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FAK mediates a compensatory survival signal parallel to PI3K-AKT in *Pten*-null T-ALL cells

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Summary

Mutations and inactivation of phosphatase and tensin homolog deleted from chromosome ten (*PTEN*) are observed in 15–25% cases of human T-cell acute lymphoblastic leukemia (T-ALL). *Pten* deletion induces myeloproliferative disorders (MPD), acute myeloid leukemia (AML), and/or T-ALL in mice. Previous studies attributed *Pten*-loss-related hematopoietic defects and leukemogenesis to excessive activation of PI3K/AKT/mTOR signaling. Although inhibition of this signal dramatically suppresses the growth of *PTEN*-null T-ALL cells *in vitro*, treatment with inhibitors of this pathway do not cause a complete remission *in vivo*. Here we report that focal adhesion kinase (Fak), a protein substrate of Pten, also contributes to T-ALL development in *Pten*-null mice. Inactivation of FAK signaling pathway by either genetic or pharmacologic methods significantly sensitizes both murine and human *PTEN*-null T-ALL cells to PI3K/AKT/mTOR inhibition when cultured *in vitro* on feeder layer cells or a matrix and *in vivo*.

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Contributions

D.Y, J.X, A.V, W.W, R.S, G.S, S.N, E.B, P.K, A.K, P.B, M.N, N.Z-Le, and J.Z performed the research and analyzed the data. J.Z and D.Y designed the research. J.Z, D.Y, P.B, N.Z-L. and J.X wrote the paper.

Disclosure of Potential Conflicts of Interest

The authors declare no conflicting financial interests.

Introduction

PTEN mutations are detected in 15-25% of T-ALL patients (Gutierrez et al., 2009; Jotta et al., 2010; Zurbier et al., 2012). Inactivation of *PTEN* and constitutive activation of its downstream signaling pathways are associated with early treatment failure, drug resistance, and poor prognosis (Jotta et al., 2010; Piovan et al., 2013). Thus, it should be useful to completely elucidate the roles of the *PTEN* downstream signaling pathways in the pathogenesis of T-ALL for development of less toxic targeted therapies for this fatal disease.

PTEN possesses both lipid and protein phosphatase activities (Lee et al., 1999; Tamura et al., 1998). It represses growth factor/cytokine-stimulated cell growth and survival by repressing PI3K/AKT/mTOR signaling through dephosphorylation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) (Stambolic et al., 1998). It also dephosphorylates FAK, thus inhibiting integrin-induced adhesion and migration by suppressing the turnover of focal adhesions and cytoskeletal reorganization (Gu et al., 1998; Guo and Giancotti, 2004; Tamura et al., 1998). It was reported that T-ALL development in *Pten*-null mice could be repressed by inactivation of the PI3K/Akt/mTOR signaling (Kalaitzidis et al., 2012; Subramaniam et al., 2012). Preclinical studies showed that PI3K/AKT/mTOR inhibitors induce apoptosis and repress the growth of T-ALL cell lines and primary human T-ALL cells, suggesting that this signaling pathway might be a promising target for T-ALL therapy (Evangelisti et al., 2011; Subramaniam et al., 2012). However, PI3K/AKT/mTOR inhibition did not cause complete remission *in vivo* (Rodon et al., 2013), suggesting that there might be an additional survival signal which compensates for inhibition of PI3K/AKT signaling in *PTEN*-null T-ALL. As an important target of *PTEN*, the role of FAK has not been studied in the etiology and progression of T-ALL induced by *PTEN* mutations.

FAK is a non-receptor tyrosine kinase. In response to integrin and/or chemokine stimulation, activated FAK promotes cell adhesion, spreading, motility, proliferation and anchorage-dependent survival by inducing the activation of its downstream signals, including p130Cas/Rac/CDC42, Rho/Gap/RhoA, ERK/MAPK, Wnt/ β -catenin and NF- κ B (Desgrosellier and Cheresch, 2010; Guan, 2010; Mitra and Schlaepfer, 2006; Schaller, 2010). Dysregulation of FAK has been detected in multiple cancers, including breast, brain, prostate and liver cancer (Guan, 2010; Mitra and Schlaepfer, 2006; Pylayeva et al., 2009). In these cancers, the activity of FAK generally correlates with increased tumor malignancy, drug resistance and metastasis (Golubovskaya, 2014; Lechertier and Hodivala-Dilke, 2012). FAK also plays an essential role in regulating the self-renewal of cancer stem cells (Guan, 2010). Thus, FAK has been suggested to be a potentially advantageous target for cancer therapy (Golubovskaya, 2014).

Despite the liquid nature of blood and the peripheral circulation of leukemic cells (LCs), the majority of leukemia-initiating cells (LICs) reside in the bone marrow, spleen, and other organs where they adhere to their niches; this could provide *in vivo* signals to activate FAK in LCs (Konopleva and Jordan, 2011). Clinically, increased FAK activity is correlated with high blast cell counts and shorter survival times (Despeaux et al., 2011; Recher et al., 2004). We speculated that signals emanating from niches might stimulate FAK activation in LCs, which is normally restricted by *PTEN*. In *PTEN*-mutant T-ALL cells, activated FAK may

play a critical role in disease initiation, progression and the development of drug resistance by providing a survival signal in LCs parallel to PI3K/AKT/mTOR signaling. To test this hypothesis, we inactivated FAK by genetic and pharmacologic methods to study the functions of FAK in *PTEN*-null T-ALL cells.

Results

The Activity of Fak is Significantly Increased in *Pten*^{-/-} Thymocytes

Loss of *Pten* causes MPD, AML, and/or T-ALL in *Mx-1-Cre*⁺*Pten*^{fx/fx} mice (*Pten*^{-/-}), in which *Pten* is deleted in nearly 100% of adult hematopoietic cells (HCs) after 3-5 polyinosine-polycytidine (pI:C) injections (Yilmaz et al., 2006; Zhang et al., 2006). Repression of PI3K/Akt/mTor signaling by inhibitors or genetic deletion largely suppresses the defects of HSCs and leukemia development, but none of these inhibitors or deletions can completely block leukemia development in *Pten*^{-/-} mice (Kalaitzidis et al., 2012; Yilmaz et al., 2006). Thus, it is possible that another potential downstream target(s) of *Pten* which can be activated *in vivo* compensates for PI3K/Akt/mTor inhibition in the development of leukemia in *Pten*^{-/-} mice. *Pten* dephosphorylates both the lipid substrate PIP3, which is activated by PI3K, and protein targets such as Fak (Tamura et al., 1998), which is activated by adhesion signals (Mitra and Schlaepfer, 2006). We predicted that FAK might mediate such compensatory signaling. To test this hypothesis, we determined the expression of *Fak* in *wild-type* (*WT*) hematopoietic stem/progenitor cells from bone marrow (BM) and differentiated HCs from spleen (SP) and thymus (TH) (Fig.1A). *Fak* was expressed at low levels in Lin⁻Sca1⁺c-Kit⁺ cells (LSK), and very low in progenitor cells (CMP, GMP, MEP, and CLP), myeloid cells (Gr1⁺Mac1⁺), macrophages (F4/80⁺), B-cells (B220⁺), and erythroid lineage cells (Ter119⁺). *Fak* was highly expressed in platelet precursors (CD41⁺), as well as CD4⁻CD8⁻, CD4⁺CD8⁺, CD4⁺CD8⁻, and CD4⁻CD8⁺ T-lymphocytes. Although the expression of *Fak* was comparable between *WT* and *Pten*^{-/-}, *Pten* deletion enhanced the activation of both Akt and Fak in thymocytes, as shown by the increased levels of pAkt/pS6k, and pFak/pSrc, respectively. Neither Cre expression by itself nor pI:C treatment activated either signal (Fig.1B). In the four subtypes of thymocytes (Fig.1C,D; Fig.S1), the expression of *Fak* was not changed by *Pten* deletion. However, *Pten* deletion enhanced Fak activation in all thymocytes, especially in CD4⁺CD8⁻ cells, which consist of T-ALL cells in *Pten*^{-/-} mice (Zhang et al., 2011). These data suggest that hyper-activated Fak in CD4⁺CD8⁻ cells may contribute to T-ALL development in *Pten*^{-/-} mice.

Fak Deletion Significantly Delays T-ALL Development but does not Affect MPD in *Pten*^{-/-} Mice

Mx-1-Cre⁺*Pten*^{fx/fx} mice were crossed with *Fak*^{fx/fx} mice to generate *Mx-1-Cre*⁺*Pten*^{fx/fx}*Fak*^{fx/fx} mice to study the role of Fak in hematopoietic defects in *Pten*^{-/-} mice. Initially, the mice received five pI:C injections as reported (Yilmaz et al., 2006; Zhang et al., 2006). The hematopoietic alterations among *Mx-1-Cre*⁺*Pten*^{fx/fx}, *Mx-1-Cre*⁺*Fak*^{fx/fx}, *Mx-1-Cre*⁺*Pten*^{fx/fx}*Fak*^{fx/+}, and *Mx-1-Cre*⁺*Pten*^{fx/fx}*Fak*^{fx/fx} mice were analyzed 20-25 days after pI:C treatments. *Mx-1-Cre*⁺, *Mx-1-Cre*⁺*Pten*^{fx/+}*Fak*^{fx/+}, *Mx-1-Cre*⁺*Pten*^{fx/+}, *Mx-1-Cre*⁺*Fak*^{fx/+} or *Pten*^{fx/fx}*Fak*^{fx/fx} mice from the same or a comparable litter were used as *WT* controls (no phenotypic differences among these mice, data not shown). Consistent with

previous reports, *Mx-1-Cre⁺Fak^{fx/fx}* mice (*Fak*^{-/-} hereafter) had no significant hematopoietic defects (Fig.2 E, F, G, H; Fig.3 A, B, C; Fig.S2 A, B) except for a small but significant increase in platelet numbers (Fig.S2 C) (Vemula et al., 2010). Twenty to 25 days after 5×pI:C injections, both *Mx-1-Cre⁺Pten^{fx/fx}* or *Mx-1-Cre⁺Pten^{fx/fx}Fak^{fx/fx}* mice (*Pten*^{-/-} hereafter, no differences between them) and *Mx-1-Cre⁺Pten^{fx/fx}Fak^{fx/fx}* mice (*Pten*^{-/-}*Fak*^{-/-} hereafter) developed MPD, as shown by a significant increase in Gr-1⁺Mac-1⁺ cells in BM, PB, and SP. The percentage of B220⁺ and Gr1⁺Mac1⁺ cells (Fig.S2 A, B) or nucleated cells in PB (Fig.S2 F, G) were not affected by *Fak* deletion. Interestingly, compared to *Pten*^{-/-} mice, there was a mild, but statistically significant impact on white blood cell counts (WBC), platelets and red blood cell counts (RBC) (Fig.S2 C), and a subtle increased survival, though without statistical significance, in *Pten*^{-/-}*Fak*^{-/-} mice (Fig.S2 D). These data suggest that *Fak* is less essential for MPD development in *Pten*^{-/-} mice.

