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# Regulation of T cell alloimmunity by PI3K $\gamma$ and PI3K $\delta$

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Phosphatidylinositol-3-kinases (PI3K)  $\gamma$  and  $\delta$  are preferentially enriched in leukocytes, and defects in these signaling pathways have been shown to impair T cell activation. The effects of PI3K $\gamma$  and PI3K $\delta$  on alloimmunity remain underexplored. Here, we show that both PI3K $\gamma^{-/-}$  and PI3K $\delta^{D910A/D910A}$  mice receiving heart allografts have suppression of alloreactive T effector cells and delayed acute rejection. However, PI3K $\delta$  mutation also dampens regulatory T cells (Treg). After treatment with low dose CTLA4-Ig, PI3K $\gamma^{-/-}$ , but not PI3K $\delta^{D910A/D910A}$ , recipients exhibit indefinite prolongation of heart allograft survival. PI3K $\delta^{D910A/D910A}$  Tregs have increased apoptosis and impaired survival. Selective inhibition of PI3K $\gamma$  and PI3K $\delta$  (using PI3K $\delta$  and dual PI3K $\gamma\delta$  chemical inhibitors) shows that PI3K $\gamma$  inhibition compensates for the negative effect of PI3K $\delta$  inhibition on long-term allograft survival. These data serve as a basis for future PI3K-based immune therapies for transplantation.

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Current immunosuppressive drugs (ISD) commonly suppress both effector and regulatory axes of adaptive immunity and fail to induce immune regulation, which is critical for long-term graft acceptance. Furthermore, ISDs contribute to microvascular toxicity and organ failure, as well as major long-term complications such as malignancy, metabolic disorders, and infections<sup>1, 2</sup>. Therefore, the development of targeted immunomodulatory agents with improved efficacy and safety profiles<sup>3</sup> in solid organ transplantation is highly desirable.

PI3Ks belong to the family of lipid kinases that phosphorylate the 3'-OH-group of phosphatidylinositols to generate phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P<sub>3</sub>), which then interacts with the pleckstrin-homology (PH)-domains of various signal transduction proteins including AKT<sup>4, 5</sup>. Class I PI3Ks are the best characterized of the different PI3K classes and are composed of regulatory subunits (p85) and catalytic subunits (p110 $\alpha$ , p110 $\beta$ , p110 $\delta$ , and p110 $\gamma$ ). Class I PI3Ks are further classified as class IA or IB according to their modes of activation: class IA PI3Ks are activated downstream of tyrosine kinase receptors, whereas the class IB PI3K has only one subunit (PI3K $\gamma$ ) and is activated by G protein-coupled receptors<sup>5, 6</sup>.

The  $\gamma$  and  $\delta$  catalytic forms of PI3K are preferentially enriched in leukocytes and, through their capacity to regulate the function of immune cells<sup>5, 7–10</sup>, represent a promising drug target for the treatment of inflammatory diseases. Although PI3K $\gamma$  is activated by G protein-coupled receptors including the chemokine receptors, our data and others indicate that PI3K $\gamma$ -deficient T cells also have a diminished anti-CD3 proliferative response<sup>11–15</sup>. The importance of p110 $\gamma$  in alloimmune responses also lies in its capacity to regulate innate immune cells and inflammatory responses<sup>8</sup>. PI3K $\delta$  is a sister isoform and lies downstream of tyrosine kinase-associated receptors, T cell receptor (TCR), co-stimulatory and cytokine receptors<sup>16–22</sup>. The lack of PI3K $\delta$  is detrimental to effector T cells (Teff)<sup>23, 24</sup>.

Despite an accumulating body of data on the role of PI3K $\gamma$  and  $\delta$  in immunity, the mechanisms by which the PI3K $\gamma$  and  $\delta$  signaling pathways control alloimmune responses remains to be explored. Here, we show for the first time the role of PI3K $\gamma$  and PI3K $\delta$  pathways in determining the fate of alloimmune responses.

## Results

**PI3K $\gamma$  or PI3K $\delta$  deletion suppresses T cell alloreactivity.** To study the effect of PI3K $\gamma$  and PI3K $\delta$  deletion on alloimmune responses *in vivo*, we injected  $6 \times 10^6$  CD3<sup>+</sup>CD25<sup>−</sup> T cells isolated from splenocytes of PI3K $\delta^{D910A/D910A}$ , PI3K $\gamma^{-/-}$ , or WT naive mice, into RAG<sup>−/−</sup> recipients of BALB/c skin allograft at day 1 post-transplant (Fig. 1a). Graft survival in recipients of PI3K $\gamma^{-/-}$  or PI3K $\delta^{D910A/D910A}$  T cells significantly exceeded that in recipients of control T cells ( $*p < 0.05$ , *t*-test,  $n = 5/\text{group}$ ) (Fig. 1b, c). Interestingly, there was a significant decrease in regulatory T cell (Treg) induction in mice receiving PI3K $\delta^{D910A/D910A}$  compared to WT CD3<sup>+</sup>CD25<sup>−</sup> T cells ( $27.60\% \pm 4.07\%$  vs.  $39.35\% \pm 5.05\%$ , respectively,  $p = 0.05$ , *t*-test,  $n = 3/\text{group}$ ) (Fig. 1d). Similarly, under Treg polarizing conditions, there was a marked decrease in Treg induction *in vitro* in the PI3K $\delta^{D910A/D910A}$  group compared with WT ( $11.07\% \pm 0.26\%$  vs.  $14.07\% \pm 0.88\%$ , respectively,  $*p < 0.05$ , *t*-test,  $n = 3–4/\text{group}$ ) (Fig. 1e).

**PI3K $\gamma$  or PI3K $\delta$  deletion impairs T cell activation *in vitro*.** PI3K $\gamma^{-/-}$  splenocytes proliferated significantly less when stimulated with allogeneic cells, as measured by thymidine incorporation (Supplementary Fig. 1A). Similarly, alloantigen stimulation of PI3K $\gamma^{-/-}$ -deficient T cells led to a significantly lower frequency of interferon (IFN) $\gamma$  producing T cells than WT controls (Supplementary Fig. 1B). PI3K $\delta^{D910A/D910A}$  CD4<sup>+</sup> and CD8<sup>+</sup> T cells

also proliferated significantly less than WT T cells upon stimulation with anti-CD3 and anti-CD28 antibodies, as measured by thymidine incorporation (Supplementary Fig. 1C, D).

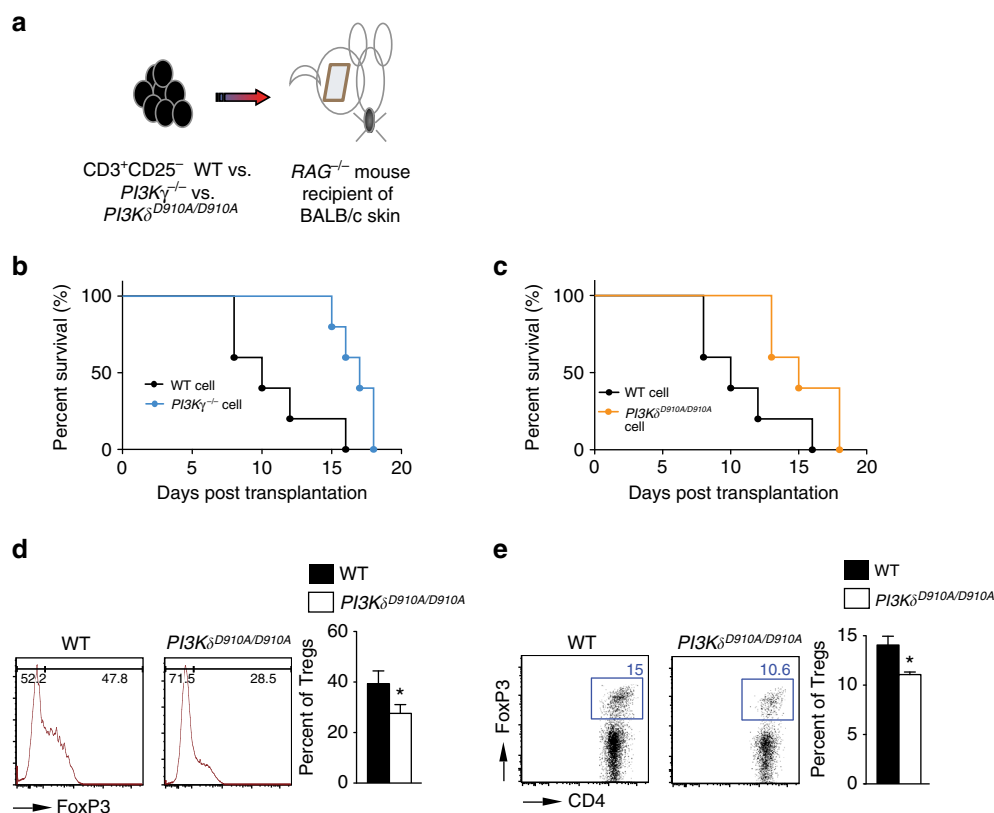
**PI3K $\gamma$  or PI3K $\delta$  deletion prolongs heart allograft survival.** We first used a model of acute cardiac transplant rejection as a pre-clinical model to study the immunosuppressive role of PI3K $\gamma$  and PI3K $\delta$  deletion. Naive BALB/c heart allografts were transplanted into fully allogeneic C57BL/6 (WT) recipients, PI3K $\gamma^{-/-}$  C57BL/6 (PI3K $\gamma^{-/-}$ ) recipients or PI3K $\delta^{D910A/D910A}$  C57BL/6 (PI3K $\delta^{D910A/D910A}$ ) recipients. PI3K $\gamma^{-/-}$  and PI3K $\delta^{D910A/D910A}$  recipients exhibited prolonged allograft survival compared to WT recipients (mean survival time, (MST) PI3K $\gamma^{-/-}$  vs. WT: MST of 11 vs. 7 days, respectively,  $*p < 0.05$ , *t*-test,  $n = 10/\text{group}$ , PI3K $\delta^{D910A/D910A}$  vs. WT: MST of 14 vs. 7 days, respectively,  $*p < 0.05$ , *t*-test,  $n = 10–14/\text{group}$ ) (Fig. 2a). Accordingly, PI3K $\gamma^{-/-}$  and PI3K $\delta^{D910A/D910A}$  recipients showed marked reduction in the severity of acute rejection of the heart allograft as assessed by histological analysis (Fig. 2b, c). We also observed a significant decrease in the number of CD4<sup>+</sup> and CD8<sup>+</sup> effector cells (CD44<sup>High</sup>CD62L<sup>Low</sup>) in the secondary lymphoid tissues of PI3K $\gamma^{-/-}$  recipients compared to WT (at day 7 post-transplant) ( $*p < 0.05$ , *t*-test,  $n = 6/\text{group}$ ) (Supplementary Fig. 2A–D). Furthermore, PI3K $\gamma^{-/-}$  recipients showed reduced T cell proliferative responses and suppression of inflammatory cytokine production at 7 days post-transplantation as assessed by MLR and luminex-assays ( $*p < 0.05$ , *t*-test,  $n = 4–6/\text{group}$ ) (Supplementary Fig. 2E–G).

