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Inactivation of the PI3K p110δ breaks regulatory T cell-mediated immune tolerance to cancer

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Abstract

Inhibitors against the p110δ isoform of PI3K have shown remarkable therapeutic efficacy in some human leukaemias[1,2]. Since p110δ is primarily expressed in leukocytes[3], drugs against p110δ have not been considered for the treatment of solid tumours[4]. We report here that p110δ inactivation in

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Author Contributions K.A., D.S., R.P., E.L.L, H.B. and W.P. performed experiments and data analyses with input from K.O. and B.V. D.S. and K.O. generated the FoxP3$^{YFP\text{-}Cre}$x$\delta^{flox/flox}$ mice and helped to design, perform and interpret experiments. R.P. and W.P. helped design experiments, T. Hagemann and his team carried out the KPC experiments and performed analysis with help of K.A. M.T. generated the p110$\delta^{flox/flox}$ mice. T. Hancox performed chemistry for small molecule inhibitor development. H.M. helped design and performed *in vivo* pharmacologic cancer experiments. L.F. helped design and interpret pharmacologic data. C.S. performed and interpreted histopathology. K.A., K.O. and B.V. wrote the paper.

Author Information K.O. is consultant to GSK (Stevenage) and B.V. is a consultant to Retroscreen (London, UK) and Karus Therapeutics (Oxford, UK).

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mice protects against a broad range of cancers, including non-haematological solid tumours. We demonstrate that p110δ inactivation in regulatory T cells (Treg) unleashes $CD8^+$ cytotoxic T cells and induces tumour regression. Thus, p110δ inhibitors can break tumour-induced immune tolerance and should be considered for wider use in oncology.

PI3K $p110\delta^{D910A}$ (δ^{D910A}) mice in which endogenous p110δ kinase is inactive, present specific immune deficiencies[5,6] but are not predisposed to cancer. To test if host p110δ activity affects tumour growth, we inoculated weakly immunogenic syngeneic cancer cell lines into δ^{D910A} mice. Compared to wild-type (WT) mice, δ^{D910A} mice were more resistant to B16 melanoma, with reduced tumour incidence and almost abrogated lymph node metastasis in those mice that developed tumours (Fig. 1a). Growth of Lewis lung carcinoma (LLC) and EL4 thymoma cells was also suppressed in δ^{D910A} mice (Fig. 1b,c). Similar observations were made with luciferase-labelled 4T1 breast cancer cells injected into the mammary fat pad. At sacrifice, δ^{D910A} mice showed reduced mass and luciferase activity of the primary 4T1 tumour (Fig. 1d) and lower metastasis (Fig. 1e). In WT mice, 4T1 tumours were detected by day 10 and grew progressively until day 30, at which point the mice became moribund (Fig. 1f). In some δ^{D910A} mice, 4T1 tumours grew initially, but then started to regress from day 15-20 onwards (Fig. 1f). Across 10 independent experiments, 97% (71/73) of WT mice had an observable cancer mass at the end of study, compared to 65% (43/66) of δ^{D910A} mice, with a median survival time of 23 and 40 days in WT and δ^{D910A} mice, respectively (Fig. 1g).

Effective tumour immunity is limited by Treg-mediated immune suppression[7]. δ^{D910A} mice show enhanced $FoxP3^+CD4^+$

Depletion of $CD8^+$ T cells but not of $CD4^+$ T cells on day 10 after 4T1 inoculation in δ^{D910A} mice eliminated cancer protection (Fig. 3a,b). These data show that $CD8^+$ T cells are responsible for restricting tumour growth in δ^{D910A} mice, but do not exclude an accessory role for $CD4^+$ T cells. In line with published data[5], naive WT mice had higher relative numbers of activated/memory $CD44^{high}CD4^+$ and $CD44^{high}CD8^+$ T cells than δ^{D910A} mice (Extended Data Fig. 1a). Upon 4T1 inoculation in WT mice, the relative numbers of these cells were either enhanced (tumour-draining lymph nodes) or reduced (blood and spleen), but in δ^{D910A} mice showed a trend towards expansion (Extended Data Fig. 1a), indicating that δ^{D910A} mice are capable of mounting both $CD4^+$ and $CD8^+$ T cell responses against 4T1 tumours. WT and δ^{D910A} splenocytes from tumour-bearing mice, incubated *in vitro* with mitomycin C-treated 4T1 cells, generated equivalent cytotoxic activity against 4T1, with no specific lysis of LLC (Fig. 3c). Compared to WT cultures, δ^{D910A} cultures contained similar proportions of CD4 and CD8 T cell subsets (Extended Data Fig. 1b), with a reduced frequency of activated/memory $CD44^{high}CD4^+$ cells (Fig. 3d) and unaffected frequency of $CD44^{high}$ $CD8^+$ cells (Fig. 3d). Interestingly, despite this reduced proportion of δ^{D910A} $CD44^{high}CD4^+$ cells, the frequency of $IFN\gamma^+$ $CD4^+$ cells in PMA/ionomycin-stimulated cultures of splenocytes from 4T1 tumour-bearing mice was unaffected by p110δ inactivation

IFNγ *in vitro* and *in vivo*[12,13]. Therefore, in the context of an otherwise normal immune system, δ^{D910A} $CD4^+$ cells show inferior anti-tumour immunity. However, the production of IFNγ by CD4 and CD8 T cells from 4T1 tumour-bearing δ^{D910A} mice, where Treg are also defective, appeared to be intact (Fig. 3e), suggesting that p110δ inhibition can affect the balance between regulatory and effector $CD4^+$ T cells such that the effector cells prevail in the context of anti-tumour responses.

