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Essential role for the p110δ phosphoinositide 3-kinase in the allergic response

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Inflammatory substances released by mast cells induce and maintain the allergic response^{1,2}. Mast cell differentiation and activation are regulated, respectively, by stem cell factor (SCF; also known as Kit ligand) and by allergen in complex with allergen-specific immunoglobulin E (IgE)^{2,3}. Activated SCF receptors and high-affinity receptors for IgE (FcεRI) engage phosphoinositide 3-kinases (PI(3)Ks) to generate intracellular

lipid second messenger signals^{2–5}. Here, we report that genetic or pharmacological inactivation of the p110δ isoform of PI(3)K in mast cells leads to defective SCF-mediated *in vitro* proliferation, adhesion and migration, and to impaired allergen–IgE-induced degranulation and cytokine release. Inactivation of p110δ protects mice against anaphylactic allergic responses. These results identify p110δ as a new target for therapeutic intervention in allergy and mast-cell-related pathologies.

PI(3)Ks activated by the FcεRI and SCF receptors include the heterodimeric class IA PI(3)Ks, which consist of a p110α, p110β or p110δ catalytic subunit bound to either one of five regulatory proteins (p85α, p55α, p50α, p85β or p55γ) that link the p110 subunits to tyrosine kinase signalling pathways⁶. Mast cells express high levels of the p110δ isoform⁷ which, unlike the ubiquitously expressed p110α and p110β, is primarily found in leukocytes^{8,9}. Whereas mice lacking p110α or p110β are embryonic lethal⁶, p110δ-null mice are viable, with specific defects in B and T cells¹⁰. To investigate the role of p110δ in mast cells, we derived bone marrow mast cells (BMMCs) from mice expressing either wild-type p110δ or p110δ^{D910A}, a loss-of-function allele of p110δ (ref. 11). Culture of bone marrow precursor cells from both genotypes in the presence of interleukin-3 (IL-3) gave rise to mature BMMCs, which expressed identical levels of FcεRI and Kit receptor (as assessed by fluorescence-activated cell sorting analysis; data not shown) and were morphologically indistinguishable (as assessed by alcian blue and safranin staining; data not shown). However, the number of BMMCs obtained from p110δ^{D910A/D910A} cultures was substantially lower (up to 60%; data not shown). Similar observations were made when precursor cells were cultured in the presence of IL-3 plus SCF, giving rise to up to 50% less BMMCs in p110δ mutant cells (data not shown).

The level of class I PI(3)K isoform expression in p110δ^{D910A/D910A} BMMCs is similar to that of wild-type cells (Fig. 1a). p110δ lipid