Without pI:C treatment, *Mx-1-Cre⁺Pten^{fx/fx}* mice develop T-ALL 3-4 months after birth, but rarely develop MPD (Zhang et al., 2011). *VEC-Cre⁺Pten^{fx/fx}* mice, in which *Pten* is deleted in about 40% of HCs, also develop only T-ALL (Guo et al., 2008). Thus, to study the role of *Fak* in T-ALL induced by *Pten* deletion we used a reduced regimen of pI:C to induce deletion in a fraction of HCs. We injected *WT*, *Pten*^{-/-}, *Pten*^{-/-}*Fak*^{-/-}, and *Fak*^{-/-} mice once with 25 mg/kg pI:C. Ten days after injection, c-Kit⁺ HSPCs and thymocytes were isolated. We used colony PCR to determine the efficiency of *Pten* and *Fak* deletion in these cells. This pI:C regimen induced *Pten* deletion in 55% of c-Kit⁺ HSPCs and approximately 25% of thymocytes, both in *Pten*^{-/-} and *Pten*^{-/-}*Fak*^{-/-} mice. *Fak* was co-deleted with *Pten* in *Pten*^{-/-}*Fak*^{-/-} thymocytes (Fig.2 A). This result was confirmed by measuring protein levels of Pten and Fak (Fig.2 B). Thus, a reduced regimen of pI:C induced both *Pten* and *Fak* deletion to a lesser degree than with 5×pI:C treatments. A relatively limited *Pten* deletion may enable sufficient time for *Pten*-null T-ALL cells accumulation, in contrast to death from MPD after complete *Pten* deletion in mice (Zhang et al., 2006).

After a single pI:C injection, all *Pten*^{-/-} mice died of T-ALL, diagnosed by measuring CD4⁺CD8^{mid/-}CD45^{high} LCs (Guo et al., 2008) in BM and SP (Fig.S2E), within 70 days; some *Pten*^{-/-}*Fak*^{-/-} mice began dying of T-ALL on day 50, but most *Pten*^{-/-}*Fak*^{-/-} mice survived significantly longer than *Pten*^{-/-} mice. About 25% of *Pten*^{-/-}*Fak*^{-/-} mice were leukemia-free on day 200 post-pI:C treatment. *Fak*^{-/-} mice are grossly normal. We confirmed Pten and Fak deletion in T-ALL cells recovered from moribund mice (Fig.2 C, D).

On day 45 after a single pI:C treatment, most *Pten*^{-/-} had obvious disease phenotypes, including significantly enlarged lymph nodes (LNs), TH and SP compared to *Pten*^{-/-}*Fak*^{-/-} mice. *Pten*^{-/-} mice had pale-colored livers, which are typically observed during leukemia development (Fig.2 E, F, G). *Pten*^{-/-} mice had significantly greater LC infiltration into both livers and kidneys (Fig.2 H: b, h), and showed considerable destruction of splenic histologic structure compared to *WT* or *Pten*^{-/-}*Fak*^{-/-} mice (Fig.S2 H); At this stage, *Pten*^{-/-}*Fak*^{-/-} mice did not show such a high degree of disease phenotypes, having larger SP and LNs than *WT* or *Fak*^{-/-} mice (Fig.2 E, F, G), very low liver or kidney infiltration (Fig.2H: c, i), less destruction of splenic histologic structure (Fig.S2 H). After T-ALL developed, though still smaller than *Pten*^{-/-} mice, *Pten*^{-/-}*Fak*^{-/-} mice had significantly enlarged SP, LNs, TH (Fig.

2G) and high infiltration of LCs into livers and kidneys (Fig.2H:f, l), as well as significant structural destruction of the SP (Fig.S2 H). To test whether delayed T-ALL development with *Fak* deletion was cell intrinsic, we transplanted CD3⁺c-Kit^{low} cells, which are enriched for T-ALL initiating cells (Guo et al., 2008), isolated from the BM of *Pten*^{-/-} or *Pten*^{-/-}*Fak*^{-/-} mice (CD45.1⁺) on day 10 after 5×pI:C treatments into lethally-irradiated recipient mice (CD45.2⁺). After transplantation, all of the *Pten*^{-/-}-recipient mice died of T-ALL within 125 days; only about 55% of *Pten*^{-/-}*Fak*^{-/-}-recipient mice died of T-ALL within 150 days, with the remainder of these mice surviving over 180 days (Fig.2 I). These data suggest that *Fak* deletion delays T-ALL development in *Pten*^{-/-} mice.

***Fak* Deletion Partially Restores Apoptosis in *Pten*^{-/-} T-lymphocytes, but not in *Pten*^{-/-} T-ALL Cells.**

To investigate the mechanisms by which *Fak* deletion delays T-ALL development in *Pten*^{-/-} mice, we analyzed thymocyte apoptosis and proliferation on day 10 after 5×pI:C injection. *Pten*^{-/-} mice had fewer apoptotic thymocytes than *WT*. Increased apoptosis in *Pten*^{-/-}*Fak*^{-/-} thymocytes, especially CD4⁺CD8⁻ cells, suggests that *Fak* contributes to the enhanced survival of *Pten*^{-/-} thymocytes (Fig.3 A, B). However, combined *Pten* and *Fak* deletion had no significant effect on apoptosis in CD4⁻CD8⁻, CD4⁺CD8⁺ and CD4⁻CD8⁺ T lymphocytes (Fig.S3 A, B). *Fak* deletion did not change the proliferation of *Pten*-null cells, which was higher than *WT* and *Fak*^{-/-} controls (Fig.3 C). Consistent with this, there was no significant cell-cycle difference between *Pten*^{-/-} and *Pten*^{-/-}*Fak*^{-/-} thymocytes, but both had more cycling cells than did controls (Fig.S3 C). After T-ALL development, *Pten*^{-/-}*Fak*^{-/-} LCs did not differ from *Pten*^{-/-} LCs in apoptosis or proliferation (Fig.3 D). These data suggest that *Fak* deletion delays T-ALL development in *Pten*^{-/-} mice by partially restoring apoptosis in thymocytes, especially in CD4⁺CD8⁻ T-lymphocytes, but this effect disappears after T-ALL development.

***Fak* Promotes a PI3K/Akt/mTOR/Mcl-1-Independent Survival Signal in *Pten*^{-/-} Thymocytes and T-ALL Cells By Enhancing the NF-κB/Bcl-2/Bcl-xL Pathway**

To study the molecular mechanisms whereby *Fak* mediated thymocyte apoptosis, we tested survival-related signals in *Pten*^{-/-} and *Pten*^{-/-}*Fak*^{-/-} thymocytes freshly recovered from mice on day 10 after 5×pI:C treatment. Activation of Akt and *Fak* signaling in *Pten*^{-/-} thymocytes was verified by increased levels of p-Akt/p-Gsk3β and pFAK/p-Src/p-P130Cas/p-Paxillin, respectively. *Fak* deletion reduced the level of the latter but not the former (Fig.4A). Over-activated PI3K/Akt/mTor signaling provides a sustained survival signal by up-regulating *Mcl-1* in AML, T-ALL and other types of cancer cells (Hsieh et al., 2010; Inuzuka et al., 2011; Jebahi et al., 2014). Consistent with these studies, a significant increase of *Mcl-1* was detected in *Pten*^{-/-} thymocytes compared to *WT* (Fig.4 A). This was not affected by *Fak* deletion. Comparable *Mcl-1* mRNA levels among *WT*, *Pten*^{-/-} and *Pten*^{-/-}*Fak*^{-/-} thymocytes (Fig.4 D) supported the idea that *Mcl-1* is regulated by PI3K/Akt/mTor through a post-transcriptional mechanism (Hsieh et al., 2010; Inuzuka et al., 2011).

NF-κB mediates a critical survival signal in many types of cancer by enhancing the expression of several key survival genes, such as *Bcl-2* and *Bcl-xL* (Karin, 2006). To determine whether NF-κB mediates a *Fak*-stimulated PI3K/AKT-independent survival signal

in *Pten*^{-/-} thymocytes, we measured NF- κ B activity in these cells by assessing whole cell pP65 and nuclear P65. NF- κ B activation was greatly enhanced in *Pten*^{-/-} thymocytes compared to *WT*. However, in *Pten*^{-/-}*Fak*^{-/-} thymocytes, NF- κ B activity was reduced compared to *Pten*^{-/-} (Fig.4 A, C), suggesting Fak-dependent NF- κ B activation in *Pten*^{-/-} cells. As a consequence, mRNA and protein levels of Bcl-xL and Bcl-2, known downstream target genes of NF- κ B, were up-regulated in *Pten*^{-/-} thymocytes, and repressed by *Fak* deletion (Fig.4 A, D). The comparable expression of other known NF- κ B target genes such as *cIAP1/2*, *Cyclin D1/2* and *Cyclin A2* in *WT* and *Pten*-null thymocytes suggested that not all NF- κ B target genes were induced by *Pten* deletion. The expression of p57, p21, p27 and p-Rb, all negative cell cycle regulators, were not altered in *Pten*-null cells (Fig.S4 A, B). Enhanced c-Myc and p-H3 (a mitotic marker) in both *Pten*^{-/-} and *Pten*^{-/-}*Fak*^{-/-} thymocytes might explain the increased proliferation of *Pten*-null cells compared to *WT* (Fig.3 C; Fig.S4 A, B). Moreover, Pyk2 activity, a homolog of Fak with a potential compensatory effect for *Fak* deletion, and Jnk1/2, another potential survival pathway to neutralize the inactivation of NF- κ B (Volk et al., 2014), were measured. There was a negligible difference between *Pten*^{-/-} and *Pten*^{-/-}*Fak*^{-/-} thymocytes, yet p-Jnk1/2 was at a higher level than *WT* (Fig.S4 B).