A salient feature of CD4 and CD8 T cells is the ability to raise a more potent and rapid immune response to subsequent exposure to cognate antigen. Upon surgical removal of 4T1 primary tumours when they had reached 9 mm diameter and established metastatic foci[14], WT mice all succumbed to re-growth of the primary tumour and metastatic disease. In contrast, >50% of post-surgical δ^{D910A} mice showed survival extension beyond 100 days (Fig. 3i), demonstrating that p110δ inhibition can suppress cancer relapse and presumably metastatic cancer after surgery. δ^{D910A} mice which had remained tumour-free >200 days after surgery were cancer-resistant upon rechallenge with a higher 4T1 dose (Fig. 3j), suggesting that surgical intervention in δ^{D910A} mice supports the development of an effective memory anti-tumour response.

p110δ in these cells or an indirect consequence of a reduced tumour burden in δ^{D910A} mice (Fig. 4b). In support of the former, WT PMN-MDSCs suppressed T cell proliferation *in vitro*, whereas MDSCs from δ^{D910A} mice with regressing tumours did not (Fig. 4f; Extended Data Fig. 2b). Neutrophils from both genotypes did not suppress T cell responses (Fig. 4f). Moreover, splenocytes from tumour-bearing δ^{D910A} mice showed reduced *in vitro* production of TGFβ, VEGF and IL-6 (Fig. 4g), each of which can contribute to immune suppression and/or tumour growth[15,16].

Administration of PI-3065, a small molecule inhibitor with selectivity for p110δ (Extended Data Fig. 3a,b and Extended Data Table 1), also suppressed 4T1 tumour growth and metastasis, to a similar extent as genetic inactivation of p110δ, marked by initial tumour progression, followed by tumour regression (Fig. 5a; Extended Data Fig. 3c,d). Of interest, 4T1 do not express detectable levels of p110δ (Extended Data Fig. 3e) and are not growth-inhibited *in vitro* by PI-3065 (Extended Data Fig. 3f). Long-term administration of PI-3065 to mice was well-tolerated and did not induce weight loss (Extended Data Fig. 3g).

We next tested the impact of PI-3056 in the KPC model of pancreatic ductal adenocarcinoma (PDAC), which expresses endogenous mutant $Kras^{G12D}$ and $p53^{R172H}$ genes in $Pdx1^{+}$ pancreatic cells. KPC mice were left to develop palpable disease before treatment with vehicle or PI-3065 was commenced. Under these therapeutic conditions, PI-3065 prolonged survival and reduced the incidence of metastases and other disease-associated pathologies (Fig. 5b). The relative abundance of peripheral Treg in lymph nodes after 7 days of treatment was reduced (Fig. 5c), correlating with higher levels of $CD44^{high}CD8^{+}$ lymphocytes in the draining lymph nodes (Fig. 5d) and relatively more infiltrating $CD8^{+}$ T cells in pancreatic lesions 14 days after treatment (Fig. 5e). These data indicate that therapeutic targeting of p110δ can promote immune-mediated elimination of cancer.

Concerns have been raised about inhibiting p110δ in cancer as this might impair CTLs and negatively impact on cancer immune surveillance[4,18]. Our data show that although p110δ blockade reduces the effectiveness of CTLs, it also overrides Treg- and probably also MDSC-mediated suppression of anti-tumour immune responses, enabling even weakened CTLs to successfully attack tumours. Thus, p110δ is apparently more essential for regulatory rather than effector T cell responses against cancer cells. In addition, inhibition of the PI3K pathway in CD8 T cells may help maintain them in a stem-cell like state[19] with enhanced potential for generating durable anti-tumour responses. Consistent with this notion, δ^{D910A} mice resisted tumour rechallenge following surgical removal of the first tumour. The p110δ inhibitor Idelalisib has shown impressive therapeutic impact in chronic lymphocytic leukaemia (CLL) and non-Hodgkin's lymphoma[1,2]. In CLL, p110δ blockade interferes with stroma-derived survival and adhesion signals supporting the tumour cells[4], but it is unclear if this fully explains the effectiveness of p110δ inhibition. Our finding that p110δ inhibition can unlock adaptive anti-tumour responses provides a potential additional mechanism for the efficacy of p110δ blockade in CLL, and add to the emerging rationale for targeting PI3K in the tumour stroma[4], to dampen inflammation (p110γ)[20]