Similarly, flow cytometric analysis of secondary lymphoid tissue of PI3K $\delta^{D910A/D910A}$  recipients showed fewer CD4<sup>+</sup> and CD8<sup>+</sup> effector T cells as compared to WT recipients ( $*p < 0.05$ , *t*-test,  $n = 6/\text{group}$ ) (Supplementary Fig. 3A, B, D, and E). However, there was significant decrease in the percentage of Tregs in secondary lymphoid tissue of PI3K $\delta^{D910A/D910A}$  recipients of BALB/c allografts compared to WT recipients at day 7 post-transplant ( $*p < 0.05$ , *t*-test,  $n = 6/\text{group}$ ) (Fig. 2d and Supplementary Fig. 3C).

## PI3K $\gamma$ or PI3K $\delta$ deletion and long-term allograft survival.

While single dose CTLA4-Ig treatment (250  $\mu\text{g}$  on day 2) of WT recipients resulted in moderate prolongation of allograft survival compared to untreated WT control (MST of 41 vs. 7 days, respectively,  $*p < 0.05$ , *t*-test), PI3K $\gamma^{-/-}$  recipients treated with a single dose CTLA4-Ig showed indefinite prolongation of heart allograft survival (MST of >100 vs. 41 days, respectively,  $*p < 0.05$ , *t*-test,  $n = 7–10/\text{group}$ ) (Fig. 3a). However, PI3K $\delta^{D910A/D910A}$  recipients treated with single dose CTLA4-Ig showed relatively shorter heart allograft survival compared to WT recipients (MST of 32 vs. 41 days, respectively,  $*p < 0.05$ , *t*-test,  $n = 7–10/\text{group}$ ) (Fig. 3a). Histological examination of heart allografts (harvested at day 100 post-transplant) showed minimal signs of rejection in the PI3K $\gamma^{-/-}$  + single dose CTLA4-Ig group as compared to WT + single dose CTLA4-Ig group (heart allografts harvested at day 28 post-transplant) (Fig. 3b). PI3K $\gamma^{-/-}$  + single dose CTLA4-Ig recipients also showed fewer CD4<sup>+</sup> and CD8<sup>+</sup> T cells in heart grafts harvested at day 28 post-transplant compared to WT + single dose CTLA4-Ig group. ( $*p < 0.05$ , *t*-test,  $n = 6/\text{group}$ ) (Fig. 3c). However, histological examination of heart allografts harvested at day 28 post-transplant showed massive cell infiltration in the PI3K $\delta^{D910A/D910A}$  recipients treated with single dose CTLA4-Ig as compared to WT recipients treated with single dose CTLA4-Ig (Fig. 3d).

PI3K $\gamma^{-/-}$  + single dose CTLA4-Ig recipients also showed significant suppression of CD4<sup>+</sup> and CD8<sup>+</sup> effector T cells in the secondary lymphoid tissue as compared to control mice



**Fig. 1** PI3K $\gamma$  or PI3K $\delta$  deletion impairs T cell activation in vivo. **a** Schematics describing the methodology used in this experiment.  $RAG^{-/-}$  mice received a BALB/c skin allograft; on day 1 post-transplant, the mice were injected with  $6 \times 10^6$  CD3 $^{+}$ CD25 $^{-}$  T cells isolated from splenocytes of PI3K $\gamma^{-/-}$ , PI3K $\delta^{D910A/D910A}$  or WT naive mice. **b** Graft survival in recipients of PI3K $\gamma^{-/-}$  T cells significantly exceeded that in recipients of control T cells (MST of 17 vs. 10 days, respectively,  $*p < 0.05$ ,  $t$ -test,  $n = 5$ /group). **c** Graft survival in recipients of PI3K $\delta^{D910A/D910A}$  T cells significantly exceeded that in recipients of control T cells (MST of 15 vs. 10 days, respectively,  $*p < 0.05$ ,  $t$ -test,  $n = 5$ /group). **d** Representative figures of flow cytometry analysis of splenocytes retrieved from transplanted mice at day 7 post-transplant. Data shows significant decrease in Treg induction in the DLN of PI3K $\delta^{D910A/D910A}$  recipients of BALB/c skin and WT controls ( $*p < 0.05$ ,  $t$ -test,  $n = 3$ /group). Bar graph represents the percentage of Tregs. **e** Representative example of FACS staining of CD4 $^{+}$  PI3K $\delta^{D910A/D910A}$  and WT T cells stimulated with anti-CD3/CD28 Abs in the presence of IL-2 and TGF $\beta$ . (Data are representative of three separate experiments,  $*p < 0.05$ ,  $t$ -test,  $n = 3$ –4/group). Bar graph represents the percentage of induced Tregs. **d, e** The graphs show data as mean  $\pm$  s.e.m. (MST mean survival time; Treg regulatory T cell)

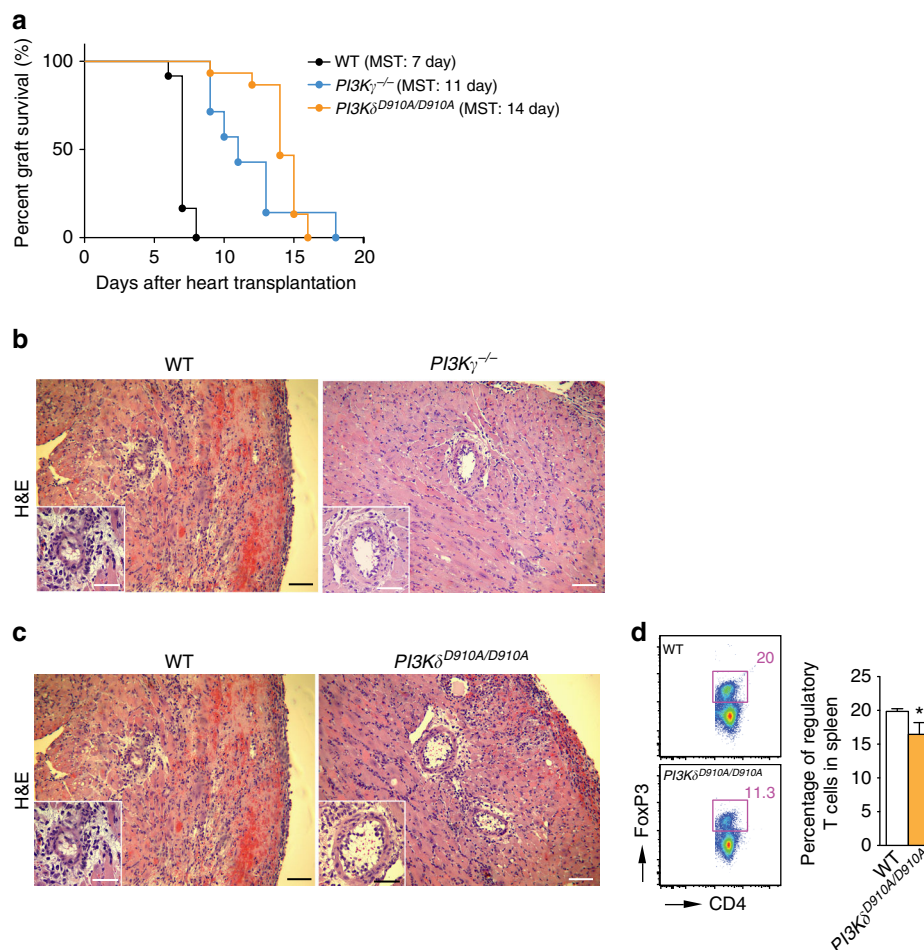
(Supplementary Fig. 4A, B). A significant reduction in T cell proliferative responses and in the level of inflammatory cytokines IFN $\gamma$  and interleukin (IL)-6 were also noted in PI3K $\gamma^{-/-}$  + single dose CTLA4-Ig recipients (Supplementary Fig. 4C–E). These data indicate that the inhibition of PI3K $\gamma$  selectively suppresses alloreactive T cells.