Although *Fak* deletion significantly enhanced survival, most *Pten*^{-/-}*Fak*^{-/-} mice still developed T-ALL after a single pI:C treatment (Fig.2 C). Inactivation of NF- κ B/Bcl-xL/Bcl-2 signaling in *Pten*^{-/-}*Fak*^{-/-} T-ALL cells might be compensated by Fak-independent signal(s). We recovered T-ALL cells from both *Pten*^{-/-} and *Pten*^{-/-}*Fak*^{-/-} mice to test this possibility. *Pten*^{-/-}*Fak*^{-/-} cells had lower NF- κ B activity and reduced Bcl-2/Bcl-xL than *Pten*^{-/-} (Fig.4 B, C). Interestingly, we found significantly enhanced PI3K/Akt/mTOR signaling and further increased Mcl-1 in *Pten*^{-/-}*Fak*^{-/-} compared to *Pten*^{-/-} T-ALL cells (Fig.4 B). This indicated that further enhancement of PI3K/Akt/mTOR/Mcl-1 signaling could compensate for inactivated Fak/NF- κ B/Bcl-xL/Bcl-2 signaling in *Pten*^{-/-}*Fak*^{-/-} cells to promote T-ALL development and progression. These data suggest that PI3K/Akt/mTOR/Mcl-1 and Fak/NF- κ B/Bcl-xL/Bcl-2 mediate two parallel survival signaling pathways, which are normally restricted by Pten, in *Pten*-null thymocytes and T-ALL cells.

Fak Deletion Restores the Sensitivity of *Pten*-null Thymocytes and T-ALL Cells to PI3K/mTOR/Mcl-1 Inhibitors *in vitro*

When cultured in suspension without feeder cells or ECM-coating (hereafter as “suspension”), both *Pten*^{-/-} and *Pten*^{-/-}*Fak*^{-/-} thymocytes and T-ALL cells were sensitive to a pan-PI3K inhibitor, LY294002 (LY), mTOR inhibitors, KU0063794 (KU)/Rapamycin (RA), and Mcl-1 inhibitor (MI); no difference was observed between *Pten*^{-/-} and *Pten*^{-/-}*Fak*^{-/-} cells (Fig.5 A, B: leftmost panels). Because Fak is activated by extracellular matrix (ECM) signals (Mitra and Schlaepfer, 2006), we speculated that Fak might not be activated in suspension culture. We measured Fak activation in freshly-recovered and suspension-cultured *Pten*^{-/-} thymocytes and T-ALL cells. Compared to freshly-recovered cells, Fak activation was much lower in suspension-cultured cells. Grown on OP9 feeder layer cells (OP9), or on MatriGel (MG)-, collagen (CLG)-, or fibronectin (FN)-coated plates, Fak could be activated to a level comparable to freshly-recovered cells (Fig.S5 A, B). Fak activation by OP9, or MG/CLG/FN-coated plates enabled *Pten*^{-/-} thymocytes and T-ALL cells to become less sensitive to PI3K/mTOR/Mcl-1 inhibitors, which were titrated to optimize treatment

concentrations (Fig.S5 C), although they were still more sensitive to these inhibitors than *WT* cells. *Pten*^{-/-}*Fak*^{-/-} demonstrated increased death compared to *Pten*^{-/-} cells upon PI3K/Akt signal inhibition by LY, KU, RA, or MI. Consistent with the genetic inactivation of *Fak*, suppression of both PI3K/Akt and *Fak* signals (by FAK inhibitor PF573228, PF) with combined pharmacological inhibitors, LY+PF, caused more death in *Pten*^{-/-} cells than with individual inhibitor LY (Fig.5 A, B; Fig.S5 D, E). To test whether the increased death induced by *Fak* deletion was due to its kinase activity, we overexpressed *Fak*^{WT} or *Fak*^{Y397F} (kinase-null) in *Pten*^{-/-}*Fak*^{-/-} thymocytes and T-ALL cells. Expression of *Fak*^{WT}, but not *Fak*^{Y397F}, increased survival of *Pten*^{-/-}*Fak*^{-/-} cells treated with LY, KU, RA, or MI (Fig.S5 F; Fig.5 C-E). These data suggest that *Fak* activation by OP9, or ECM-coated plates increases the resistance of *Pten*^{-/-} thymocytes and T-ALL cells to PI3K/Akt/mTOR/Mcl-1 inhibition.

Fak* Inactivation Increases the Sensitivity of *Pten*-null T-ALL Cells to Apoptosis Induced by PI3K/mTOR Inhibitors *in vivo

We transplanted CD3⁺c-Kit^{low} cells from *Pten*^{-/-} T-ALL mice (CD45.1⁺) into lethally-irradiated recipient mice (CD45.2⁺). Beginning on day 14 after transplantation, we treated recipient mice with KU at 30 mg/kg, PF at 50 mg/kg, alone or together, every other day until analysis or death of the animal (maximum: 15 injections). Either KU or PF alone significantly enhanced the survival of recipient mice compared to vehicle treatment. KU+PF treatment further increased the survival compared to either single inhibitor treatment (Fig.S5 G). We next tested whether *Fak* inhibition could enhance the therapeutic effects of PI3K/Akt/mTOR inhibitors on *Pten*-null T-ALL mice. Immediately following T-ALL development, diagnosed by >30% blasts (Fig.S2 F, G) and 35,000-60,000 μl^{-1} WBC (Fig.5 H) in their PB, we treated *Pten*^{-/-}, *Pten*^{-/-}*Fak*^{-/-} mice with LY at 40 mg/kg (Hu et al., 2002; Nagata et al., 2004) or KU at 30 mg/kg every other day until analysis or a maximum 15 injections. After five drug treatments, we analyzed T-ALL cell apoptosis. *Pten*^{-/-} T-ALL cells underwent apoptosis to a greater extent with LY or KU than with vehicle treatment. *Pten*^{-/-}*Fak*^{-/-} T-ALL cells underwent apoptosis to an even greater extent than *Pten*^{-/-} (Fig.5 F, G). *Pten*^{-/-} mice survived longer with LY or KU compared to vehicle treatment; *Pten*^{-/-}*Fak*^{-/-} mice survived even longer than *Pten*^{-/-}, and about 25% were still alive more than 60 days after the first drug treatment. To rule out the treatment toxicity, we treated *WT* mice with LY or KU at the same time; no *WT* mice died (Fig.5 H). To determine whether the role of *Fak* in *Pten*-null T-ALL was cell-intrinsic, we transplanted CD3⁺c-Kit^{low} cells from either *Pten*^{-/-} or *Pten*^{-/-}*Fak*^{-/-} T-ALL, or *WT* mice (CD45.1⁺) into lethally-irradiated recipient mice (CD45.2⁺). Recipient mice were treated with vehicle, LY or KU from day 14 post-transplantation every other day until analysis or maximum 15 injections. LY and KU enhanced the survival of *Pten*^{-/-}-recipient mice compared to vehicle treatment. *Pten*^{-/-}*Fak*^{-/-}-recipient mice survived longer than *Pten*^{-/-}-recipient mice, whether treated with vehicle, LY, or KU. With LY or KU treatment, 25% or 40% of *Pten*^{-/-}*Fak*^{-/-}-recipient mice survived more than 120 days after the first drug treatment, respectively (Fig.5 I). To further rule out *in vivo* toxicity of LY, we treated *Pten*^{-/-} and *Pten*^{-/-}*Fak*^{-/-} transgenic or transplanted T-ALL mice with another pan-PI3K inhibitor, GDC-0941 (GDC) (Folkes et al., 2008). Similar results were observed in treatments with GDC as with LY (Fig.S5 H, I). These data suggest

that *Fak* deletion makes *Pten*-null T-ALL cells more sensitive to PI3K/AKT/mTOR inhibition *in vivo*.

FAK/NF- κ B/Bc1-xL/Bc1-2 and PI3K/AKT/mTOR/Mcl-1 Provide two Independent Survival Signals in *PTEN*-mutant Human T-ALL Cell Lines

When cultured on MG-coated plates, both FAK and AKT signals were activated in *PTEN*-null human T-ALL cell lines (JURKAT, CCRF-CEM, MOLT-4, and LOUCY), but not in *PTEN*-WT cell lines (CUTLL1, HPB-ALL, or KOPT-K1) (Fig.6A). This result was confirmed in JURKAT and HBP-ALL cells, when grown on OP9 cells, or on CLG/FN-coated plates (Fig.S6 A). *Pten* regulates the activity of *Fak* in NIH 3T3 cells (Tamura et al., 1998). To test whether FAK activation was also regulated by *PTEN* in human T-ALL cells, we expressed *PTEN*-WT, *PTEN*^{C124S}, *PTEN*^{Y138L}, and *PTEN*^{G129E} in JURKAT cells cultured on MG-coated plates. *PTEN*^{WT} repressed the activities of both AKT and FAK; *PTEN*^{C124S}, a phosphatase-null mutant, did not inhibit either signal; *PTEN*^{Y138L}, a protein phosphatase-inactive mutant, suppressed only AKT activity; the lipid phosphatase-null mutant, *PTEN*^{G129E}, restrained only FAK activity (Fig.6 B). Immunoprecipitation using *PTEN* or FAK antibodies in these transduced JURKAT cells demonstrated that *PTEN* could bind to FAK in a phosphatase activity-independent manner (Fig.6 C). The interaction of endogenous *PTEN* and FAK was verified in HPB-ALL cells (Fig.6 D). Though *PTEN* can bind FAK in cells, it is possible that *PTEN* dephosphorylated FAK indirectly. These data suggest that the activities of both AKT and FAK are regulated by *PTEN* in human T-ALL cells.

