 hours for BM analysis.

Western blotting. BM cells were enriched for c-Kit⁺ cells using CD117-bound magnetic beads (Miltenyi Biotec) according to the manufacturer's protocol. The cells were serum starved in IMDM containing 2% FBS for 1 hour at 37°C. Cells were stimulated with 10 ng/mL TNF- α or 100 ng/mL IL-1 β at 37°C for 10 or 20 minutes, respectively. Cells were centrifuged immediately after stimulation, media was aspirated, and the cell pellets were stored at –80°C. Cell lysates were prepared in NP-40 low-salt lysis buffer (Boston BioProducts) containing Protease and Phosphatase Inhibitor (Thermo Fisher Scientific), 100 μ g/mL PMSF (Roche), and 1 mM sodium orthovanadate (Sigma-Aldrich). Lysates were kept on ice for 30 minutes and then cleared by centrifugation at 14,000 g for 10 minutes at 4°C. Protein concentration was determined with Bradford assay using Bio-Rad Protein Assay Dye Reagent. Briefly, Western blotting was performed by loading 15 μ g cell lysate per well on a NuPAGE Bis-Tris 10% polyacrylamide gel (Thermo Fisher Scientific). Nitrocellulose 0.45- μ m membrane (Millipore) was used for transfer. Blocking was done in TBST 5% nonfat milk. The primary antibodies were as follows: phospho-p38 MAPK (Thr180/Tyr182) 1:1000 (catalog 4511S, Cell Signaling Technology), phospho-AKT 1:1000 (catalog 4060S, Cell Signaling Technology), vinculin 1:5000 (catalog V9131, Sigma-Aldrich); β -actin 1:5000 (A2228, Sigma-Aldrich). The secondary antibodies, anti-mouse 680 and anti-rabbit 800 (LI-COR), were used at dilutions of 1:5000 or 1:10,000, respectively. Membranes were developed on the LI-COR Odyssey instrument. Signal quantification and background adjustments were performed using LI-COR Image Studio Lite 5.2 software.

BM and HSC transplantation. For noncompetitive BM transplantation, 1×10^6 whole BM CD45.2⁺ donor-derived cells from C57BL/6 inbred mice were transplanted into lethally irradiated B6.SJL (CD45.1⁺) mice at 6–8 weeks of age. For lethal irradiation, mice were given a single dose of 950 cGy at least 3 hours prior to transplantation. Donor BM was injected into the retro-orbital plexus. Recipient mice were given Bactrim water for 3 weeks after transplantation. For competitive BM transplantation, 1×10^6 donor-derived whole BM CD45.2⁺ cells and 1×10^6 competitor CD45.1⁺ BM cells were transplanted into lethally irradiated B6.SJL mice. After confirmation of engraftment by peripheral blood counts, recipient mice were injected once i.p. with pIpC at 4 weeks after transplantation. For secondary HSC transplantation, 250 sorted CD45.2⁺ LT-HSCs (CD150⁺CD48⁺Flk2⁺Lin[–]c-Kit⁺Sca-1⁺) from the BM of primary noncompetitive transplant recipient mice were mixed with 250,000 WT CD45.1⁺/CD45.2⁺ whole BM cells from C57BL/6-B6.SJL F₁ hybrid mice and transplanted into lethally irradiated 6- to 8-week-old B6.SJL mice. Peripheral blood

was collected from recipient mice every 4 weeks, and animals were sacrificed after at least 16 weeks of observation after transplantation, or for 16 weeks after pIpC injection.

Microarray analysis. Lethally irradiated 6- to 8-week-old female B6.SJL mice were transplanted with 2×10^6 whole BM cells from *Pik3ca-lox-lox*;Mx1-Cre, *Pik3cd*;Pik3ca-lox-lox, *Pik3cd*;Pik3ca-lox-lox;Mx1-Cre, or WT;Mx1-Cre mice ($n = 7$ per group). Peripheral blood was collected at 4 weeks after transplantation to confirm engraftment. All transplant recipients were injected once i.p. with 250 μ g pIpC. Excision of *Pik3ca* was confirmed by examining the blood counts at 3 weeks after pIpC administration. At 8 weeks after transplantation, BM cells were harvested from femurs, tibiae, ilia, and vertebrae. Bones were gently crushed in RPMI medium (Life Technologies). Low-density BM mononuclear cells (BMMNCs) were isolated by density gradient centrifugation using Ficoll Histopaque-1083 (Sigma-Aldrich). BMMNCs were stained with anti-mouse biotin-labeled lineage antibodies (CD3e, CD4, CD8a, B220, Ter119, CD19, and Gr-1) for 30 minutes at 4°C. The lineage-stained cells were then incubated with Biotin Binder Dynabeads (Thermo Fisher Scientific, catalog 11047) and depleted with a magnet according to the manufacturer's protocol. To purify HSC and LMPP populations, Lin[−] cells were stained with a panel of fluorochrome-conjugated monoclonal anti-mouse cell surface antibodies (CD45.2–PerCP, CD45.1-FITC, Flk2-PE, IL-7R–PECy7, Sca-1–BV605, c-Kit–APC, CD150–Pacific blue, CD48–Alexa Fluor 700, Streptavidin–APC Cy7; see Supplemental Table 7). The donor-derived HSC and LMPP cell populations were sorted according to the gating strategy in Figure 3A using the Aria II instrument (BD Biosciences). Cells were directly sorted into 50 μ l RNA extraction buffer (ARCTURUS PicoPure RNA Isolation Kit; Life Technologies, Invitrogen) and stored at -80°C . RNA isolation was performed using the ARCTURUS PicoPure RNA Isolation Kit according to the manufacturer's protocol. RNA quantification was performed using the RNA Quantification Kit for SYBR Green I and ROX Passive Reference Dye (Thermo Fisher Scientific, catalog 902905). Total RNA was amplified and hybridized to the Gene Chip Mouse Transcriptome Pico Assay 1.0 (Affymetrix, catalog 902663) using the GeneChip Hybridization, Wash, and Stain Kit (catalog 900720). Raw data were analyzed for quality control using Expression Console software (Affymetrix). After quality control, microarray data were analyzed by GSEA using MSigDB software (<http://software.broadinstitute.org/gsea/index.jsp>), Transcriptome Analysis Software (Affymetrix), Gene ontology enrichment analysis (Gene Ontology Consortium; <http://geneontology.org>), and by IPA software (QIAGEN). Venn diagrams were generated using Bioinformatics & Evolutionary Genomics software (<http://bioinformatics.psb.ugent.be/webtools/Venn>). The microarray data were deposited in the NCBI's Gene Expression Omnibus database (GEO GSE118053).