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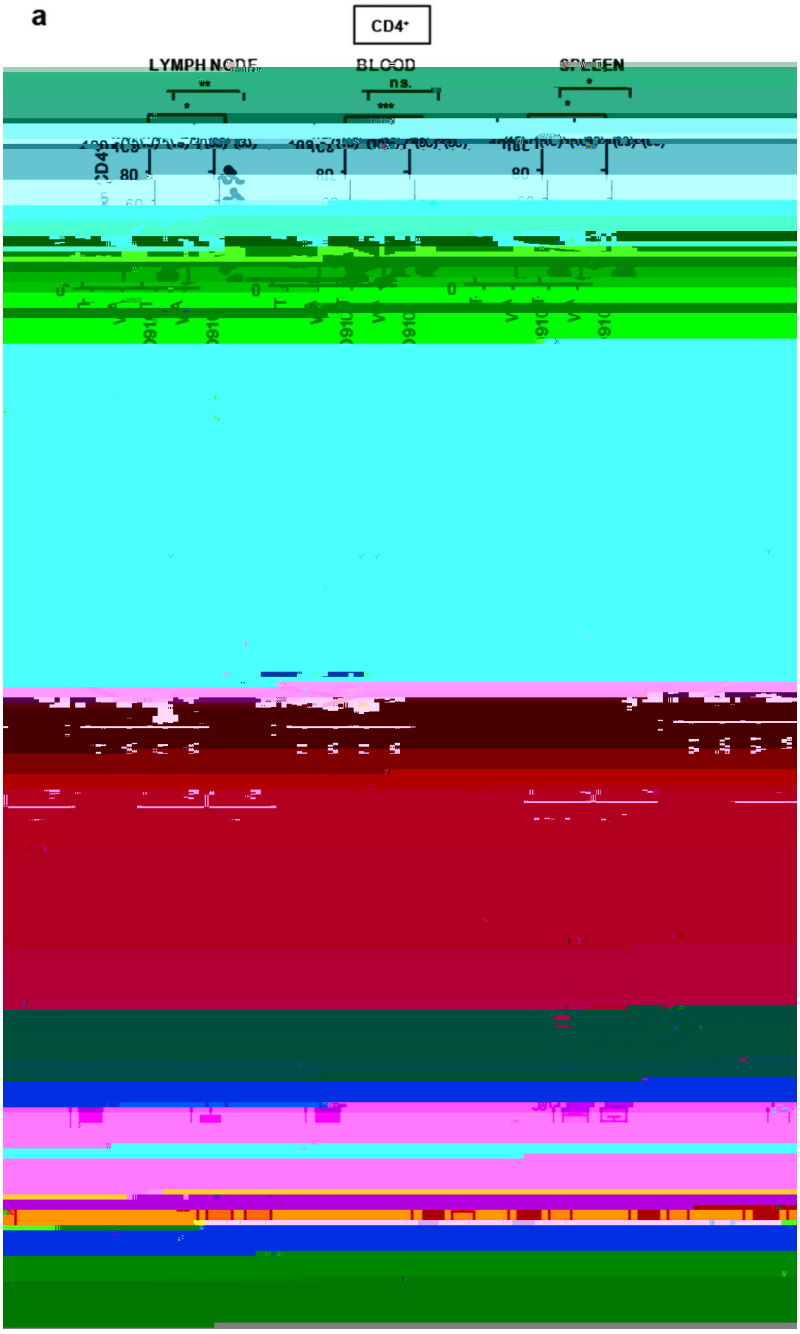
Tumour-induced immune suppression constitutes an important barrier for effective anti-tumour immunity and immunotherapy in cancer. Our work suggests that p110δ inhibitors, by disrupting the function of Treg and possibly of MDSCs, have the potential to shift the balance from immune tolerance towards effective anti-tumour immunity. This provides a rationale for p110δ inhibition both in solid and haematological cancer, possibly as an adjuvant to cancer vaccines, adoptive cell therapy, or other strategies that promote tumour-specific immune responses.

METHODS SUMMARY

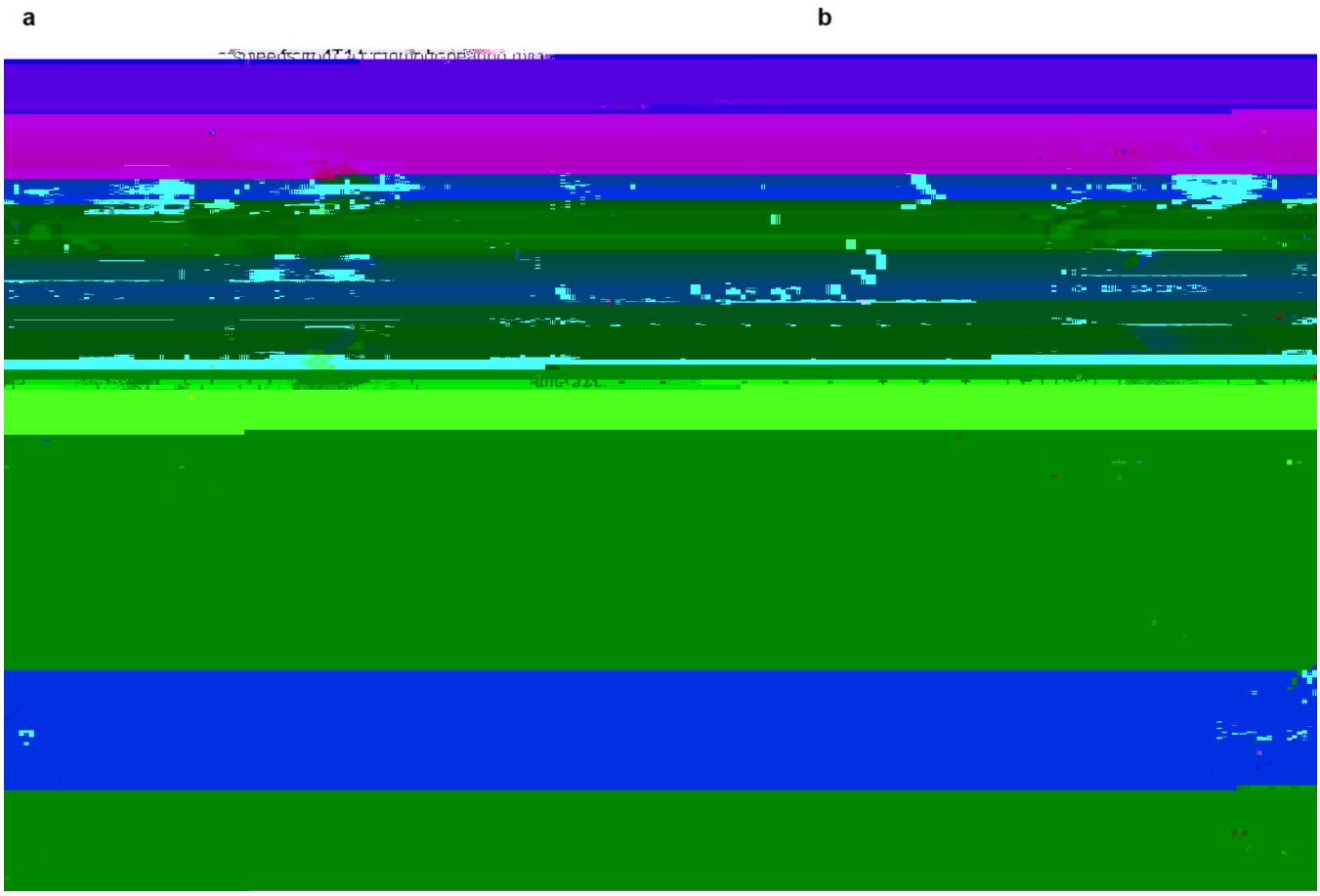
All animal procedures were in compliance with institutional animal care and use committee guidelines. Details of procedures and reagents are described in Methods.