To explore the effect of AKT and/or FAK signal inhibition on human T-ALL cell lines cultured in suspension, or on OP9, or MG/CLG/FN-coated plates, we treated the cells with PI3K/mTOR/MCL-1 and FAK/SRC inhibitors, which were titrated to optimize concentrations (Fig.S6 B, C), alone, or in combination. In suspension culture the survival of JURKAT (*PTEN*-null) cells was highly restrained by PI3K, mTOR, or MCL-1 inhibitors, but there was no further restrained effect with combined inhibition of both AKT and FAK signaling. When cultured on OP9 or MG/CLG/FN-coated plates, JURKAT cells were less sensitive to AKT signaling inhibition; which was further suppressed with combined AKT and FAK signaling inhibition. However, CUTLL1 (*PTEN*-WT) cells did not respond to the inhibition of AKT, FAK alone, or both (Fig.6 E). We confirmed this result with other *PTEN*-null (CCRF-CEM, MOLT-4, LOUCY) and *PTEN*-WT (HPB-ALL, KOPT-K1) human T-ALL cell lines (Fig.S6 D). The colony-forming ability of JURKAT cells was more dramatically reduced by the combined inhibition of AKT and FAK signaling compared to the inhibition of either alone (Fig.6 F). To study the molecular mechanisms of survival signals in *PTEN*-null human T-ALL cells, we treated JURKAT cells with PI3K/AKT/mTOR/MCL-1 inhibitors (LY, KU, RA, MI), or FAK signal inhibitors (PF, SRC inhibitor (SI)) cultured on MG-coated plates. Inhibition of PI3K/AKT signal did not change the expression of BCL-xL or BCL-2, but significantly reduced MCL-1. Inhibition of FAK signal reduced the expression of both BCL-xL and BCL-2; so did the restriction of NF- κ B by Bay 11-7085 (BA). Similar to what we observed in *Pten*^{-/-}*Fak*^{-/-} T-ALL mouse cells, enhanced AKT signaling and further increased MCL-1 were also detected when FAK activity was suppressed in JURKAT cells (Fig.6 G).

As observed with pharmacologic inhibitors treatment, shRNA restriction of FAK, suppressed NF- κ B activity, as shown by reduced protein level (Fig.6 H) and P65 nuclear localization (Fig.S6 E). Suppression of FAK by shRNA made JURKAT cells more sensitive to the inhibition of PI3K/AKT signal by LY, KU, RA, or MI treatment, displaying reduced viability on MG-coated plates (Fig.6 I) or OP9 (Fig.S6 F), and colony forming ability (Fig.6 J).

Activated FAK promotes NF- κ B activation in a Rho/Rac-GTPase- or RIP1-dependent manner in cancers (Kamarajan et al., 2010; Tong and Tergaonkar, 2014). To determine which pathway mediated the activated Fak promoting NF- κ B activity in *Pten*-null T-ALL cells, we tested the expression and activity of Rac1/Cdc42 (assessed by p-Pak1), which were not affected by *Fak* deletion (Fig.S4 B). Rac GTPase inhibitor, EHop-016 (EH), failed to repress NF- κ B activity in JURKAT (*PTEN*-null) cells. However, a Smac mimetic (inhibiting NF- κ B activity by repressing c-IAP1/2-mediated RIP1 ubiquitination (Wang et al., 2008)), birinapant (BI), restrained NF- κ B activity in JURKAT cells. Neither inhibitor changed NF- κ B activity in HBP-ALL (*PTEN*-WT) cells (Fig.6 K). This indicated that FAK activated NF- κ B through RIP1, not Rho/Rac in *PTEN*-null T-ALL cells. To address whether the reduction in BCL-xL and BCL-2 expression was caused by inhibition of NF- κ B, we treated JURKAT, KOPT-K1, and CCRF-CEM with BA (inhibiting NF- κ B) or ABT-737 (BCL-xL/BCL-2 inhibitor) on MG-coated plates. In *PTEN*-mutant cell lines, JURKAT and CCRF-CEM, inhibition of NF- κ B reduced BCL-xL/BCL-2, but inhibition of BCL-xL/BCL-2 did not affect NF- κ B activity, as measured by pP65 and nuclear P65 (Fig.S7 A, B). This indicated that BCL-xL and BCL-2 were regulated by NF- κ B in *PTEN*-null human T-ALL cell lines.

***PTEN*-mutant Human Primary T-ALL Cells are more Sensitive to Combined Treatment Using PI3K/mTOR and FAK Inhibitors than to Treatment with Either Inhibitor Alone**

We collected and cultured primary T-ALL patient samples and CD3⁺ lymphocytes from healthy subjects on MG-coated plates, and assessed the activation of AKT and FAK signals. In two of three *PTEN* positive samples, patients 1 and 4, neither signal was activated. All *PTEN*-negative samples and one *PTEN* positive (patient 7) showed increased activation of both AKT and FAK signals (Fig.7 A). We considered that patient 7 might contain a loss-of-function *PTEN*-mutation, which is commonly associated with human T-ALL (Zuurbier et al., 2012), but this could not be determined due to limited cell availability. Cells were treated with PI3K/mTOR, FAK inhibitors alone, or in combination. The specific inhibitory activities of these chemicals on primary T-ALL cells were verified by western blot (Fig.7 B). Unlike healthy CD3⁺ cells, the survival of all primary T-ALL cells could be restricted to some extent by either PI3K/mTOR or FAK inhibition; however, *PTEN*-negative and patient 7 cells were significantly more sensitive to combined treatment (LY+PF/KU+PF) than to treatment with either single inhibitor, but *PTEN*-WT or healthy controls were not (Fig.7 C). These data suggest that simultaneous inhibition of both PI3K/AKT/mTOR and FAK/NF- κ B signaling might represent a more effective treatment strategy for *PTEN*-null T-ALL.

Discussion

In *PTEN*-null, but not in *PTEN*-WT murine and human T-ALL cells, both AKT and FAK signals are activated *in vivo* or when cultured on a feeder layer or ECM-coated plates. We determined that PI3K/AKT acts through mTOR, and FAK works via NF- κ B, up-regulating distinct survival genes, *MCL-1* and *BCL-2/BCL-xL*, respectively. Thus, two parallel survival signaling pathways are stimulated in *PTEN*-mutant T-ALL cells (Fig.7 D). Inhibition of PI3K/AKT/mTOR signaling induces apoptosis and represses the growth of T-ALL cells, which sensitizes drug-resistant T-ALL cells to conventional chemotherapy (Evangelisti et al., 2011; Piovani et al., 2013; Subramaniam et al., 2012). These inhibitors are currently in clinical trials in T-ALL patients, but no complete remission was reported with these inhibitors treatment (Rodon et al., 2013). We found that suppression of FAK further enhanced the inhibitory effects of PI3K/AKT signal restriction on *PTEN*-null T-ALL cells. FAK inhibitors are also in clinical trials for treatment of solid tumors and have been approved as relatively safe and promising drugs (Golubovskaya, 2014). Our study provides a strong rationale to treat *PTEN*-mutant T-ALL by co-inhibition of both AKT and FAK-stimulated parallel survival signals.

Both the lipid and protein phosphatase activities of PTEN were well demonstrated in earlier studies (Lee et al., 1999; Tamura et al., 1998). However, the pathophysiologic role downstream of PTEN's protein phosphatase activity has been underestimated in leukemogenesis because inactivation of its phosphatase or constitutive activation of its lipid substrates is sufficient to induce tumors in mice (Kharas et al., 2010; Myers et al., 1998; Wang et al., 2010). Nevertheless, transgenic over-expression of active PI3K or AKT fails to faithfully replicate the *Pten*^{-/-} phenotype, suggesting a PI3K/AKT-independent function of PTEN (Blanco-Aparicio et al., 2007). Several direct substrates of PTEN's protein phosphatase, such as IRS1 and CREB, have been defined (Gu et al., 2011; Shi et al., 2014; Tibarewal et al., 2012). We determined that the ECM activates FAK in *PTEN*-mutant T-ALL cells, providing an additional layer of survival/proliferation signaling to promote the initiation and progression of leukemia, together with PI3K/AKT signaling. *PTEN*-mutant T-ALL cells are sensitive to inhibition of non-canonical PI3K signaling, both when cultured on MS5-DL feeder cells and *in vivo* (Subramaniam et al., 2012). Consistent with this report, we found that PI3K/AKT inhibitors are effective in killing *PTEN*-null but not *PTEN*-WTT-ALL cells both *in vitro* and *in vivo*. However, *PTEN*-null T-ALL cells are relatively resistant to these inhibitors when grown on stromal feeder cells or ECM-coated plates compared to suspension culture. Further study suggested that inactivation of both FAK and PI3K/AKT signals might provide a better treatment for *PTEN*-null T-ALL than inhibition of PI3K/AKT alone. Furthermore, in our study of primary human T-ALL cells, we began with 14 samples, of which 8 were able to grow on MG-coated plates. Of these 8 samples, 6 of them (75%) showed PTEN inactivation. Given that *PTEN* mutations are generally reported in 15-25% of T-ALL patients (Gutierrez et al., 2009; Jotta et al., 2010; Zuurbier et al., 2012), we believe that MG-coated culture selectively favors the growth of *PTEN*-null T-ALL cells.