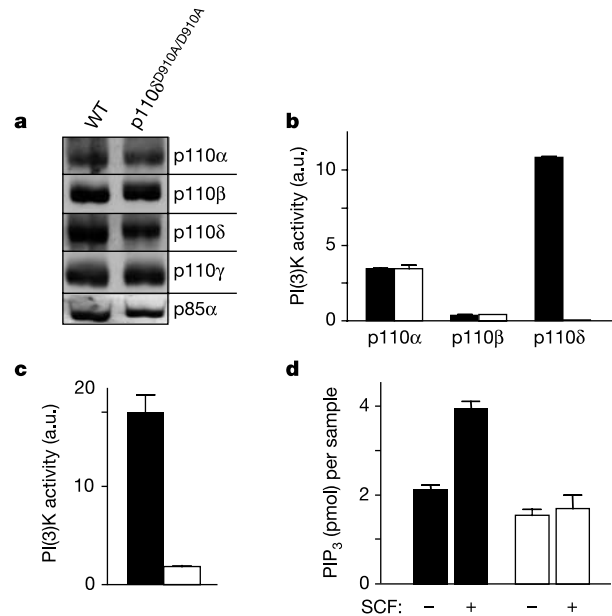


Figure 1 Effect of p110δ inactivation on PI(3)K expression and activity. **a**, Expression of class I PI(3)K isoforms in total cell lysates (50 μg per lane), assessed by immunoblotting. WT, wild type. **b**, *In vitro* lipid kinase activity of class IA PI(3)Ks. p110 isoforms were immunoprecipitated using isoform-specific antibodies, and tested for associated lipid kinase activity (presented as arbitrary units (a.u.)). **c**, *In vitro* lipid kinase activity of BMMC class IA PI(3)Ks, isolated using Y_pVPMLG (where Y_p is phosphotyrosine) peptide complexes. **d**, *In vivo* PIP₃ levels in BMMCs stimulated with or without 100 ng ml⁻¹ SCF for 10 min. Filled bars, wild type; open bars, p110δ^{D910A/D910A}.

kinase activity was abrogated in p110 $\delta^{D910A/D910A}$ BMMCs, with no alterations in the kinase activities of p110 α and p110 β (Fig. 1b). Total *in vitro* class IA PI(3)K lipid kinase activity, isolated from mutant BMMCs, was reduced by up to 90% (Fig. 1c), indicating that p110 δ contributes substantially to the overall class IA PI(3)K activity in mast cells. In agreement with this, there was no increase in *in vivo* phosphatidylinositol 3,4,5-trisphosphate (PIP₃) above resting levels in p110 $\delta^{D910A/D910A}$ cells upon stimulation with SCF (Fig. 1d).

To assess the impact of p110 δ inactivation on intracellular signalling, we monitored Ser 473 phosphorylation of protein kinase B (PKB, also known as Akt) and Tyr 204 phosphorylation of Erk as indirect measures of activation of these kinases. SCF-induced PKB phosphorylation was almost completely abrogated in p110 $\delta^{D910A/D910A}$ BMMCs, in contrast with Erk activation, which was not affected (Fig. 2a). These results could be mimicked in wild-type cells by the p110 δ -selective inhibitor IC87114 (ref. 12) (Fig. 2c, d). It has been reported¹² that IC87114 is selective for p110 δ in cells at doses up to 5–10 μ M; this is in line with our observation that such doses of this compound do not affect cells in which p110 δ is already genetically inactivated (data not shown and Figs 2f and 3c). IL-3-induced PKB phosphorylation was severely reduced but not completely abrogated in mutant mast cells (Fig. 2b). IL-3-stimulated PKB phosphorylation could be completely blocked by treatment with LY294002, a broad-spectrum PI(3)K inhibitor

(Fig. 2e and ref. 13), indicating that isoforms other than p110 δ contribute to PI(3)K signalling downstream of the IL-3 receptor. The residual IL-3-induced PI(3)K activity in p110 $\delta^{D910A/D910A}$ cells may explain why mature BMMCs can be derived from p110 $\delta^{D910A/D910A}$ bone marrow precursors in the presence of this cytokine. As was seen for SCF, IL-3-induced Erk activation was not affected by inactivation of p110 δ (Fig. 2b), and pharmacological inhibition of p110 δ using IC87114 on wild-type cells mirrored the impact of genetic inactivation of p110 δ on IL-3 signalling (Fig. 2e).

We next assessed *in vitro* functional responses of p110 $\delta^{D910A/D910A}$ mast cells to SCF or IL-3. DNA synthesis induced by each cytokine was severely reduced (Fig. 2f, left and middle panels). The synergistic effect on DNA synthesis of co-stimulation with IL-3 and SCF was also substantially diminished in p110 $\delta^{D910A/D910A}$ BMMCs (Fig. 2f, right panel). Treatment with IC87114 reduced DNA synthesis of wild-type BMMCs without significantly affecting p110 $\delta^{D910A/D910A}$ BMMCs (Fig. 2f). Other SCF-stimulated responses that were severely decreased in p110 $\delta^{D910A/D910A}$ mast cells include the ability to adhere to fibronectin upon SCF stimulation (Fig. 2g) and migration towards SCF (Fig. 2h). Taken together, these data indicate a critical contribution of p110 δ in signalling of SCF towards physiological functions of this cytokine such as proliferation, stimulation of adhesion to the extracellular matrix and chemotactic activity for homing of mast cells to tissues¹⁴.