#### PI3K $\delta$ deletion abrogates the tolerogenic effect of CTLA4-Ig.

We then escalated the dose of CTLA4-Ig to induce indefinite allograft survival in WT recipients. Multiple dose CTLA4-Ig treatment (500  $\mu$ g on day 0 then 250  $\mu$ g on day 2, 4, 6, 8, and 10) of WT recipients resulted in indefinite prolongation of heart allograft survival compared to the untreated WT control (MST of >100 vs. 7 days, respectively,  $*p < 0.05$ ,  $t$ -test). However, PI3K $\delta^{D910A/D910A}$  + multiple dose CTLA4-Ig group showed significantly earlier rejection as compared to WT recipients (MST of >100 vs. 96 days, respectively,  $*p < 0.05$ ,  $t$ -test,  $n = 10$ /group) (Fig. 4a). Heart allografts from PI3K $\delta^{D910A/D910A}$  + multiple dose CTLA4-Ig treated mice recovered at day 100 post-transplant showed much more severe chronic rejection with lymphocyte infiltration and vasculopathy (Fig. 4b). Average scores for lymphocyte infiltration and vasculopathy were higher in PI3K $\delta^{D910A/D910A}$  + multiple dose CTLA4-Ig group compared to control (Fig. 4b below the H&E pictures) with increased intima-media thickness (Supplementary Fig. 5). PI3K $\delta^{D910A/D910A}$  + multiple

dose CTLA4-Ig group showed significantly greater CD4 $^{+}$  and CD8 $^{+}$  infiltration of the allografts compared to WT + multiple dose CTLA4-Ig group ( $*p < 0.05$ ,  $t$ -test,  $n = 6$ /group) (Fig. 4c). Furthermore, a significantly lower percentage of Tregs was observed in the spleen of treated PI3K $\delta^{D910A/D910A}$  mice compared to treated WT ( $*p < 0.05$ ,  $t$ -test,  $n = 6$ /group) (Fig. 4d). A much higher number of CD8 $^{+}$  effector T cells were also observed in the secondary lymphoid organs of treated PI3K $\delta^{D910A/D910A}$  mice compared to treated WT ( $*p < 0.05$ ,  $t$ -test,  $n = 6$ /group) (Supplementary Fig. 6). Interestingly, splenic CD8 $^{+}$  CD44 $^{high}$  T cells from PI3K $\delta^{D910A/D910A}$  mice showed much higher expression of Granzyme B (GrB) as compared to WT recipients, as measured by flow cytometry ( $52.38\% \pm 2.0\%$  vs.  $26.83\% \pm 1.77\%$ , respectively,  $*p < 0.05$ ,  $t$ -test,  $n = 4$ /group) (Fig. 4e). Finally, PI3K $\delta^{D910A/D910A}$  recipient splenocytes also significantly increased inflammatory cytokine and chemokine production upon stimulation by irradiated donor cells, as measured by luminex assay ( $*p < 0.05$ ,  $t$ -test,  $n = 4$ /group) (Fig. 4f).

**PI3K $\delta$  deletion reduces Treg survival.** Two groups of lethally irradiated BALB/c mice were reconstituted with  $2 \times 10^6$  CD3 $^{+}$ CD25 $^{-}$  T cells derived from WT C57BL/6 (Thy1.1) mice. Mice were co-injected with  $1 \times 10^6$  WT and PI3K $\delta^{D910A/D910A}$  natural regulatory T cells (nTreg) (Thy1.2). As shown in Fig. 5a, flow cytometric analysis of splenocytes showed that PI3K $\delta^{D910A/D910A}$



**Fig. 2**  $PI3K\gamma$  or  $PI3K\delta$  inhibition suppresses acute rejection. Naive BALB/c heart allografts were transplanted into fully allogeneic C57BL/6 (WT),  $PI3K\gamma^{-/-}$ , or  $PI3K\delta^{D910A/D910A}$  recipients. **a**  $PI3K\gamma^{-/-}$  recipients exhibited prolonged allograft survival compared to WT recipients (MST of 11 vs. 7 days respectively,  $*p < 0.05$ ,  $t$ -test,  $n = 10$ /group).  $PI3K\delta^{D910A/D910A}$  recipients of BALB/c hearts showed significant prolongation of heart allograft survival compared to control (MST of 14 vs. 7 days, respectively,  $*p < 0.05$ ,  $t$ -test,  $n = 10$ –14/group). **b** Representative examples of H&E staining of cardiac allograft histology show well-preserved myocytes and lower cellular inflammatory infiltrate at 7 days post-transplant in the  $PI3K\gamma^{-/-}$  mice compared to WT controls (Scale bar 50  $\mu$ m, inset scale bar 25  $\mu$ m). **c** Representative examples of cardiac allograft histology show well preserved myocytes and lower cellular inflammatory infiltrate at 7 days post-transplant in the  $PI3K\delta^{D910A/D910A}$  mice compared to WT controls (H&E stain, scale bar 50  $\mu$ m, inset scale bar 25  $\mu$ m). **d** Representative dot plots of Tregs ( $CD4^{+}FoxP3^{+}$ ) in splenocytes of WT and  $PI3K\delta^{D910A/D910A}$  recipients at day 7 post-transplant. Bar graph represents the percentage of Tregs in splenocytes in  $PI3K\gamma^{-/-}$  recipients compared to WT at day 7 post-transplant ( $*p < 0.05$ ,  $t$ -test,  $n = 6$ /group, the graph shows data as mean  $\pm$  s.e.m.). (MST mean survival time, Treg regulatory T cell)

nTregs had higher rate of apoptosis compared to WT nTregs ( $*p < 0.05$ ,  $t$ -test,  $n = 3$ /group). No difference was seen in the proliferation of the transferred WT and  $PI3K\delta^{D910A/D910A}$  nTregs as measured by Ki67 using flow cytometry. ( $p = ns$ ,  $t$ -test,  $n = 3$ /group) (Fig. 5b).

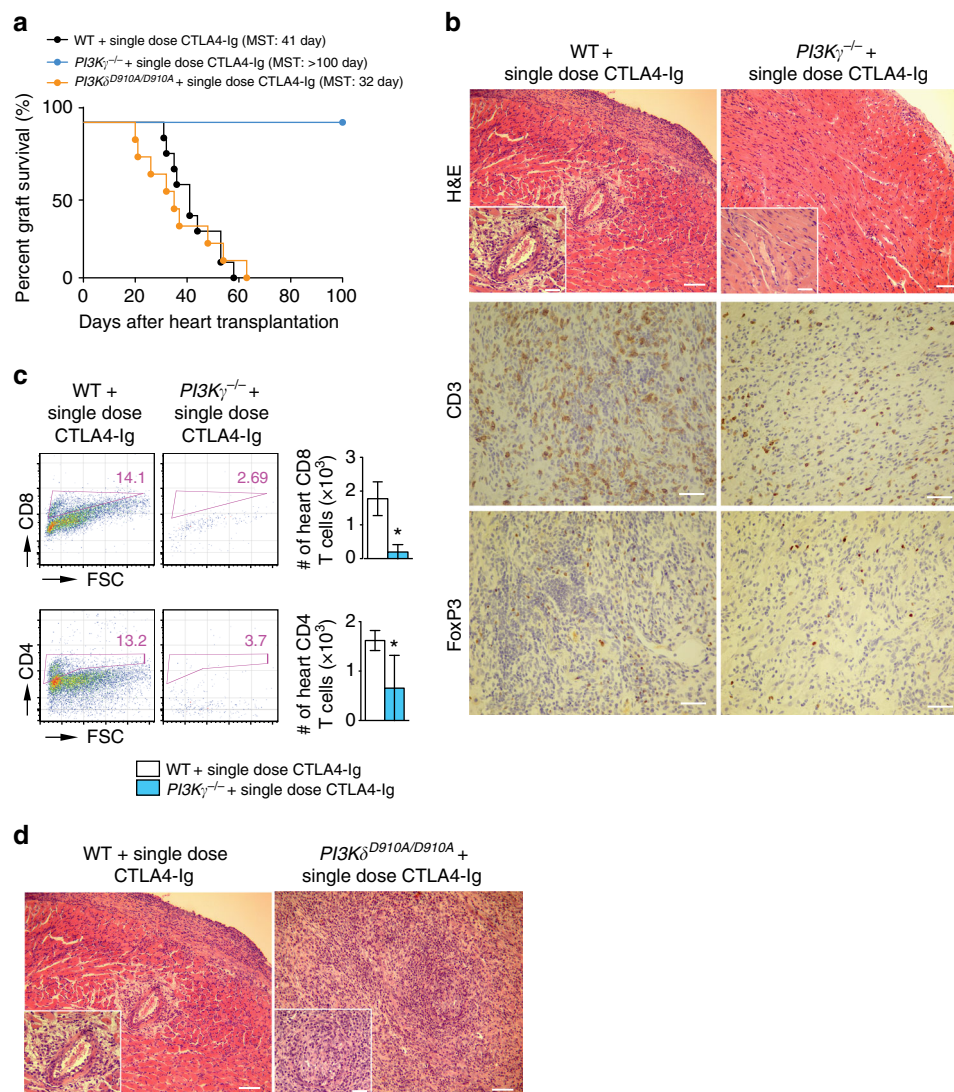
**$PI3K\gamma$  deletion protects from chronic rejection.** Chronic rejection remains a major cause of graft failure in clinical solid organ transplants<sup>25, 26</sup>. We next investigated the role of  $PI3K\gamma$  inhibition in chronic rejection using class II MHC-mismatch heart transplant model (B6.H-2<sup>bm12</sup> donor hearts into C57BL/6 recipient). Heart allografts recovered from  $PI3K\gamma^{-/-}$  recipients day 28 showed significantly less infiltration, fibrosis and intima-medial thickness as compared to WT recipients (Supplementary Fig. 7).