Extended Data

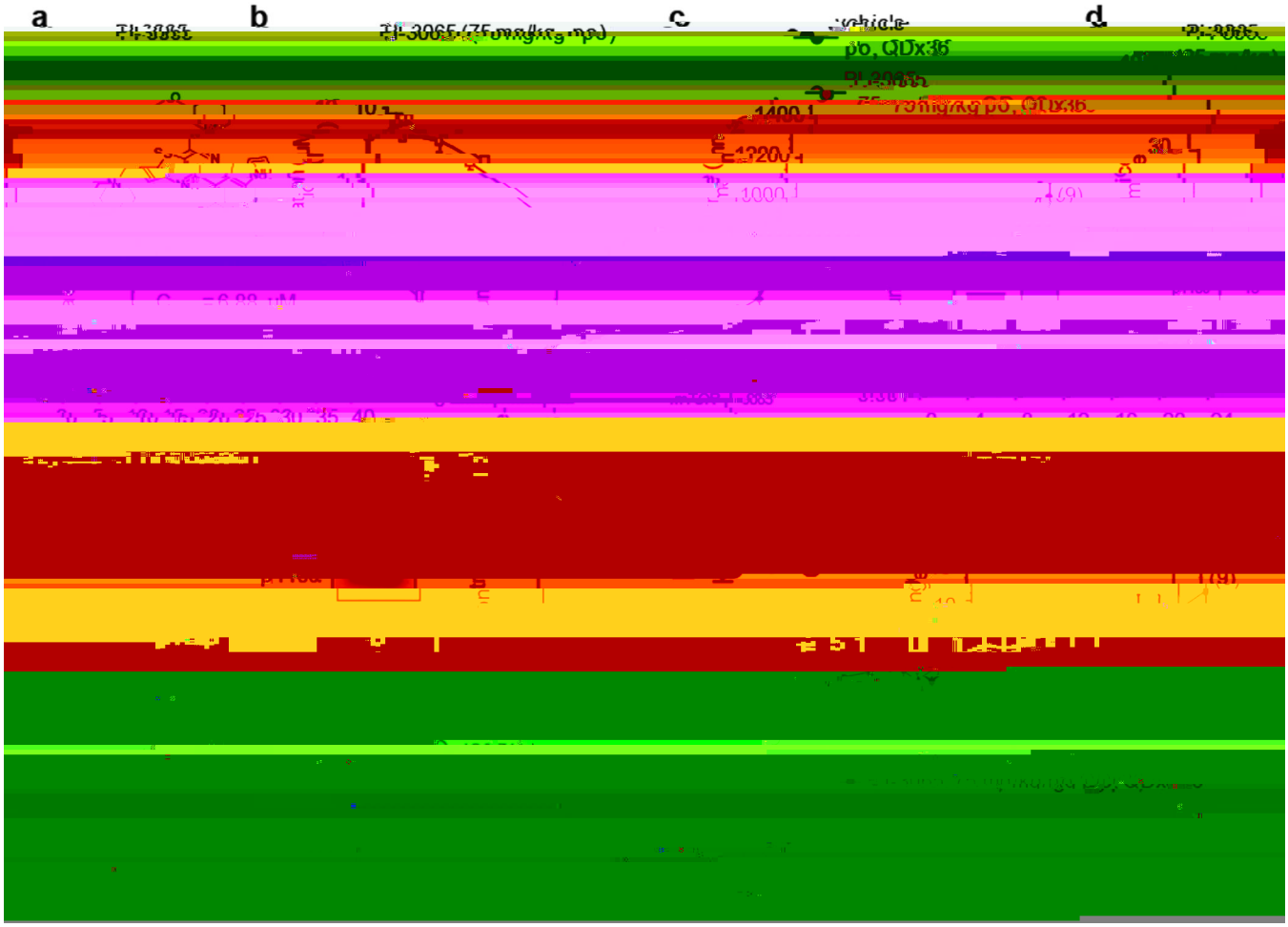


Extended Data Figure 1. Impact of p110δ inactivation on CD4 and CD8 T cells in mice with 4T1

mice, cultured in the presence of SIINFEKL OVA peptide and IL2. GzmA, granzyme A; GzmB, granzyme B, Prf1, perforin and (FasL or CD95L) Fas ligand. Expression levels are presented relative to β2-microglobulin. a-b, Statistically significant differences are indicated by * ($P < 0.05$) or ** ($P < 0.01$), as determined by the non-parametric Mann-Whitney *t* test. Between brackets: number of mice used per experiment. Each dot represents an individual mouse.