We next compared the capacity of BMMCs from p110 $\delta^{D910A/D910A}$

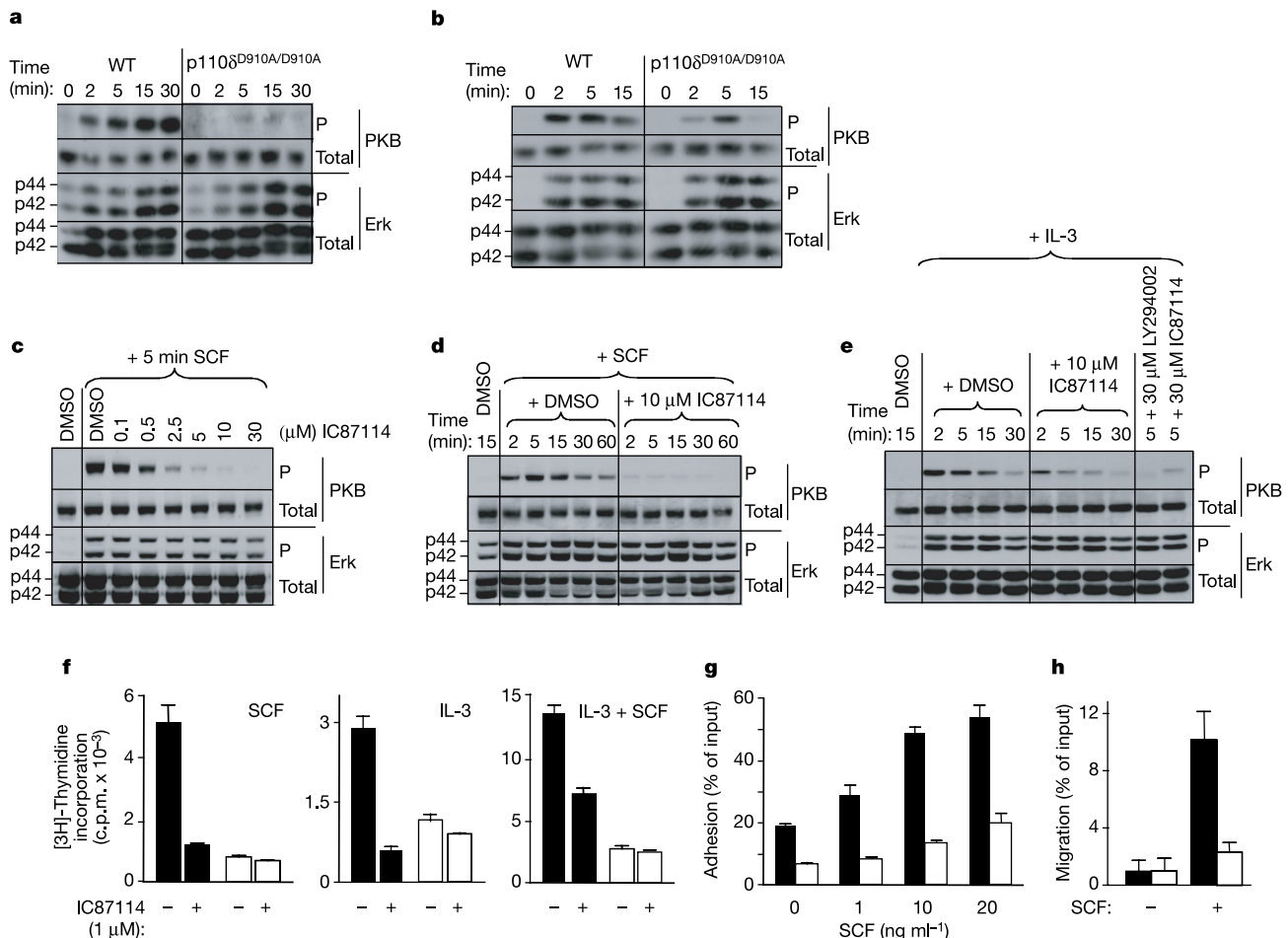


Figure 2 Effect of p110 δ inactivation on SCF- or IL-3-induced BMMC responses. **a**, Signalling in BMMCs stimulated with SCF. **b**, Signalling in BMMCs stimulated with IL-3. **c**, **d**, Effect of IC87114 on SCF signalling in wild-type BMMCs. **e**, Effect of IC87114 and LY294002 on IL-3 signalling in wild-type BMMCs. Inhibitors were added 20 min before