**$PI3K\delta$  deletion has no effect on *FoxP3* methylation.** We assessed the expression of the genes controlling the *FoxP3* promoters and the status of *FoxP3* demethylation on multiple CpG

motifs of the first intron of *FoxP3*, which have been shown to control its transcription<sup>27, 28</sup>. We performed quantification analysis of the *FoxP3* gene methylation at the five CpG sites of the proximal promoter known to control its transcription (location: -6750 to -6714 from ATG) in WT and  $PI3K\delta^{D910A/D910A}$  Tregs at 24 h post stimulation. Our analysis did not show any statistically significant difference between the different groups (Supplementary Fig. 8). Total percent methylation for in WT and  $PI3K\delta^{D910A/D910A}$  were  $58.3 \pm 6.6$  and  $59.29 \pm 11.2$ , respectively, ( $p = 0.8$ ,  $t$ -test,  $n = 4$ /group).

**Generation of  $PI3K\delta$  and  $PI3K\gamma\delta$  selective inhibitors.** To examine the effect of pharmacologic inhibition of PI3K isoforms, the selective inhibitors IPI-1828 and INK-055 were synthesized and utilized in our transplant model. To mimic the high ATP concentrations found within cells, IPI-1828 and INK-055 were evaluated in PI3K isoform specific biochemical assays in the presence of 3 mM ATP. IPI-1828 is a  $PI3K\delta$  selective inhibitor with activity against the targeted isoform of 19 nM against  $PI3K\delta$

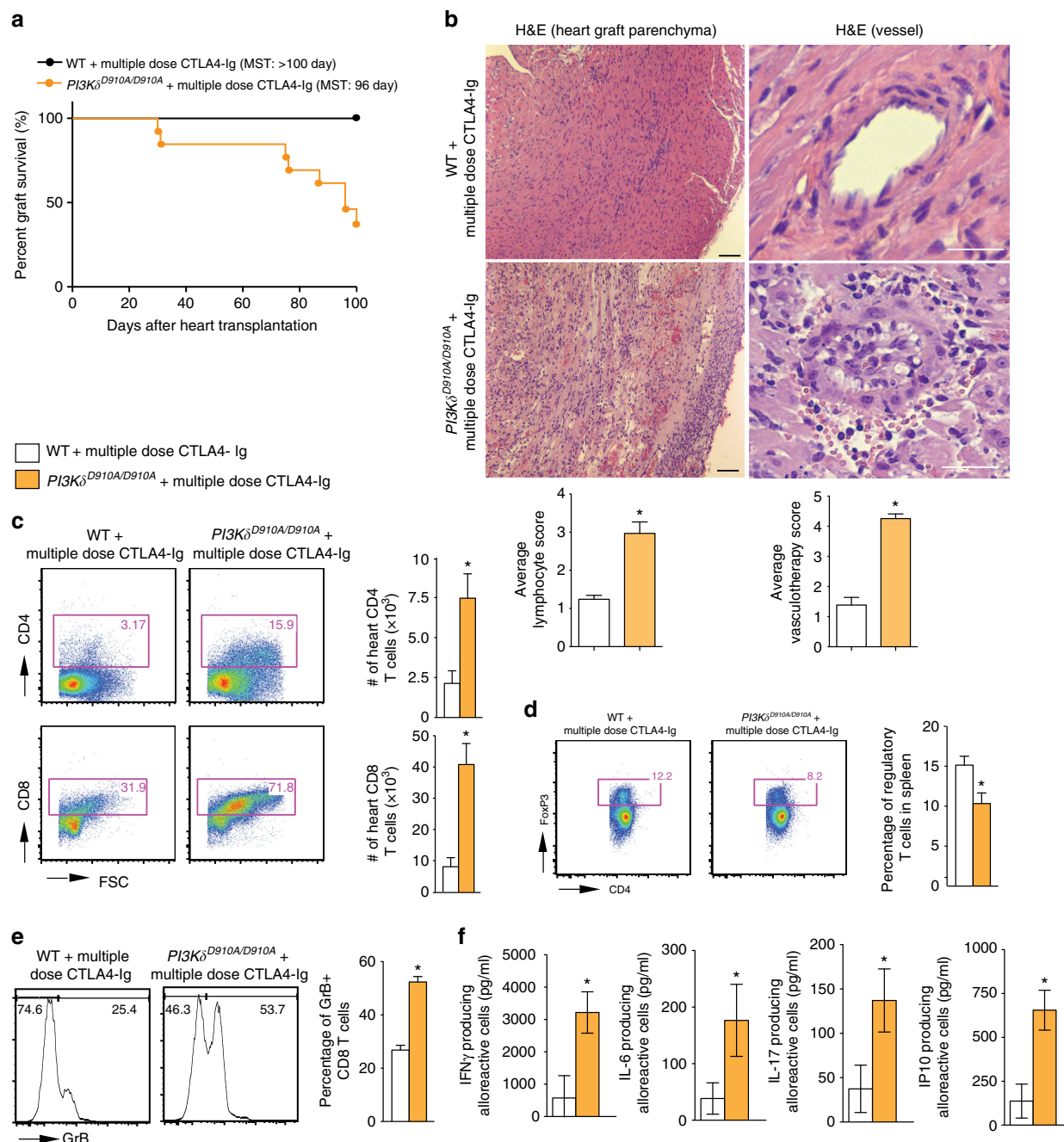




**Fig. 3**  $PI3K\gamma$  or  $PI3K\delta$  deletion and long-term allograft survival. **a** single dose CTLA4-Ig, injected into  $PI3K\gamma^{-/-}$  recipients induced long-term acceptance of fully mismatched BALB/c cardiac allografts compared to the controls (MST of 41 vs. >100 days,  $*p < 0.05$ ,  $t$ -test,  $n = 10$ /group). However, single dose CTLA4-Ig, injected into  $PI3K\delta^{D910A/D910A}$  recipients of fully mismatched BALB/c cardiac allografts showed reduced allograft survival compared to the controls (MST: 41 vs. 32 days,  $*p < 0.05$ ,  $t$ -test,  $n = 7$ –10/group). **b** Representative examples of cardiac allograft histology at day 100 post-transplant show lower grades of acute cellular rejection in the  $PI3K\gamma^{-/-}$  hearts treated with single dose CTLA4-Ig compared to cardiac allograft at day 28 post-transplant from WT mice treated with single dose CTLA4-Ig ( $n = 4$ /group). Inset show worse vasculopathy in the WT recipients treated with single dose CTLA4-Ig compared to  $PI3K\gamma^{-/-}$  hearts treated with single dose CTLA4-Ig (H&E stain, scale bar 50  $\mu$ m, inset scale bar 25  $\mu$ m). Immunohistochemistry analysis of CD3 and FoxP3 shows fewer CD3<sup>+</sup> infiltrating cells in the  $PI3K\gamma^{-/-}$  hearts treated with single dose CTLA4-Ig compared to WT but relatively more FoxP3 (scale bar 50  $\mu$ m). **c** Representative dot plots of leukocytes isolated from the allograft of  $PI3K\gamma^{-/-}$  and WT recipients treated with single dose CTLA4-Ig show significantly less CD4<sup>+</sup> and CD8<sup>+</sup> infiltration in the  $PI3K\gamma^{-/-}$  allografts compared to WT. Bar graph represents the absolute count of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the heart allografts of  $PI3K\gamma^{-/-}$  and WT recipients treated with single dose CTLA4-Ig. ( $*p < 0.05$ ,  $t$ -test,  $n = 6$ /group, the graphs show data as mean  $\pm$  s.e.m.). **d** Representative examples of cardiac allograft histology at day 28 post-transplant show higher grades of acute cellular rejection in the  $PI3K\delta^{D910A/D910A}$  hearts treated with single dose CTLA4-Ig compared to WT mice treated with single dose CTLA4-Ig ( $n = 4$ /group). Inset show more severe vasculitis in the  $PI3K\delta^{D910A/D910A}$  treated with single dose CTLA4-Ig compared to WT-recipient hearts treated with single dose CTLA4-Ig (H&E stain, scale bar 50  $\mu$ m, inset scale bar 25  $\mu$ m). (MST mean survival time)

and 5900, 3200, and 7900 nM against  $PI3K\alpha$ ,  $PI3K\beta$ , and  $PI3K\gamma$ , respectively. IPI-1828 was evaluated in isoform selective cell based assays with a 13 nM IC<sub>50</sub> of the phosphorylation of AKT (S473) in the  $PI3K\delta$  selective assay, and greater than 200-fold less activity in the  $PI3K\alpha$ ,  $PI3K\beta$ , and  $PI3K\gamma$  isoform selective cell based assays. INK-055 is a previously characterized  $PI3K$ -inhibitor that has potent cellular activity against  $PI3K\delta$  and  $PI3K\gamma$  with IC<sub>50</sub>s of 13 and 5 nM, respectively, (Fig. 6a–c) and is a dual  $PI3K\delta$  inhibitor in animal models<sup>29</sup>.