Both MCL-1 and BCL-xL/BCL-2 are highly expressed in T-ALL cells, and they functionally compensate for each other to support LCs survival. Simultaneously inhibiting both MCL-1 and BCL-2/BCL-xL shows an additive effect in killing T-ALL LCs (Inuzuka et al., 2011).

The activated AKT signal increases MCL-1 (Hsieh et al., 2010; Inuzuka et al., 2011; Jebahi et al., 2014), and we demonstrated that FAK/NF- κ B signal activated by ECM of niches enhances BCL-xL/BCL-2 in *PTEN*-null T-ALL cells. Further studies of these signaling mediators could produce favorable drug target(s) for T-ALL treatment.

Experimental Procedures

Mice and drug treatments:

Fak^{fx/fx} mice (Stock Number: 009967-UCD) were purchased from Mutant Mice Regional Resource Center. *Mx-1-Cre⁺ Pten^{fx/+}* mice were maintained in our laboratory (Zhang et al., 2011). Drug treatments were conducted as indicated. (See Supplementary Information)

T-ALL transplantation and *in vivo* treatments:

CD3⁺c-Kit^{Low} cells were isolated from *Pten^{-/-}* or *Pten^{-/-} Fak^{-/-}* T-ALL, or *WT* mice (CD45.1⁺) and transplanted into lethally-irradiated recipient mice (CD45.2⁺) together with support BM cells (CD45.2⁺). Each mouse received 1×10⁴ T-ALL cells and 2×10⁵ support BM cells. Drug treatments were conducted as indicated. (See Supplementary Information).

Flow cytometry and antibodies:

Bone marrow, spleens, thymuses and peripheral blood were collected after the mice had been sacrificed. MNC were isolated from these tissues after red blood cell lysis. Cells were suspended in FACS buffer (1×PBS supplemented with 2% FBS) at a concentration of 1×10⁷ cells/ml and aliquotted into flow cytometry tubes (100 μ l per tube) for antibody staining. Antibodies used in this study were purchased from eBioscience Inc. (See Supplementary Information).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

We determined that focal adhesion kinase (FAK), another downstream target of PTEN, promotes a survival signal parallel to PBK/AKT/mTOR in *PTEN*-null T-ALL cells. Our data suggest that targeting both PI3K/AKT/mTOR and FAK may provide a more effective therapeutic strategy against T-ALL in patients with *PTEN* mutations.

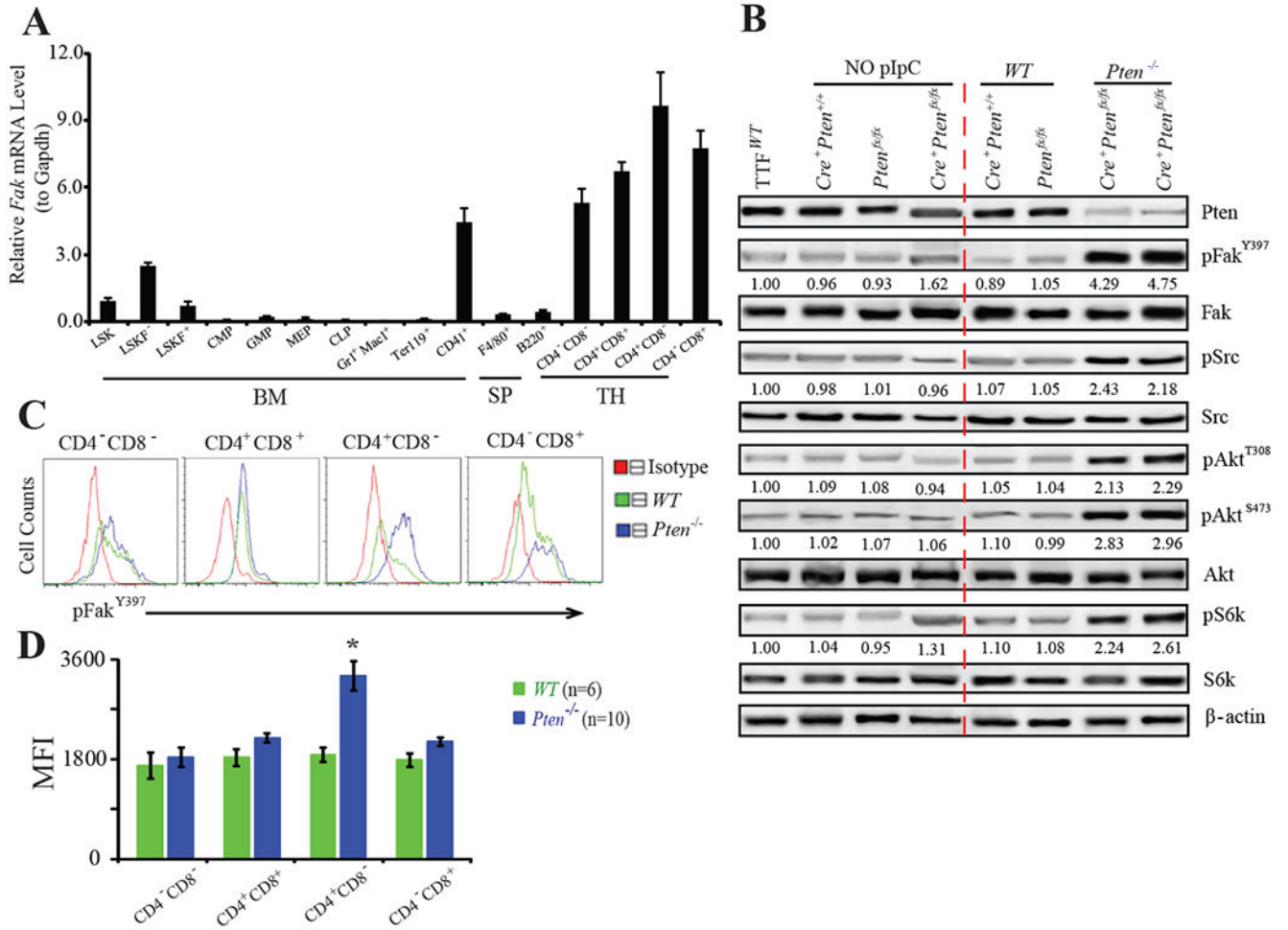


Figure 1. Akt and Fak Signals are Highly Activated in *Pten*^{-/-} Thymocytes
(A) *Fak* expression in Lin⁻Sca1⁺c-Kit⁺ (LSK), LSKFik2⁻, LSKFik2⁺, CMP, GMP, MEP, CLP, Gr1⁺Mac1⁺, Ter119⁺, and CD41⁺ cells isolated from BM; F4/80⁺, B220⁺ cells from SP; and CD4⁻CD8⁻, CD4⁺CD8⁺, CD4⁺CD8⁻, CD4⁻CD8⁺ cells from TH of *WT* mice. **(B)** Fak and Akt activities in *WT* and *Pten*^{-/-} thymocytes. Controls: thymocytes from mice without pI:C injection; *WT* tail tip fibroblasts (TTF). Images representative of three independent experiments. Quantification (*Multi Gauge 3.0*) relative to *WT* control after normalized to loading controls. **(C&D)** pFak^{Y397} in T-lymphocytes measured by FACS **(C)**, and quantified by mean fluorescence intensity (MFI) **(D)**. Data shown are Mean ± S.E.M. * compared to *WT*, P < 0.001, by *Student's t-test*.