Extended Data Figure 2. Impact of p110δ inactivation on myeloid cells in 4T1 tumours
a, Gating strategy used to identify myeloid cell subsets. Splenic cells were gated on CD11b^{high}



Extended Data Figure 3. Characterisation of the p110

by * ($P < 0.05$) or ** ($P < 0.01$), as determined by the non-parametric Mann-Whitney *t* test. Between brackets: number of mice used per experiment.

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Extended Data Table 1

Comparison of PI-3065 with Idelalisib (formerly called GS-1101 or CAL-101) and IC87114

Human whole blood was stimulated with anti-IgM followed by FACS for CD69 as described[1,2]. Human B cell lymphoma Ri-1 cells were preincubated for 30 min with vehicle or compound prior to stimulation with anti-IgM for 1 h at 37°C, followed by determination of Akt-Ser473 phosphorylation, as described[1,2].

Compound	Ki(nM)		IC50 (nM)	
	p110δ	p110α	anti-IgM stimulated whole blood	anti-IgM-stimulated human R1 B cell lymphoma

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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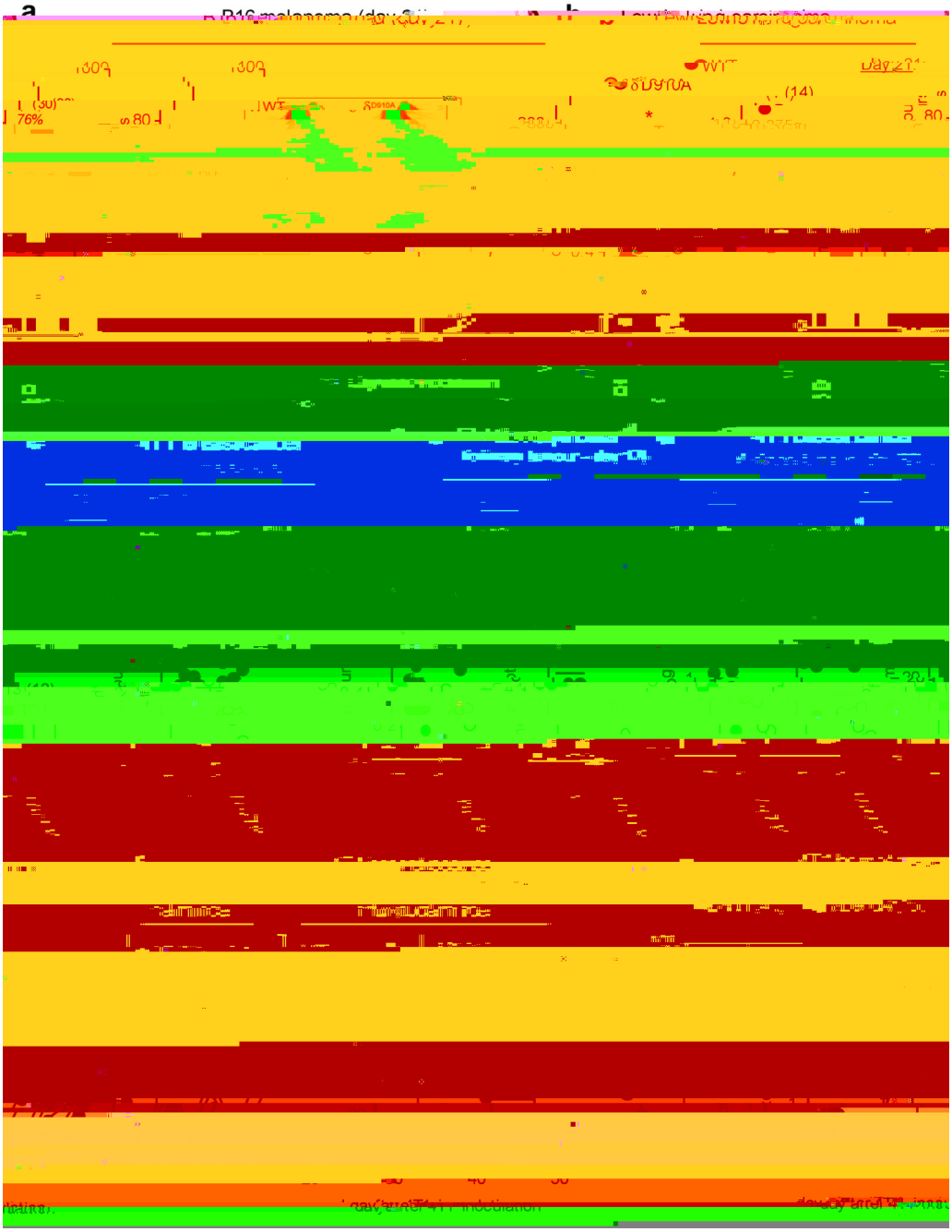

parametric Mann-Whitney *t* test. Between brackets: number of mice used per experiment. Each dot represents an individual mouse.