**Synergistic effect of  $PI3K\gamma$  and  $PI3K\delta$  inhibition.** We then tested whether the presence of  $PI3K\gamma$  inhibition would rescue the deleterious effect of  $PI3K\delta$  inhibition and whether there is synergism in further prolonging heart allograft survival. Naive BALB/c allografts were transplanted into WT C57BL/6 recipients and treated with a specific  $PI3K\delta$  inhibitor (IPI-1828: 50 mg/kg twice daily via gavage day 0 to 6). As shown in Fig. 6d, WT recipients treated with  $PI3K\delta$  inhibitor (IPI-1828) exhibited prolonged allograft survival compared to the WT recipients



**Fig. 4**  $PI3K\delta$  deletion abrogates the tolerogenic effect of CTLA4-Ig. **a** Multiple dose CTLA4-Ig treatment of  $PI3K\delta^{D910A/D910A}$  recipients abrogates the long-term acceptance of fully mismatched BALB/c cardiac allografts seen in treated WT controls (MST of >100 vs. 96 days,  $*p < 0.05$ , t-test,  $n = 10$ /group). **b** Representative examples of cardiac allograft histology harvested 100 days after transplant show higher grades of cellular infiltration (H&E stain, scale bar 50  $\mu$ m) and vasculopathy (H&E stain, scale bar 25  $\mu$ m) in the  $PI3K\delta^{D910A/D910A}$  hearts treated with multiple dose CTLA4-Ig compared to WT mice treated with multiple dose CTLA4-Ig ( $n = 4$ /group). The bar graphs show the average of lymphocyte infiltration score and vasculopathy score. The  $PI3K\delta^{D910A/D910A}$  hearts treated with multiple dose CTLA4-Ig shows significantly higher score compared to WT mice treated with multiple dose CTLA4-Ig ( $*p < 0.05$ , t-test,  $n = 4$ /group, the graphs show data as mean  $\pm$  s.e.m.). **c** Representative examples of dot plots of leukocytes isolated from the allograft of  $PI3K\delta^{D910A/D910A}$  and WT recipients treated with multiple dose CTLA4-Ig shows more CD4 $^{+}$  and CD8 $^{+}$  infiltration in the allografts of  $PI3K\delta^{D910A/D910A}$  recipients compared to WT control. Graph represents the absolute count of graft-infiltrating CD4 and CD8 in  $PI3K\delta^{D910A/D910A}$  recipients treated with multiple dose CTLA4-Ig compared to WT control treated with multiple dose CTLA4-Ig ( $*p < 0.05$ , t-test,  $n = 6$ /group). **d** Representative examples of dot plots of Tregs in the DLN of  $PI3K\delta^{D910A/D910A}$  and WT recipients treated with multiple dose CTLA4-Ig show significantly fewer Tregs in the  $PI3K\delta^{D910A/D910A}$  recipients compared to WT control ( $*p < 0.05$ , t-test,  $n = 6$ /group). **e** Representative examples of histograms of GrB expression by CD8 $^{+}$  CD44 $^{high}$  T cells in the spleen of  $PI3K\delta^{D910A/D910A}$  and WT recipients treated with multiple dose CTLA4-Ig show significantly more GrB expression in CD8 $^{+}$  of the  $PI3K\delta^{D910A/D910A}$  recipients compared to WT ( $*p < 0.05$ , t-test,  $n = 4$ /group). **f** WT and  $PI3K\delta^{D910A/D910A}$  recipient splenocytes were stimulated with donor splenocytes. Bar graphs represent the level of IFN $\gamma$ , IL-6, IL-17, and IP10 in the supernatant collected from the MLR assay as measured by luminex ( $*p < 0.05$ , t-test,  $n = 4$ /group). **c–f** The graphs show data as mean  $\pm$  s.e.m. (MST mean survival time; DLN draining lymph node; Treg regulatory T cell; GrB GranzymeB)

treated with vehicle only (MST of 13.5 vs. 7 days, respectively,  $*p < 0.05$ ,  $t$ -test,  $n = 5/\text{group}$ ). However, mice treated with dual PI3K $\gamma\delta$  inhibitor (INK-055: 100 mg/kg daily, day 0 to 6) exhibited an additional allograft survival compared to either the WT recipients treated with PI3K $\delta$  inhibitor (IPI-1828) or vehicle only (MST of 35 vs. 13.5 vs. 7 days, respectively,  $*p < 0.05$ ,  $t$ -test,  $n = 5/\text{group}$ ) (Fig. 6d, e). Furthermore, PI3K $\gamma^{-/-}$  recipients treated with PI3K $\delta$  inhibitor (IPI-1828) exhibited a significantly prolonged allograft survival compared to the PI3K $\gamma^{-/-}$  recipients treated with vehicle only (MST of 20 vs. 11 days, respectively,  $*p < 0.05$ ,  $t$ -test,  $n = 5\text{--}10/\text{group}$ ) (Fig. 6d). Interestingly, while PI3K $\delta$  inhibitor (IPI-1828) abrogated the tolerogenic effect of multiple dose CTLA4-Ig on WT C57BL/6 recipients of BALB/c hearts (MST of 91 days), adding PI3K $\delta$  inhibitor (IPI-1828) to PI3K $\gamma^{-/-}$  recipients treated with single or multiple dose CTLA4-Ig induced indefinite heart allograft survival and did not abrogate tolerance (Fig. 6d).

## Discussion

To study the role of PI3K $\gamma$  and PI3K $\delta$  in alloimmunity, first we showed that PI3K $\gamma^{-/-}$  and PI3K $\delta^{D910A/D910A}$  T cells have impaired alloimmune reactivity; demonstrating suppressed alloreactive T cell proliferative responses coupled with a reduction in the frequency of IFN $\gamma$  producing alloreactive cells in vitro. These findings were confirmed in vivo in an adoptive transfer model. These data are in accordance with previously published

work showing that, along with activation via G protein coupled receptors, TCR cross-linking with anti-CD3 Abs also activates p110 $\gamma$  and induces its association with Ick and ZAP70, resulting in T cell activation<sup>14</sup>. PI3K $\delta$  is also downstream of TCR and its absence from this pathway explains the T cell defect observed in vitro and in vivo. However, PI3K $\delta$  deletion had a significant deleterious effect on regulatory T cell (Treg) induction.

Prolongation of heart allograft survival in PI3K $\gamma^{-/-}$  recipients was associated with a marked suppression of alloreactive T cells in a model of acute heart allograft rejection. Accumulating experimental data point to the importance of targeting innate immunity and inflammation to improve transplant outcomes, particularly to prevent the development of chronic rejection, which remains one of the main barriers to long term allograft survival<sup>30–35</sup>. PI3K $\gamma^{-/-}$  recipients showed a notable decrease in inflammatory cytokines known to play a key role in the pathogenesis of chronic rejection<sup>36, 37</sup>. This reduction in inflammatory cytokines may, in part, explain the marked reduction in chronic rejection noted in the PI3K $\gamma^{-/-}$  recipients.

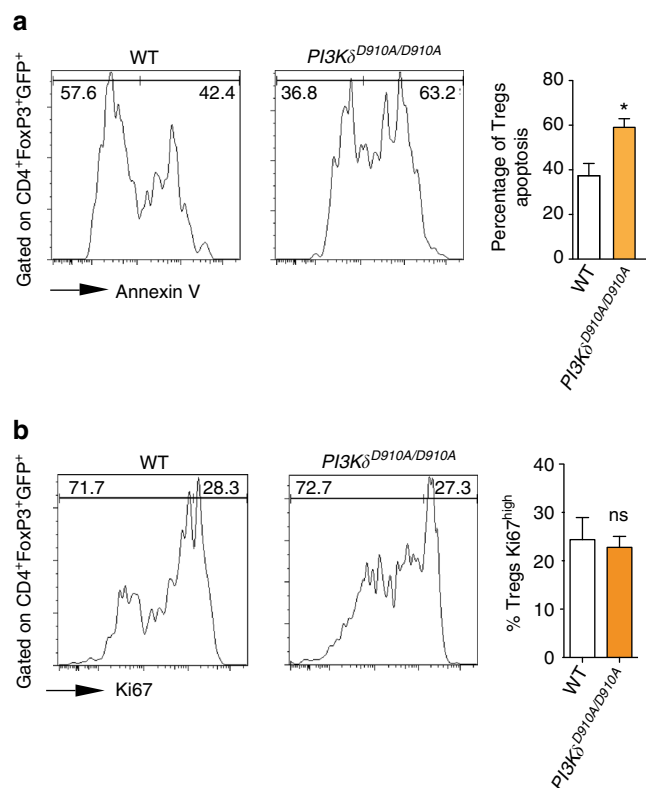
One of our key findings was that the inhibition of PI3K $\delta$  effectively suppressed alloreactive T effectors while it also reduced Tregs. The balance of Tregs to T effectors is well established as a key determinant of the long-term outcome of transplantation. Similar to PI3K $\gamma$  inhibition, PI3K $\delta$  inhibition prolonged allograft survival in an acute heart allograft rejection model with significant suppression of effector CD4 $^{+}$  and CD8 $^{+}$  T cells post-transplantation. However, to study the impact of PI3K $\delta$  signaling on the maintenance of long-term tolerance via its effect on Treg homeostasis, we used a combinatorial strategy, treating PI3K $\delta^{D910A/D910A}$  recipients with single dose CTLA4-Ig. In this long-term transplantation model, graft survival is dependent on the presence of Tregs. PI3K $\delta$  inhibition abrogated the tolerogenic effect of CTLA4-Ig with a significant reduction in Tregs. This decrease in Tregs may explain the upregulation of the CD8 $^{+}$  T effector cells in PI3K $\delta^{D910A/D910A}$  recipients and the observed increase in allograft-infiltrating inflammatory cells.