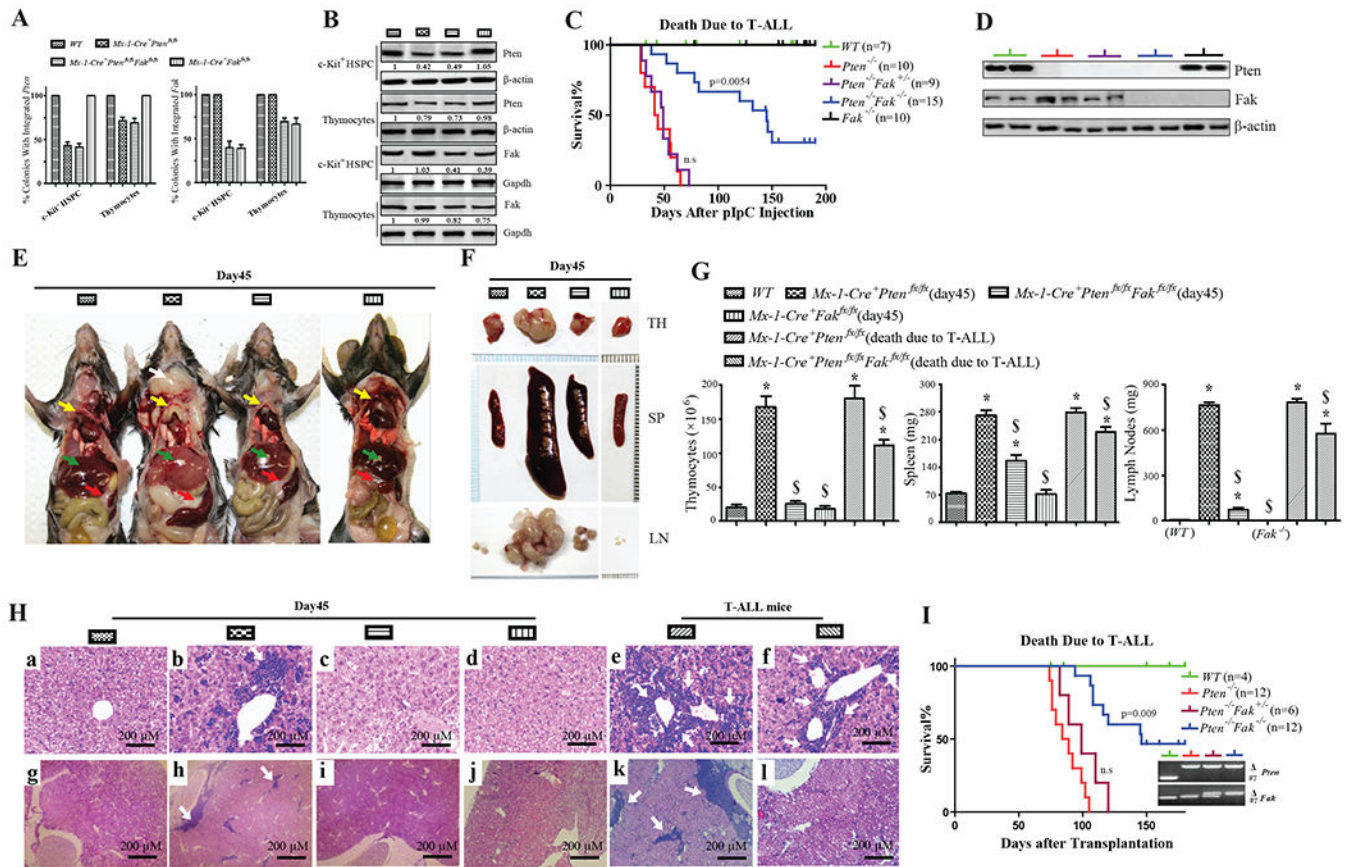


Figure 2. *Fak* Deletion Significantly Delays the Development of T-ALL in *Pten*^{-/-} Mice (A&B) *Pten* and *Fak* induced deletion efficiency (A), and *Pten* and *Fak* protein (B) in c-Kit⁺ HSPCs and thymocytes on day 10 treated with single pI:C. (C) Kaplan-Meier survival curve of indicated mice with a single pI:C injection; compared to *Pten*^{-/-}, P=0.0054. (D) *Pten* and *Fak* protein in thymocytes recovered from moribund T-ALL mice. (E&F) Phenotypes on day 45 after single pI:C injection. Anatomic mice images: white arrow, lymph nodes; yellow arrows, thymuses; green arrows, livers; and red arrows, spleens (E). TH, SP, and LN (F). Images shown are representative of five independent experiments. (G&H) Phenotypes on day 45 and moribund T-ALL mice after single pI:C injection. Thymocyte number, SP and LN weight: data shown are Mean ± S.D. * compared to *WT*, \$ compared to *Pten*^{-/-}, P<0.001, by 2-way ANOVA tests (G). Images of H&E staining for lymphocytes infiltrated into livers and kidneys (H). (I) Kaplan-Meier survival curve of recipient mice (CD45.2⁺) transplanted with CD3⁺c-Kit^{low} cells from BM of indicated mice (CD45.1⁺) on day 10 after 5×pI:C injections. Compared to *Pten*^{-/-}-recipient mice, P= 0.009. Inset: donor cell genotype immediately prior to transplantation.

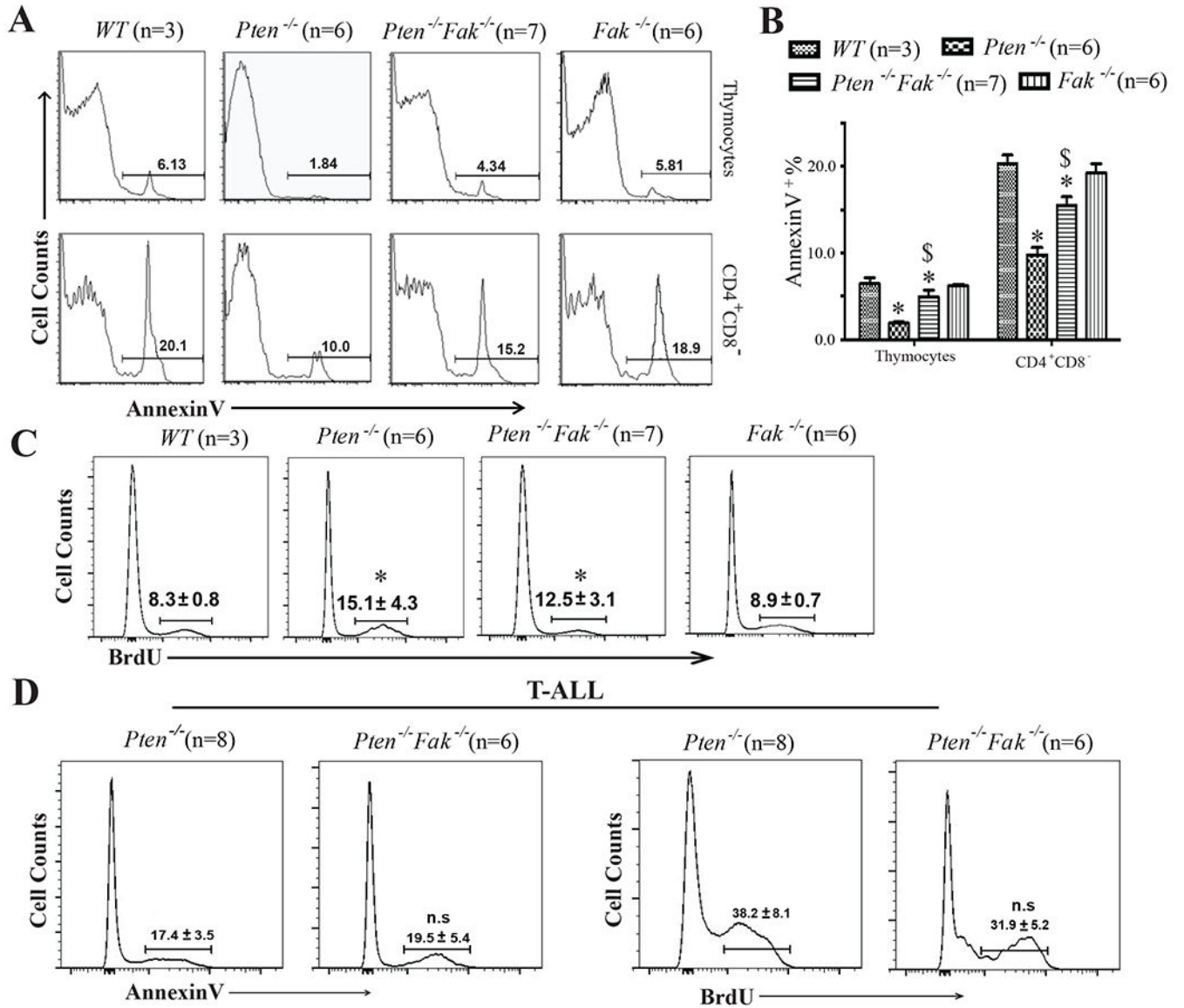


Figure 3. *Fak* Deletion Partially Restores Apoptosis of *Pten*^{-/-} Thymocytes but does not Affect the Survival or Proliferation of *Pten*-null T-ALL Cells. (A&B) Apoptosis by AnnexinV staining: total thymocytes and CD4⁺CD8⁻ T-lymphocytes before T-ALL development after a single pI:C injection. (C) BrdU pulse-labelled thymocytes prior to T-ALL development. (D) Apoptosis and proliferation analysis of T-ALL cells from *Pten*^{-/-} or *Pten*^{-/-} *Fak*^{-/-} mice. Data shown are Mean ± S.D. * compared to WT, § compared to *Pten*^{-/-}, P<0.001; n.s, no significant difference, by 2-way ANOVA tests.