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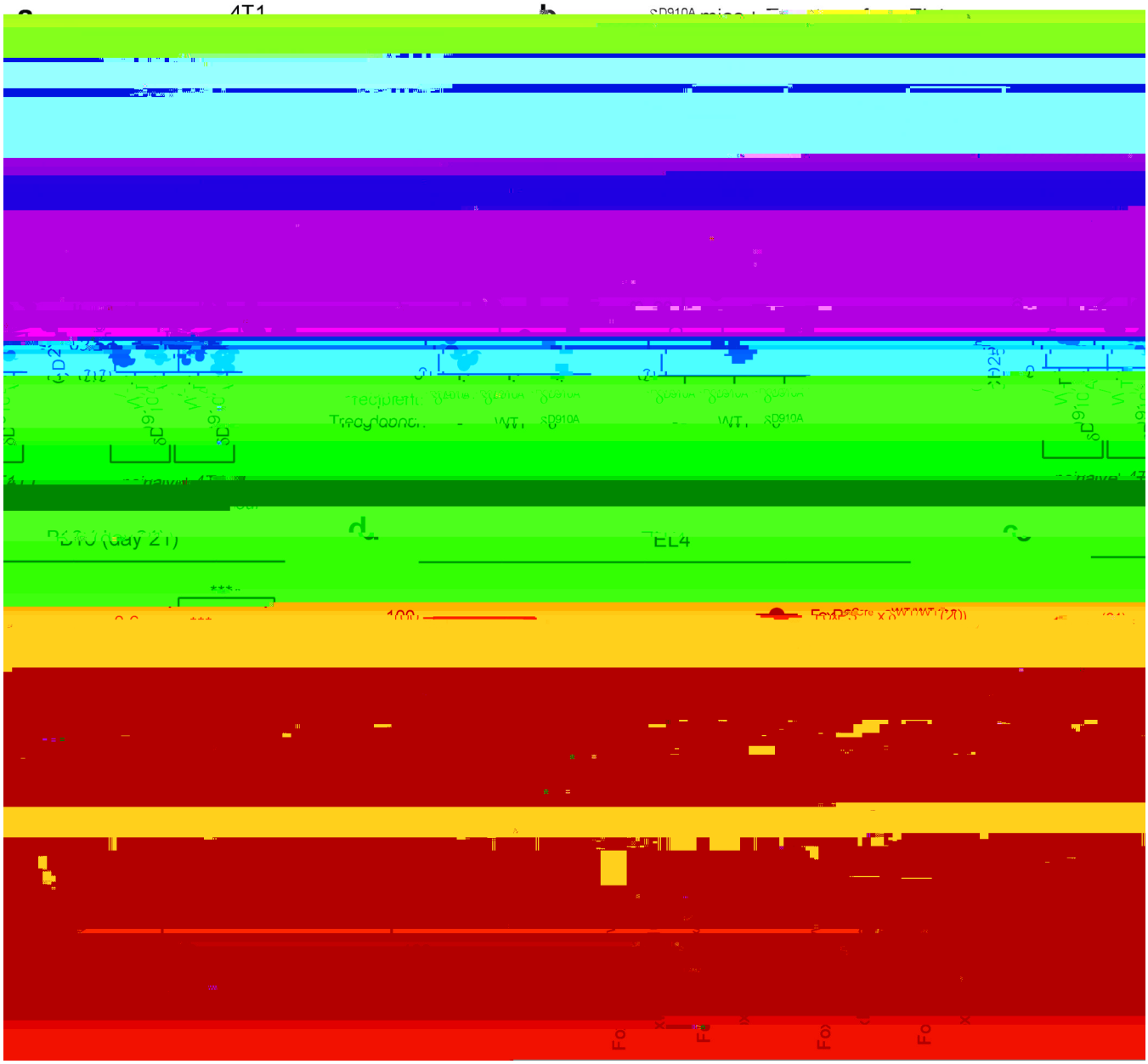

Figure 2. Inactivation of p110δ in Treg is sufficient to confer cancer resistance

a, Relative and total numbers of Treg in the draining lymph nodes of naive and 4T1 tumour-bearing mice. **b**, Impact of adoptive transfer of Treg into δ^{D910A} mice on EL4 tumour wet weight and tumour-infiltrating $CD8^+$ T cells. **c**, Number of mice with visible B16 tumours and B16 tumour weight in mice of the indicated genotype. **d**, Survival of EL4 tumour-bearing mice of the indicated genotype. a-c, Statistically significant differences are indicated by * (P<0.05) or ** (P<0.01), as determined by the non-parametric Mann-Whitney t test or Anova. Between brackets: number of mice used per experiment. Each dot represents an individual mouse.