We then investigated whether this reduction in Tregs in the periphery is due to decreased proliferation or increased apoptosis. Transfer of natural Tregs (nTreg) from WT, and PI3K $\delta^{D910A/D910A}$  mice into an allogeneic host showed a higher rate of PI3K $\delta^{D910A/D910A}$  nTreg apoptosis compared to WT nTregs. No difference in proliferation was observed between the different groups, as assessed by Ki67 expression of transferred Tregs. Our data is in accordance with previous work by Ali et al.<sup>38</sup>, where p110 $\delta$  inhibition induced a Treg defect, leading to tumor regression through enhanced CD8 cytotoxic T cell activity.

We also investigated the mechanisms responsible for the deleterious effect of PI3K $\delta$  on Treg induction. The transcriptional activity of the CpG-motif containing element of the *FoxP3* first intron, which is located within TSDR (Treg-specific demethylated region) is known to be methylation-sensitive. Methylation of this island inversely correlates with CREB binding and *FoxP3* expression<sup>28</sup>. Furthermore, DNA methylation in TSDR has been shown to be critically involved in maintaining stable *FoxP3* expression<sup>39</sup>. We observed no differences in the demethylation of the *FoxP3* gene in PI3K $\delta^{D910A/D910A}$  CD4 $^{+}$  Tregs.

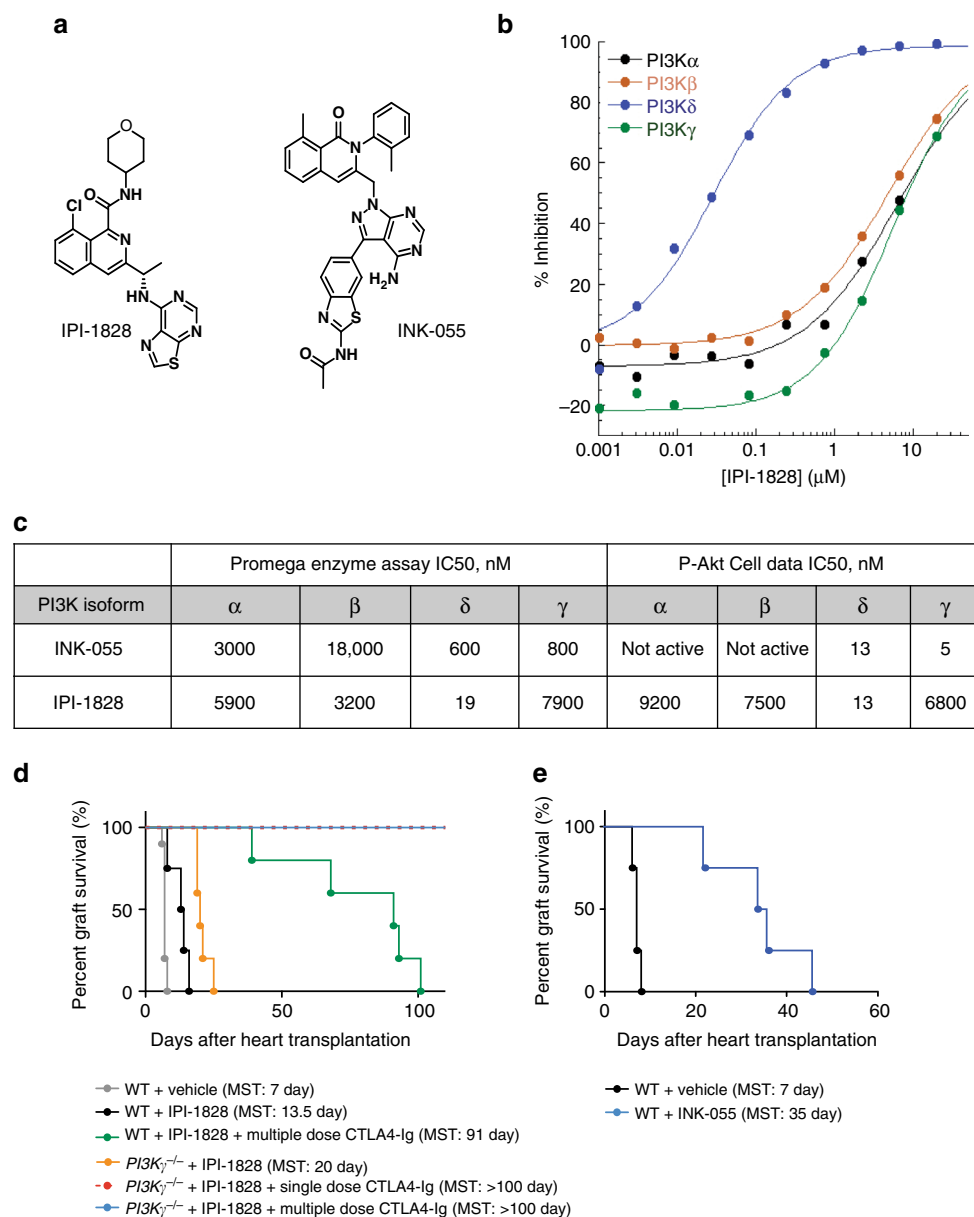
Currently, the importance of PI3K pathways in the development of Tregs is under intense investigation<sup>40</sup>, and dramatic anti-tumor effects have been seen in trials using PI3K $\delta$  inhibition in patients with chronic lymphocytic leukemia<sup>41</sup>. This effect has been ascribed to the inhibition of Treg in these patients and our data are in concordance with these findings.

Altogether, our data indicate that the inhibition of PI3K $\gamma$  and PI3K $\delta$  both restrict the expansion of alloreactive T cells, however, PI3K $\delta$  has a counter-regulatory effect on the homeostasis of Treg. Recent studies have highlighted the potential importance of the



**Fig. 5** PI3K $\delta$  inhibition abrogates Treg survival. **a** Representative examples of Annexin staining of WT and PI3K $\delta^{D910A/D910A}$  nTregs transferred in the spleen of C57BL/6 mice induced with GVHD. Bar graph represents the different percentages. ( $*p < 0.05$ ,  $t$ -test,  $n = 3/\text{group}$ ). **b** Representative examples of Ki67 expression of WT and PI3K $\delta^{D910A/D910A}$  nTregs transferred into C57BL/6 mice induced with GVHD. Bar graph represents the different percentages. ( $*p < 0.05$ ,  $t$ -test,  $n = 3/\text{group}$ ). **a, b** The graphs show data as mean  $\pm$  s.e.m. (Treg regulatory T cell; GVHD graft vs. host disease; nTreg natural regulatory T cell)





**Fig. 6** Synthesis of new PI3K  $\delta$  and  $\gamma\delta$  inhibitor. The characterization of IPI-1828, a PI3K $\delta$  selective compound, and INK-055, a PI3K $\gamma\delta$  inhibitor. **a** The chemical structure of IPI-1828 and INK-055. **b** Isotherms of IPI-1828 inhibitory activity in enzymatic assays for Class 1 PI3Ks were determined by monitoring the hydrolysis of ATP with a luminescence-based end point assay as described in methods section. This assay was run in the presence of 3 mM ATP and 500  $\mu$ M PIP<sub>2</sub> substrates. The ATP concentration of 3 mM was used to more closely resemble cellular concentrations. **c** Summary of IC<sub>50</sub>s in a biochemical assay run in the presence of 3 mM ATP, and isoform selective cell-based assays for class 1 PI3Ks. **d** Synergism of PI3K $\delta$  and PI3K $\gamma$  inhibition in prolonging allograft survival. Kaplan-Meier survival of heart allografts in WT and PI3K $\gamma^{-/-}$  recipients treated with PI3K $\delta$  inhibitor (IPI-1828) and single dose CTLA4-Ig or multiple dose CTLA4-Ig. ( $n = 5$ /group). **e** Kaplan-Meier survival of heart allograft in WT recipients treated with PI3K $\gamma\delta$  inhibitor (INK-055) showed significant prolongation of heart allograft survival compared to control (MST of 35 vs. 7 days,  $*p < 0.05$ ,  $t$ -test,  $n = 5$ /group). (MST mean survival time)

level of AKT (activated by the PI3K pathway) on the fate of Treg and differentiation of effector T cells<sup>42, 43</sup>. Modulation of AKT activity in Treg is essential for their optimal function and excessive AKT activation has been shown to be detrimental to their suppressive activity<sup>40, 44, 45</sup>.