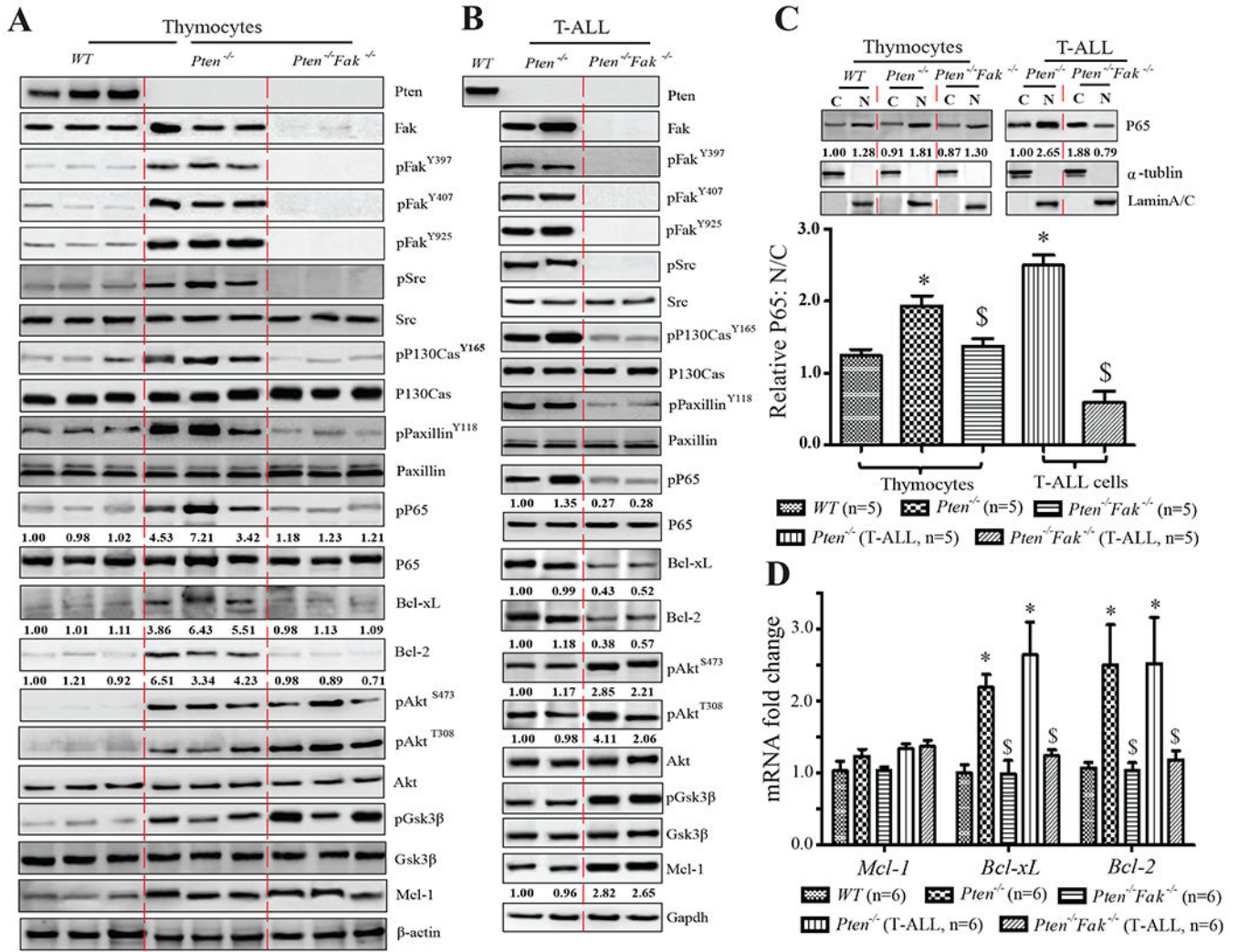


Figure 4. Two Parallel Survival Signaling Pathways, PI3K/Akt/mTOR/Mcl-1 and Fak/NF-κB/Bcl-xL/Bcl-2, in *Pten*^{-/-} Thymocytes and T-ALL Cells. (A&B) pFak, pSrc, pP130Cas, pPaxillin, pP65, pAkt, pGsk3β, Bcl-xL, Bcl-2, and Mcl-1 expression in thymocytes before T-ALL development (A) and T-ALL cells (B). (C) Nuclear (N) and cytoplasmic (C) P65 in thymocytes (left) or T-ALL cells (right). Images shown representative of five independent experiments. (D) mRNA levels of *Mcl-1*, *Bcl-xL*, and *Bcl-2* in thymocytes before T-ALL development and T-ALL cells, with *WT* as controls. Data shown are Mean ± S.D. * compared to *WT*, \$ compared to *Pten*^{-/-}, P<0.001, determined by 2-way ANOVA tests.

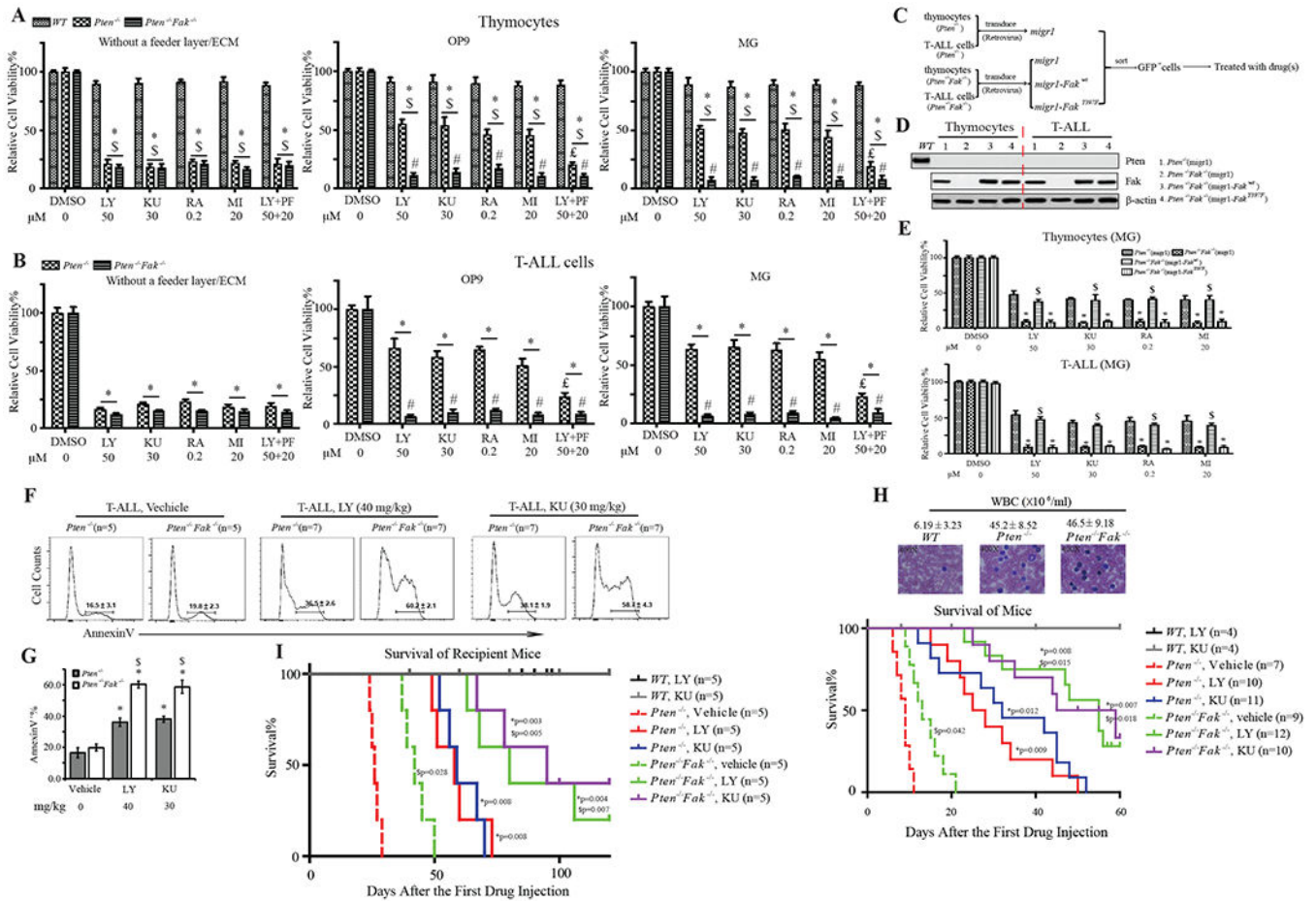


Figure 5. Inhibition of Fak/NF-κB Signaling Sensitizes *Pten*^{-/-} Thymocytes and T-ALL Cells to PI3K/mTOR/Mcl-1 Inhibition *in vitro* and *in vivo*.

(A&B) Sensitivity of thymocytes (A) and T-ALL cells (B) to DMSO (DO), LY, KU, RA, MI, and LY+PF when cultured in suspension, on OP9 feeder cells, or on MG-coated plates, respectively; *WT*, control. Data shown are Mean ± S.D. * compared to DMSO, \$ compared to *WT*, # compared to *Pten*^{-/-}, £ compared to LY treatment, P<0.001, determined by 2-way ANOVA. (C-E) Sensitivity of *Fak*-overexpressing *Pten*^{-/-}*Fak*^{-/-} thymocytes and T-ALL cells to DO, LY, KU, RA, or MI when cultured on MG-coated plates. Experimental procedure (C), *Pten* and *Fak* protein in transduced cells (D). Sensitivity of transduced cells on MG-coated plates (E), using *Migr1* (empty vector)-transduced cells as controls. Data shown are Mean ± S.D. * compared to *Migr1-Pten*^{-/-}, \$ compared to *migr1-Pten*^{-/-}*Fak*^{-/-}, P<0.001, determined by 2-way ANOVA. (F&G) Apoptosis of T-ALL cells from leukemic *Pten*^{-/-} and *Pten*^{-/-}*Fak*^{-/-} mice on day 10 with vehicle, LY, or KU treatment (F), statistical quantification of AnnexinV⁺ cells (G). Data shown are Mean ± S.E.M. * compared to *WT*, \$ compared to *Pten*^{-/-}, P<0.001, determined by Student's *t*-test. (H) Kaplan-Meier survival curve for *Pten*^{-/-} and *Pten*^{-/-}*Fak*^{-/-} T-ALL mice treated with vehicle, LY, or KU; *WT* mice treated with LY or KU to evaluate treatment toxicity. Upper panel: WBC counts and Giemsa staining of PB of *Pten*^{-/-} and *Pten*^{-/-}*Fak*^{-/-} T-ALL mice, using *WT* as control. (I) Kaplan-Meier survival curve of recipient mice (CD45.2⁺) transplanted with CD3⁺c-Kit^{low} cells isolated from BM of *Pten*^{-/-}, or *Pten*^{-/-}*Fak*^{-/-} T-ALL, or *WT* mice (CD45.1⁺) treated with

vehicle, LY, or KU. * compared to corresponding vehicle treatment; \$ compared to *Pten*^{-/-} mice with corresponding vehicle or LY or KU treatment.