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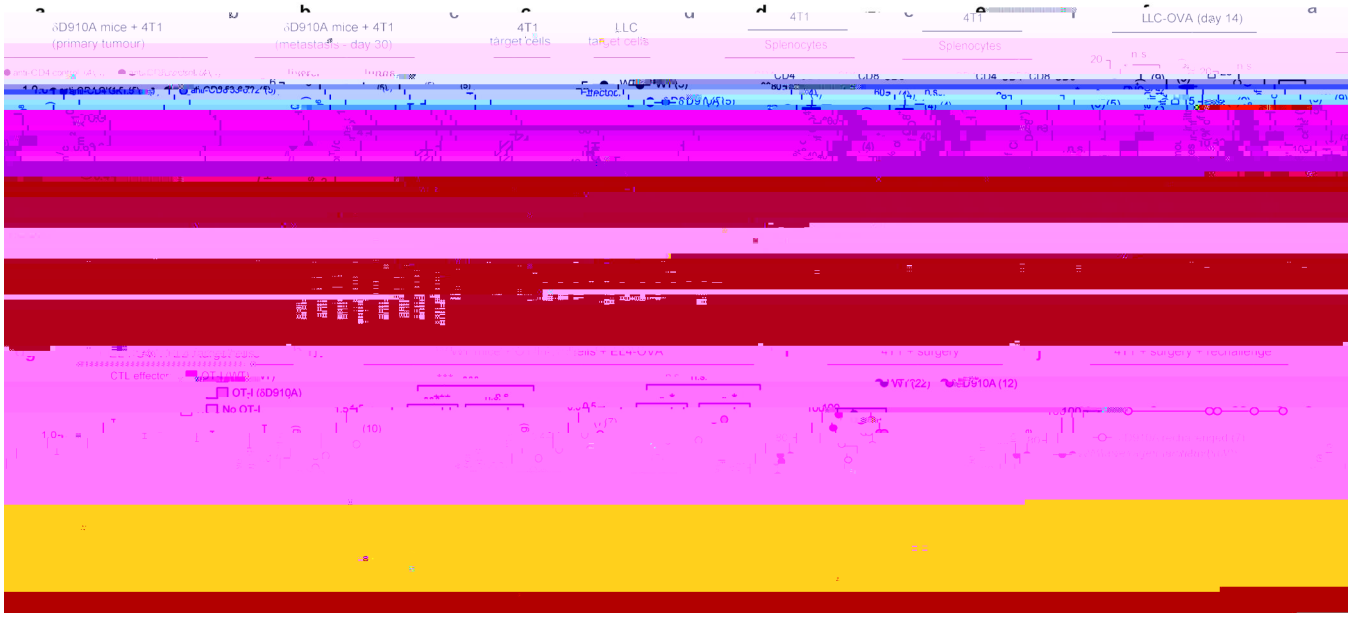

Figure 3. Impact of p110δ inactivation on T cell-mediated anti-tumour immunity

a, Growth of 4T1 in δ^{D910A} mice injected with antibodies to CD4 or CD8. Arrow indicates the time of antibody injection. **b**, Metastasis in CD4 or CD8 T cell-depleted 4T1 tumour-bearing δ^{D910A} mice. **c**, *In vitro* cytotoxic activity of splenocytes, isolated from 4T1 tumour-bearing WT and δ^{D910A} mice 21 days after inoculation and cultured for 4 days with mitomycin-treated 4T1 cells. E/T, effector to target (4T1 or LLC) ratio. **d**, Frequency of $CD44^{high}$ $CD4^+$ and $CD8^+$ T cells in splenocytes from 4T1 tumour-bearing mice cultured for 5 days with mitomycin-treated 4T1 cells. **e**, Frequency of $IFN\gamma^+$ T cells after 16h PMA +ionomycin stimulation of splenocytes from WT and δ^{D910A} 4T1 tumour-bearing mice. **f**, Relative levels of tumour-infiltrating $CD3^+$ lymphocytes and OVA-specific $CD8^+$ T cells in LLC-OVA tumours in WT or δ^{D910A} mice. **g**, *In vitro* cell killing of a 1:1 EL4-OVA:EL4 mix following 24h incubation with CTL from WT or a δ^{D910A} OT-I mice (at an E/T ratio of 10:1), incubated with or without the p110δ inhibitor IC87114 during the 8 day expansion phase, the 24h killing phase, or both. Cell killing efficiency is expressed as the ratio of EL4-OVA cells over EL4 cells remaining after incubation with effector cells. **h**, Effect of adoptive transfer in WT mice of OT-I $CD8^+$ or OT-II $CD4^+$ cells on growth of subsequently inoculated EL4-OVA. **i**, Survival of post-surgical 4T1 tumour-bearing mice. **j**, Survival of δ^{D910A} mice which had remained tumour-free >200 days after surgery, and of naïve WT mice, following injection of 10,000 4T1 cells. Statistics are as described in the legend to Figure 1.