Phospho-flow cytometry (after ex vivo stimulation). Mice were injected twice with pIpC (250 μ g) at 4–8 weeks of age on nonconsecutive days, and were sacrificed 4–6 weeks after the first pIpC injection. BM cells were harvested and RBC lysis was performed. Phospho-flow cytometry was performed based on Kalaitzidis et al., with the following modifications (43). Cells were stained with a cocktail of biotinylated lineage antibodies (CD127, CD3e, CD4, CD8a, B220, CD19, Ter119, and Gr-1) for 30 minutes. The subsequent depletion of Lin[−] cells was performed using Biotin Binder Dynabeads according to the manufacturer's protocol. Lin[−] cells were serum starved in IMDM containing 2% FBS for 1 hour at 37°C. Cells were stimulated with 10 ng TNF- α or 50 ng IL-1 β for 10 or 20 minutes, respectively, at 37°C. Cells were immediately fixed after stimulation with 16% paraformaldehyde for 10 minutes at room temperature (Electron Microscopy Sciences, catalog 15710). The cells were then centrifuged and permeabilized with cold 100% acetone for 10 minutes on ice. Fixed cells were stained with a panel of HSPC surface markers (Streptavidin–APC Cy7, c-Kit–PE, Sca-1–BV421) and intracellular phospho-p38 MAPK (Thr180/Tyr182)–Alexa Fluor 647 (Cell Signaling Technology, catalog 4552S) antibodies.

Phospho-flow cytometry (after in vivo stimulation). Mice were injected twice with pIpC. At 2–3 weeks after pIpC injection, mice were injected i.p. with 0.5 μ g IL-1 β or 5 μ g LPS and sacrificed after 24 hours. BM cells were harvested, and RBC lysis was performed. Cells were stained with a cocktail of lineage antibodies as described in the previous section. Cells were stained with a panel of HSPC surface markers (Streptavidin–APC Cy7, c-Kit–PE, Sca-1–PE–Cy7). Cells were then fixed and permeabilized using the FIX & PERM Cell Fixation & Cell Permeabilization Kit according to the manufacturer's protocol. The fixed and permeabilized cells were stained with intracellular phospho-p38 MAPK (Thr180/Tyr182)–Alexa Fluor 647 at 3:100 and phospho-AKT (Ser473)–Alexa Fluor 488 (Cell Signaling Technology, catalog 2336S) at 1:20 dilutions.

Cytokine assays. BM sera were collected from mice injected with pIpC alone, or from mice injected with pIpC and then once with LPS (5 μ g) or with 5-FU (180 mg/kg) at 4–6 weeks after pIpC administration. Animals were sacrificed at 24 hours after LPS injection or at 7 days after 5-FU injection. One femur of each mouse was flushed with 200 μ l RPMI with no additional supplements. The BM cell suspension was centrifuged, and BM

sera were collected and stored at -80°C for cytokine array analysis. The levels of cytokines in the BM sera were measured using the MILLIPLEX MAP Mouse custom Cytokine/Chemokine Magnetic Bead Panel — Immunology Multiplex Assay (MCYTOMAG-70K-15; EMD Millipore) according to the manufacturer's protocol. The data were acquired using the Luminex 200 instrument.

Microscope image acquisition. Histology images were acquired at room temperature using a Zeiss Axiovert 200M Microscope with digital camera, using a $\times 10$ (for $\times 100$ images) or $\times 40$ objective (for $\times 400$ images). Image acquisition was performed using AxioVision software.

Reagents. Supplemental Table 7 lists the flow cytometry antibodies used. All other reagents are listed in the respective sections in Methods.

Statistics. GraphPad Prism 7 and Microsoft Excel 2010 were used for all statistical analyses. For the comparison of 2 experimental groups, unpaired 2-tailed Student's *t* test was used. For comparisons of more than 2 groups, ANOVA was used with the Tukey's multiple-comparisons test, unless otherwise indicated. For survival analysis, log-rank analysis was used. The specific statistical test used is also indicated in each figure legend. In all graphs, error bars indicate mean $\pm$ SEM. A *P* value less than 0.05 was considered significant.

Study approval. All animal experiments were approved by the Institutional Animal Care and Use Committee under protocols 09-069 (DFCI), 20140412 (AECOM), and 20170206 (AECOM).

Author contributions

SH and KG planned and performed experiments, analyzed the data, and wrote the manuscript. TS, MT, ROO, KA, LO, TH, IK, and TSM performed experiments. BB assisted with data analysis. AA performed experiments and assisted with data interpretation. EMP, JJZ, and TMR assisted with the planning of experiments and data interpretation.

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