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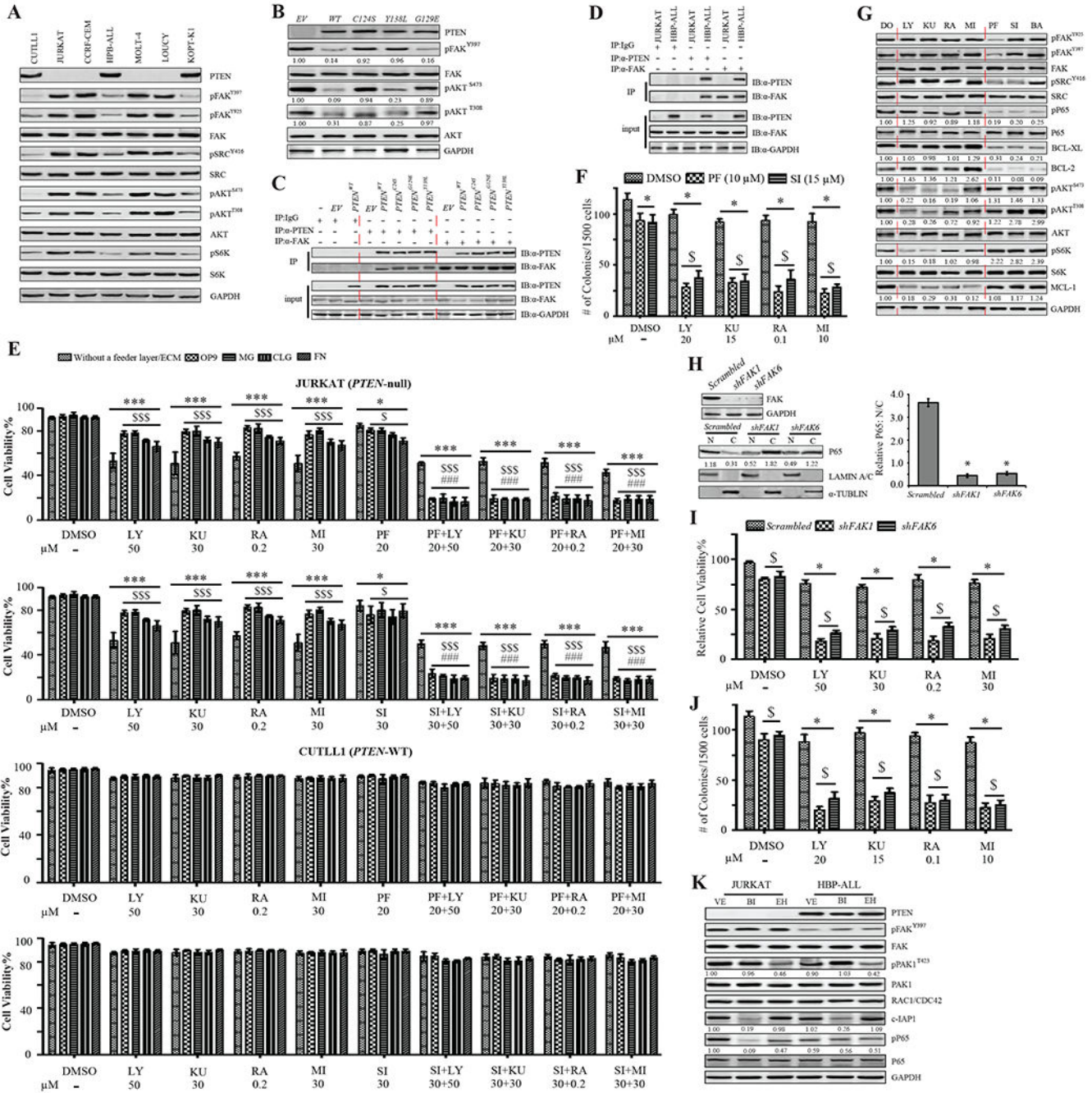


Figure 6. PI3K/AKT/mTOR/MCL-1 and FAK/NF-κB/BCL-xL/BCL-2 Represent Two Parallel Survival Signaling Pathways in *PTEN*-mutant Human T-ALL Cell Lines. (A) PTEN, pFAK, pSRC, pAKT and pS6K expression in human T-ALL cell lines, CUTLL1, JURKAT, CCRF-CEM, HPB-ALL, MOLT-4, LOUCY, and KOPT-K1, cultured on MG-coated plates. (B) PTEN, pFAK and pAKT in *PTEN*^{WT}, or *PTEN*^{C124S}, *PTEN*^{G129E}, or *PTEN*^{Y138L}-transduced JURKAT cells cultured on MG-coated plates; control: empty vector (EV) transduced cells. (C&D) The binding of PTEN and FAK in transduced JURKAT cells (C) and HBP-ALL cells (D). (E) Viability of human T-ALL cell lines in suspension, on

OP9, or on MG/CLG/FN-coated plates, treated by DMSO (DO), LY, KU, RA, MI, PF, or LY⁺PF, KU⁺PF, RA⁺PF, MI⁺PF. Data shown are Mean ± S.D. ***, * compared to DO treatment, and \$\$\$, \$ compared to suspension culture, P<0.001, <0.05, respectively; ### compared to single inhibitor treatment, P<0.001. **(F)** Colony number of JURKAT cells treated with DO, LY, KU, RA, or MI, alone or in combination with PF or SI. Data shown are Mean ± S.D. * compared DO treatment, \$ compared to single inhibitor treatment, P<0.001. **(G)** pFAK, pSRC, pP65, pAKT, pS6K, BCL-xL, BCL-2 and MCL-1 expression in JURKAT cells cultured on MG-coated plates treated by DO, LY, KU, RA, MI, PF, SI, or BA, respectively. **(H)** Nuclear (N) and cytoplasmic (C) P65 in JURKAT cells with knockdown of *FAK* by shRNA. Data shown are Mean ± S.D. * compared to scrambled, P<0.001. **(I&J)** Viability, cultured on MG-coated plates **(I)** and colony forming ability **(J)** of JURKAT cells to *FAK* inhibition by shRNA, treated with DO, LY, KU, RA, or MI. Data shown are Mean ± S.D. * compared to DO treatment, \$ compared to scrambled, P<0.001. **(K)** The levels of pFAK, pPAK1, pP65, and the expression of PTEN, RAC1/CDC42, c-IAP1 in JURKAT and HBP-ALL cells. Images shown in western blot are representative of three to five independent experiments. All statistical values were determined by 2-way ANOVA.

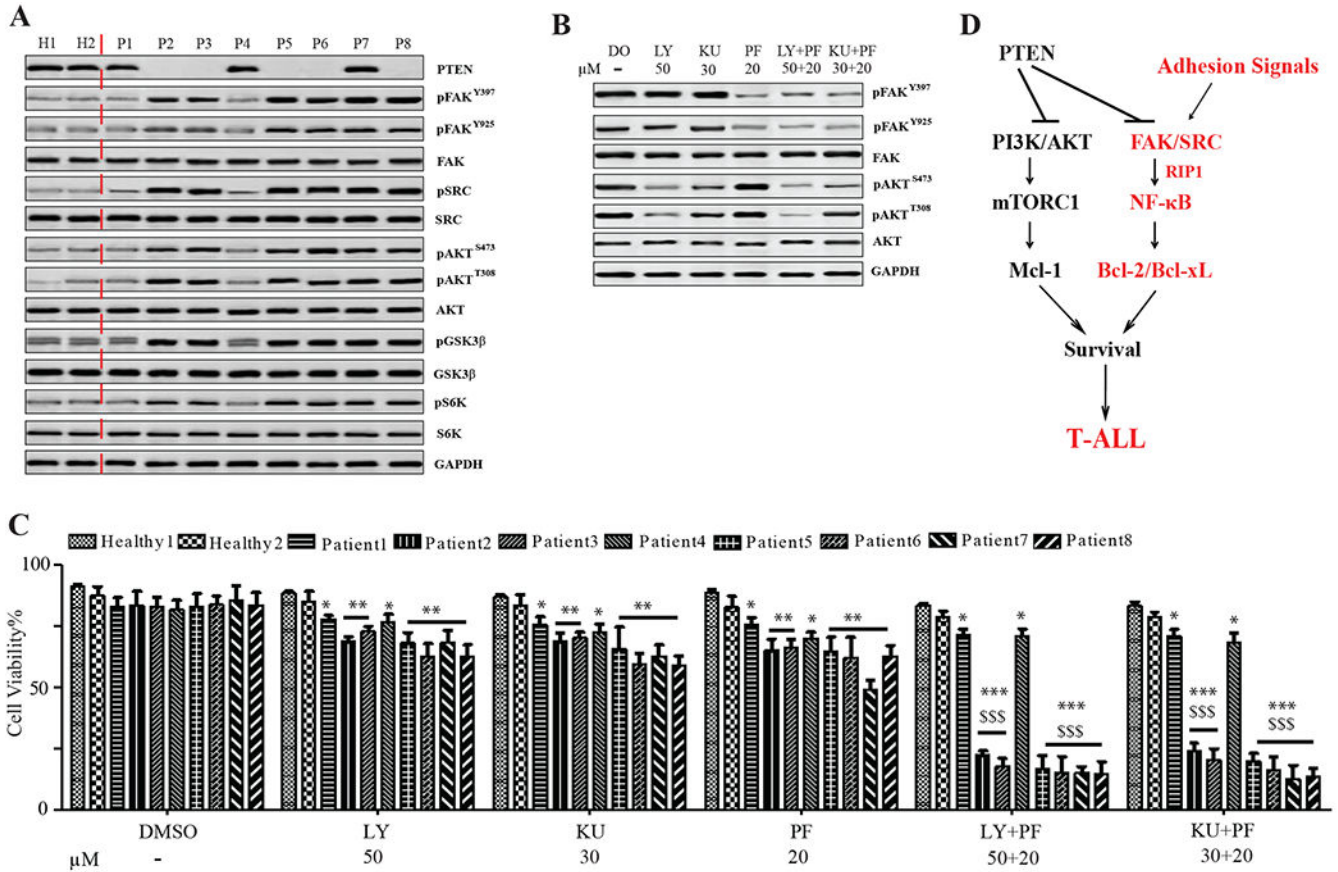


Figure 7. *PTEN*-mutant Human Primary T-ALL Cells are more Sensitive to Combined Treatment Using PI3K/mTOR and FAK Inhibitors than to Either Inhibitor Alone. (A) *PTEN*, pFAK, pSRC, pAKT, pGSK3 β and pS6K expression in primary human T-ALL cells cultured on MG-coated plates; controls: healthy CD3⁺ lymphocytes. (B) pFAK and pAKT in patient 2 samples (*PTEN*-null) treated with DMSO (DO), LY, KU, PF, or LY+PF, KU+PF. Images representative of three independent experiments. (C) Primary human T-ALL cell viability after treatment with DO, LY, KU, PF, or LY+PF, KU+PF; controls: healthy CD3⁺ lymphocytes. Data shown are Mean \pm S.D. ***, **, * compared to healthy controls, $P < 0.001$, 0.01 , 0.05 ; \$\$\$ compared to single inhibitor treatment, $P < 0.001$, determined by 2-way ANOVA. (D) Schematic model of two parallel survival signals in *PTEN*-mutant T-ALL cells.