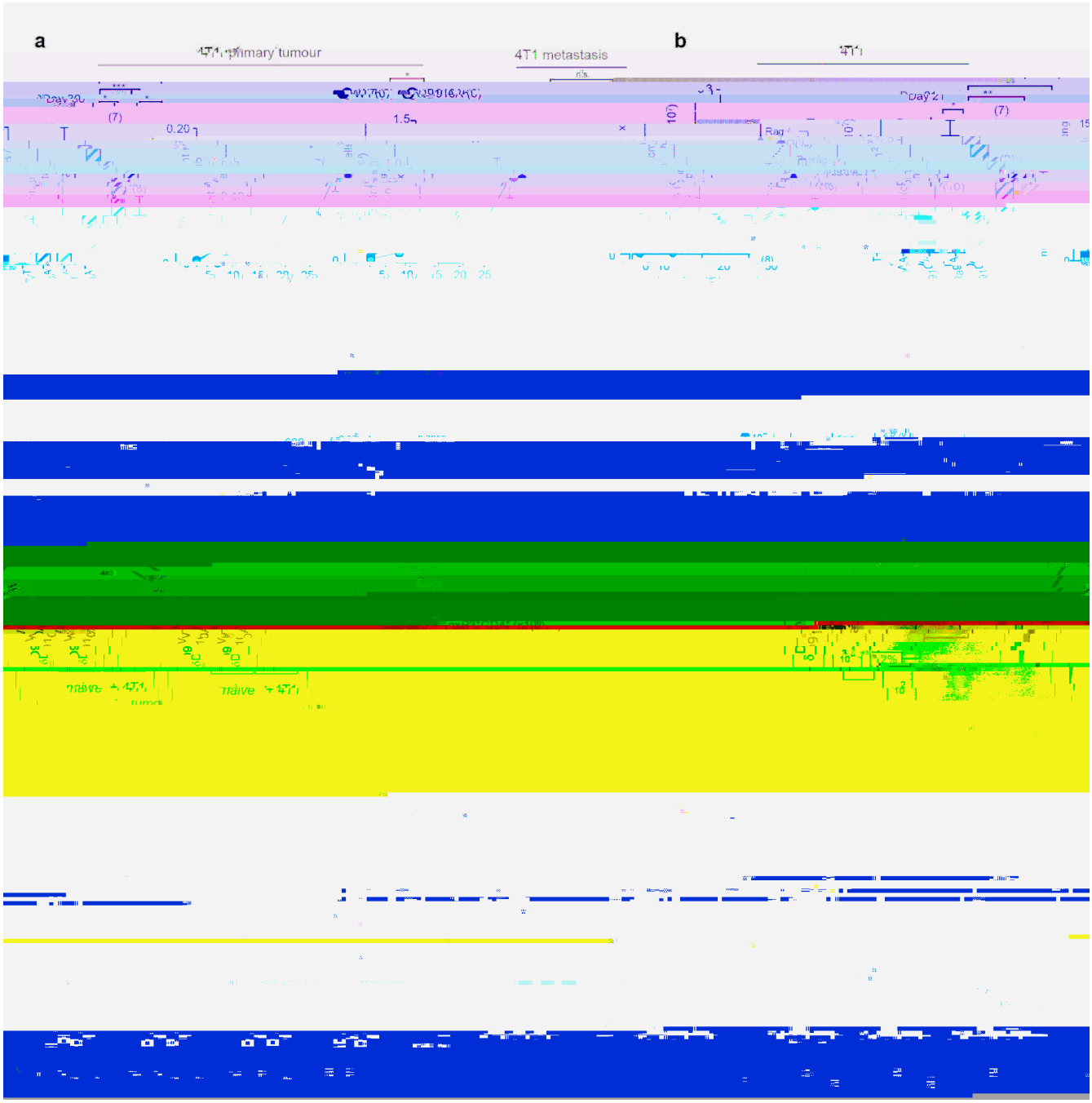

Figure 4. Impact of p110δ inactivation on myeloid cells in 4T1 tumour-bearing mice

a, 4T1 primary tumour growth and lung metastasis in WT, δ^{D910A}, $Rag^{-/-}$ and $Rag^{-/-}$ xδ^{D910A} mice. **b**, 4T1 tumour growth and total numbers of splenic $CD11b^{+}Gr1^{high}$ myeloid cells in WT and δ^{D910A} mice. **c**, Gating strategy used to identify myeloid cell subsets and frequency of splenic PMN-MDSCs and neutrophils of naïve and 4T1 tumour-bearing WT and δ^{D910A} mice. **d**, Spearman correlation between accumulation of splenic PMN-MDSCs and Treg in WT or δ^{D910A} mice. **e**, Impact of depleting $CD8^{+}$ T cells in δ^{D910A} mice on 4T1 tumour burden and presence of splenic myeloid cell populations. **f**, Impact of purified

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splenic myeloid cells on proliferation of anti-CD3-stimulated WT T cells. **g**, Cytokine production by splenocytes from 4T1 tumour-bearing (30 days after inoculation) from WT or δ^{D910A} mice, individually cultured for 4 days. Statistics are as described in the legend to Figure 1.

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Figure 5. Impact of pharmacological inactivation of p110δ on tumour growth and T cell responses

a, Mice, dosed with vehicle or PI-3065 (75 mg/kg, daily) for 36 days and inoculated with 10^5 4T1 cells 12h post first dosing, were assessed for tumour growth by luciferase imaging (*first panel*), tumour weight (*second panel)* or luciferase activity in tumours excised 35 days after inoculation *(third panel)*. Incidence of 4T1 metastasis (*fourth panel*), as detected by H&E staining and histology, expressed as percentage of the total number of tumour-bearing animals per group. **b**, Impact of PI-3065 (75 mg/kg) on KPC mouse survival *(left)* and

macrometastases (as detected by H&E staining) and cancer-associated pathology *(right)*. **c**, Proportion of Treg (% of $CD4^+$) in the draining lymph nodes of KPC mice administered vehicle or PI-3065. **d**, Proportion of $CD44^{high}$ T cells (% of $CD8^+$) in the draining lymph nodes of KPC mice administered vehicle or PI-3065. **e**, Relative numbers of $CD8^+$ T cells (% of $CD45^+$) in normal pancreas and PDAC lesions of KPC mice treated or not with PI-3065. Statistics are as described in the legend to Figure 1.