On the other hand, upon TCR activation, rapidly dividing CD8<sup>+</sup> cells demonstrate high AKT activity levels. Effector T cells with high PI3K/AKT activity are resistant to the suppressive effects of Tregs<sup>46, 47</sup>. Using mice deficient for PTEN (natural inhibitor of PI3K) in Treg, Turka's group has shown a change in the metabolic programming of Treg and decline in their

stability<sup>48</sup>. Future studies are required to better define the relative contribution of PI3K $\delta$  on controlling the balance of T<sub>eff</sub>/Treg via regulation of metabolic programming.

Finally, we have generated a PI3K $\delta$  inhibitor (IPI-1828) and a dual PI3K $\gamma\delta$  inhibitor (INK-055). PI3K $\delta$  inhibition has already entered the clinic with success, though synthesizing a PI3K $\gamma$  inhibitor has proved a more daunting task. In our studies, both PI3K $\gamma$  and PI3K $\delta$  inhibition suppressed alloreactive T cells; therefore, an important question to address was the importance of combinatorial therapy in prolonging heart allograft survival. Dual inhibition showed a further suppression of alloimmune

responses and restored the tolerogenic effect of CTLA4-Ig in the presence of PI3K $\delta$  inhibition. Dual inhibition of PI3K $\gamma$  and  $\delta$  has also previously been shown to have superior anti-inflammatory effects<sup>10</sup>. Our data indicate that PI3K $\gamma$  inhibition rescues the negative effect of PI3K $\delta$  inhibition on long-term allograft survival and identifies dual PI3K $\gamma$  and  $\delta$  inhibition as a promising and novel platform to pursue in future studies with large animals.

A role for PI3K $\gamma$  inhibition in modifying the activity of tumor-infiltrating myeloid cells toward a pro-inflammatory phenotype, leading to increased anti-tumor activity and reduced tumor growth has recently been reported<sup>49, 50</sup>. The contrasting findings observed here could be due to differences between our allogeneic transplant model versus syngeneic tumor models. In heart transplant models, rejection is predominantly a T cell mediated process<sup>51</sup>. Myeloid cells do not constitute the majority of infiltrating cells during rejection. Our T cell data is in accordance with multiple other publications showing a direct role for PI3K $\gamma$  inhibition in T cell suppression<sup>12–14, 52–54</sup>. Chronically immunosuppressed patients suffer from increased risk of malignancy and the capacity of PI3K $\gamma$  to potentially both prevent rejection and have anti-tumor activity renders it an extremely attractive target for clinical translation.

The field of transplantation has an ongoing unmet need for more selective and effective immunomodulatory molecules to improve transplantation outcomes. PI3K $\gamma$  inhibitors are being clinically studied for other immune-mediated diseases and our data indicate that these molecules also hold significant promise in controlling alloimmune activation, and show particularly impressive effects in the prevention of chronic allograft rejection, a major cause of late allograft loss. Further studies are required to fully evaluate their potential role in clinical transplantation.

## Methods

**Mice and reagents.** C57BL/6J (JAX#000664), BALB/cByJ (H-2<sup>d</sup>) (JAX#001026), B6.C-H2<sup>b</sup>m12/KhEg (bm12) (JAX#001162), *Foxp3GFP* knock-in mice on a C57BL/6 background (JAX#023800) and *Rag*<sup>−/−</sup> mice (JAX#002216) were obtained from the Jackson Laboratory. *PI3K $\gamma$ <sup>−/−</sup>* C57BL/6 mice (backcrossed 11 generations) were received from Dr Bao Lu (Boston Children's Hospital/ Harvard Medical School) and maintained in our animal facility<sup>55</sup>. *p110<sup>D910A</sup>* C57BL/6 (*PI3K $\delta$ <sup>D910A/D910A</sup>*) mice were obtained from Charles River Laboratory<sup>56</sup>. *PI3K $\gamma$ <sup>−/−</sup>* C57BL/6 and *p110<sup>D910A</sup>* C57BL/6 (*PI3K $\delta$ <sup>D910A/D910A</sup>*) mice were also backcrossed with *FoxP3GFP* knock-in mice in our facility. Male or female mice were used at 6–10 weeks of age (20–25 g) and were housed in sterilized ventilated cages in a specific pathogen-free animal facility under standard 12 h light/12 h dark cycle. Mice were fed ad libitum irradiated food and water. Each individual experiment was done using three to ten mice per group. Mice were euthanized by either CO<sub>2</sub> inhalation, intraperitoneal injection of ketamine/xylazine or isoflurane inhalation followed by observation of loss of vital signs and cervical dislocation. All animal experiments and methods were performed in accordance with the relevant guidelines and regulations approved by the Institutional Animal Care and Use Committee of Brigham and Women's Hospital, Harvard Medical School, Boston, MA.

**Murine cardiac transplantation.** Vascularized intra-abdominal heterotopic transplantation of cardiac allografts was performed using microsurgical techniques as described previously<sup>57</sup>. Briefly, 1 ml of cold heparin (BD Vacutainer Sodium Heparin #366480 143USP units/10 ml) was infused into the inferior vena cava of the donor mouse. Hearts were harvested following the ligation/dissection of superior vena cava and inferior vena cava and dissection of ascending aorta and pulmonary artery. Harvested donor hearts were stored at 4 degrees celsius, immersed in UW (University of Wisconsin) solution until transplantation. After abdominal incision of recipient mouse, abdominal aorta and inferior vena cava were clamped. Ascending aorta and pulmonary artery of donor heart were attached to abdominal aorta and inferior vena cava of recipient mouse, respectively, using 10–0 suture. Beating of transplanted heart was observed upon removal of cross clamp, and abdominal incision was closed. The survival of cardiac allografts was assessed by daily palpation. Rejection was defined as complete cessation of cardiac contractility as determined by direct visualization and confirmed by histology.

**Skin transplantation.** Full-thickness trunk skin grafts (1 cm<sup>2</sup>) harvested from BALB/c donors were transplanted onto the flank of *Rag*<sup>−/−</sup> recipient mice, sutured with 6–0 silk, and secured with dry gauze and a bandage for 7 days.

**Histological and immunohistochemical assessment.** Five micron thick formalin fixed paraffin embedded sections were stained with standard Hematoxylin and Eosin stain (H&E), CD3 stain, Foxp3 stain, Elastic Van Gieson stain and F4/80 stain. Histological evaluation was done using a score modified from the International Society for Heart and Lung Transplantation<sup>58, 59</sup>. Lymphocyte infiltration is graded 0 to 4 at 6 random fields of each heart H&E section blindly by two individual researchers (six sections/heart, three mice per group). The grades are defined as follows: grade 0 (no lymphocyte infiltration), grade 1 (less than 25% lymphocyte infiltration), grade 2 (25 to 50% lymphocyte infiltration), grade 3 (50 to 75% lymphocyte infiltration), and grade 4 (more than 75% lymphocyte infiltration and myocyte hemorrhage). Vascular score is determined by a combination of vascular occlusion score and perivascular lymphocyte infiltration. Vascular occlusion is scored from grade 0 to 4 for every vessel (six sections/heart, three mice per group). The grades are defined as follows: grade 0 (no occlusion), grade 1 (less than 50% occlusion), grade 2 (50 to 75% occlusion), grade 3 (75 to 95% occlusion) and grade 4 (more than 95% occlusion). The perivascular lymphocyte infiltration is scored as described above and was then added to the vascular occlusion score.

**Isolation of lymphocytes from hearts.** Cardiac allografts were removed, perfused with PBS, minced finely with a razor blade and digested at 37 °C with 1 mg/ml collagenase in 1 ml complete medium for 1 h. Cells in the supernatant were washed twice, centrifuged at 620xg using Percoll solutions at 33% (cell suspension) and 66%. Lymphocytes were aspirated at the interface.

**Treg generation assay.** Using a 96-well flat bottom plate, 100  $\mu$ l of anti-CD3 antibody (Ab) diluted in PBS (1  $\mu$ g/ml, BD Biosciences) was added to each well and incubated at 37 °C for 4 h. In all, 1  $\mu$ g/ml of anti-CD28 Ab, 10 ng/ml of IL-2 and soluble recombinant human TGF $\beta$  (1 ng/ml, R&D Systems) was then added to each well in the presence of  $2.5 \times 10^4$  CD4<sup>+</sup>CD25<sup>−</sup> T cells of C57BL/6 or *PI3K $\gamma$ <sup>−/−</sup>* mice.

**CD3/CD28 T cell stimulation and MLR assays.** Using a 96-well flat bottom plate, 100  $\mu$ l of anti-CD3 Ab and soluble anti-CD28 Ab (1  $\mu$ g/ml, BD Biosciences) was used in the presence of C57BL/6 or *PI3K $\gamma$ <sup>−/−</sup>* splenocytes. For the MLR assay, irradiated wild type (WT) BALB/c splenocyte stimulators and WT C57BL/6 or *PI3K $\gamma$ <sup>−/−</sup>* splenocyte responders were added to each well in a 96-well round bottom plate pulsed with 1  $\mu$ Ci of tritiated thymidine (<sup>3</sup>H) and the incorporation efficiency was determined.

**ELISpot.** ELISpot assays were performed with WT BALB/c splenocytes as stimulators and WT C57BL/6 or *PI3K $\gamma$ <sup>−/−</sup>* splenocytes as responders, as described previously<sup>60</sup>. Briefly,  $5 \times 10^5$  each of irradiated stimulating cells and responders were added to each well of Millipore Immunospot plates (Millipore Corporation, Bedford, MA). Biotinylated antibodies specific for each cytokine were added to the wells and incubated. The plates were developed and spots were counted on an Immunospot analyzer (Cellular Technology Ltd., Cleveland, OH).

**Flow cytometric analysis.** Anti-mouse monoclonal antibodies against CD62L (1:400 diluted, MEL-14, 560516), CD44 (1:400 diluted, IM7, 103027), CD4 (1:400 diluted, RM4-5, 100559), CD25 (1:400 diluted, PC61.5, 12-0251-81B), CD8 (1:400 diluted, 53-6.7, 100734), FoxP3 (1:300 diluted, FJK-16s, 11-5773-82), GrB (1:100 diluted, KGZB, 12-8898-82), H2K<sup>b</sup> (1:100 diluted, SF1-1.1, 553565), thy1.2 (1:100 diluted, 53-2.1, 17-0902-83), Annexin PE (51-65875X), 7 AAD PerCP (51-68981E), Ki67 (1:300 diluted, SolA15, 11-5698-82), cRel (1:300 diluted, IRE-LAHS, 12-6111-80) were purchased from BD Biosciences, San Jose, CA. Cells recovered from spleens and peripheral lymphoid tissues were interrogated with a FACS Canto-II flow cytometer (BD Biosciences) and analyzed using FlowJo software version 9.3.2 (Treestar, Ashland, OR). Para-aortic lymph nodes from host were harvested as secondary lymphoid organs or draining lymph nodes (DLN) of the heart allografts. Gating strategies for lymphocyte subpopulations are shown in Supplementary Fig. 9.

**Luminex assay.** A 21-plex cytokine-kit (Millipore, St Charles, MO) was used according to the manufacturer's instructions to assess cytokine production in culture supernatant and in murine serum samples.

**CFSE labeling.** Spleen and peripheral lymph nodes were recovered from C57BL/6 (WT) and *PI3K $\gamma$ <sup>−/−</sup>* mice, and a single cell suspension was prepared in HBSS. Red blood cells were lysed with ACK buffer and cells were suspended in Hanks' Balanced Salt Solution (HBSS) at  $1 \times 10^7$  cells/ml for labeling with CFSE (Molecular Probes, Portland, OR). Briefly, cells were incubated with CFSE at a final concentration of 5  $\mu$ M in serum-free HBSS at room temperature for 6 min. The labeling reaction was then terminated by the addition of FCS (10% of the total volume). Cells were then washed twice in HBSS and used for *in vitro* experiments.

**FoxP3 gene demethylation analysis.** For DNA demethylation analysis, 500 ng of extracted genomic DNA was bisulfite treated and purified according to the manufacturer's protocol. PCRs were performed using 1  $\mu$ l of bisulfite treated DNA and

0.2  $\mu$ M of each primer. PCR products were sequenced by Pyrosequencing on the PSQ96 HS System (Pyrosequencing, Qiagen) following the manufacturer's instructions. The methylation status of each CpG site was determined individually as an artificial C/T SNP using QCpG software (Pyrosequencing, Qiagen). The methylation level at each CpG site was calculated as the percentage of the methylated alleles divided by the sum of all methylated and unmethylated alleles. The mean methylation level was calculated using methylation levels of all measured CpG sites within the targeted region of each gene. Each experiment included non-CpG cytosines as internal controls to detect incomplete bisulfite conversion of the input DNA. In addition, a series of unmethylated and methylated DNA are included as controls in each PCR. Furthermore, PCR bias testing was performed by mixing unmethylated control DNA with in vitro methylated DNA at different ratios (0, 5, 10, 25, 50, 75, and 100%), followed by bisulfite modification, PCR, and Pyrosequencing analysis. CpG sites analyzed for the *FoxP3* gene were CpG 64–68 of the proximal promoter.

**PI3K inhibitors.** IPI-1828 (PI3K $\delta$  inhibitor) was prepared according the methods in US Patent No. 8,901,133 Example 85 and was obtained from Infinity Pharmaceuticals, Inc. INK-055 (PI3K $\gamma$  inhibitor) was prepared as described<sup>29</sup> and was obtained from Infinity Pharmaceuticals, Inc. IPI-1828 was stored as a 10 mM stock in dimethyl sulfoxide (DMSO) at room temperature. All human PI3K isoforms were purchased from Millipore (Billerica, MA).

**Biochemical IC<sub>50</sub> determinations.** IC<sub>50</sub> values for all PI3K isoforms were determined by measuring the dose-dependent decrease in luminescent signal with increasing concentrations of IPI-1828. PI3K was incubated for 15 min with increasing concentrations of IPI-1828 followed by addition of ATP to 3 mM concentration and 500  $\mu$ M diC<sub>8</sub>PIP<sub>2</sub> substrates. Reactions were incubated at room temperature for 2 h. Detection of PI3K activity was then carried out using the ADP-Glo Max Assay Kit. Enzyme concentrations of 10 nM were used for the PI3K $\alpha$  and PI3K $\delta$  isoforms, and 40 nM for PI3K $\beta$  and PI3K $\gamma$  isoforms. IC<sub>50</sub> measurements were calculated as described previously<sup>61</sup>.

**PI3K $\alpha$  assay design/PI3K $\beta$  assay design.** SKOV-3 cells (ATCC HTB-77) for PI3K $\alpha$  assay design and 786-O cells for PI3K $\beta$  assay design were seeded into 96-well cell culture grade plates at a density of 200,000 cells/200  $\mu$ l/well of RPMI-1640 with 10% FBS. Cells were incubated overnight at 5% CO<sub>2</sub> and 37 °C. IPI-1828 or INK-055 (diluted in 25% DMSO in threefold dilutions from 10  $\mu$ M to generate an 11 and 9 point titration curve, respectively) was added to the cells, resulting in a final DMSO concentration of 0.5%, and incubated for 30 min at 5% CO<sub>2</sub> and 37 °C.

**PI3K $\gamma$  assay design.** RAW 264.7 cells were seeded into 96-well cell culture-grade plates at a density of 200,000 cells/200  $\mu$ l/well of DMEM with no FBS added. Cells were incubated overnight at 5% CO<sub>2</sub> and 37 °C. Following 18 h of serum-starvation, IPI-1828 or INK-055 (diluted in 25% DMSO in threefold dilutions from 0.2  $\mu$ M to generate a 9-point titration curve) was added to the cells, resulting in a final DMSO concentration of 0.5%, and incubated for 30 min at 5% CO<sub>2</sub> and 37 °C. Cells were then stimulated with 25 nM C5a for 3 min in the presence of IPI-1828 or INK-055.

**PI3K $\delta$  assay design.** RAJI cells were seeded into 96-well cell culture-grade plates at a density of 200,000 cells/200  $\mu$ l/well of RPMI-1640 with no FBS added. Cells were incubated overnight at 5% CO<sub>2</sub> and 37 °C. Following 18 to 24 h of serum-starvation, IPI-1828 or INK-055 (diluted in 25% DMSO in threefold dilutions from 10  $\mu$ M to generate an 11-point titration curve) was added to the cells, resulting in a final DMSO concentration of 0.5%, and incubated for 30 min at 5% CO<sub>2</sub> and 37 °C. Cells were then stimulated with 10  $\mu$ g/ml anti-human IgM for 30 min in the presence of IPI-1828 or INK-055.

Media was aspirated and 50  $\mu$ l/well of ice-cold lysis buffer was added. Plates were incubated on ice for 5 min and then centrifuged at 3000 rpm at 4 °C for 5 min. Phospho AKT ELISA and analysis were as described for each isoform<sup>61</sup>.

**Pharmacokinetics.** The pharmacokinetics and oral bioavailability of IPI-1828 in C57BL/6 mice were studied following a single intravenous and oral doses. Blood samples were collected prior to dose administration (oral dose only) and at time points post-treatment. The blood samples were processed to plasma and assayed for IPI-1828 using a liquid chromatography method with tandem mass spectrometric (LC-MS/MS) detection. Pharmacokinetic parameters were determined using standard non-compartmental methods<sup>61</sup>.

**Statistics.** Kaplan–Meier survival curves were constructed, and a log rank comparison of the groups was used to calculate *p*-values. The unpaired *t*-test was used for comparison of experimental groups examined by ELISpot, ELISA, luminex, flow cytometry, and MLR. Differences were considered to be significant for *p* < 0.05. Prism software was used for data analysis and drawing graphs (GraphPad Software, Inc., San Diego, CA). Data represent mean  $\pm$  s.e.m.

**Data availability.** All data generated or analyzed during this study are available from the author on reasonable request.

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## Author contributions

M.U. performed experiments, microsurgery and analyzed data, and drafted the manuscript. M.M. designed experiments, interpreted data and drafted the manuscript. S.O. performed experiments and microsurgery. Z.S., N.B., S.R., and A.E. performed experiments. C.E., J.D., and D.W. developed IPI-1828 and INK-055, designed and performed PI3Kγ and δ assay. L.T., T.S., and S.T. helped with study design. J.A. and R.A. designed the study, interpreted data, analyzed data, and critically revised and finalized the manuscript.

## Additional information

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