stimulation. **f**, Effect of p110 δ inactivation on SCF- and IL-3-induced DNA synthesis after 48 h. **g**, **h**, Effect of p110 δ inactivation on SCF-induced adhesion and migration. Filled bars, wild type; open bars, p110 $\delta^{D910A/D910A}$. Data in **f–h** are expressed as mean \pm s.d.

and wild-type mice to respond to antigen in complex with IgE. Such complexes aggregate the FcεRI on mast cells, initiating biochemical signalling cascades that ultimately lead to the release of inflammatory mediators, cytokines and to much of the pathology associated with the allergic response^{1,2,4}. Ser 473 phosphorylation of PKB induced by FcεRI activation was substantially reduced in p110δ^{D910A/D910A} BMMCs (Fig. 3a). We also tested the capacity of cultured mast cells to release contents from intracellular stores into the surrounding environment upon stimulation with antigen/allergen via the FcεRI receptor. As a read-out, we measured release of β-hexosaminidase, an enzyme stored in mast cell granules¹⁵. Inclusion of 30 μM of LY294002, a broad-spectrum PI(3)K inhibitor, reduced the degranulation of wild-type mast cells by ~80% (Fig. 3b, left panel), confirming that PI(3)K is a significant component of the signalling cascade leading to mast cell degranulation^{15,16}. p110δ^{D910A/D910A} BMMCs showed a 45–55% reduction in FcεRI-induced degranulation, relative to wild-type cells (Fig. 3b, left panel). Similar results were obtained when the cells were incubated with IgE overnight, washed and then stimulated with antigen (Ag), with degranulation defects observed across a wide dose range of antigen (Fig. 3b, right panel). Degranulation in p110δ^{D910A/D910A} BMMCs could be further reduced by LY294002, as in wild-type cells (left panel of Fig. 3b). Pre-incubation of wild-type mast cells with 5 μM IC87114 reduced degranulation levels to those seen in p110δ^{D910A/D910A} cells while having no effect on p110δ mutant cells (Fig. 3c). IC87114 inhibited degranulation to the same extent in human mast cells (Fig. 3d) and also blocked the capacity of SCF to potentiate degranulation by low doses of antigen (Fig. 3e). The observation that p110δ is critical for the SCF component of mast cell degranulation is of particular physiological relevance, given that mast cells are continuously exposed to SCF in the local tissue environment. Mast cell activation thus occurs in the context of SCF signalling, especially during inflammatory response with excessive local production of SCF^{17,18}. p110δ^{D910A/D910A} BMMCs also showed defects in secretion of inflammatory cytokines such as

TNF-α and IL-6 (Fig. 3f). Overall, these data indicate that p110δ is the main component of the PI(3)K-dependent antigen–IgE signalling cascade leading to degranulation, and is essential for the SCF-mediated potentiation of FcεRI activity.

In p110δ^{D910A/D910A} mice mast cell numbers were differentially affected in distinct anatomical locations (Supplementary Table 1), with defects ranging from severe (peritoneum, jejunum, ileum and colon) to moderate (ear dermis (Fig. 4a), stomach submucosa and muscularis) to no significant differences (back dermis (Fig. 4a), mucosa of the stomach). A decrease in mast cell numbers is in line with a critical role of SCF in mast cell differentiation and survival, and the importance of p110δ in signalling by this cytokine. The residual mast cells present in p110δ^{D910A/D910A} mice may result from SCF signalling pathways unaffected by p110δ inactivation (such as the Erk pathway) and/or from developmental/homeostatic cues other than SCF.

We next investigated the impact of p110δ inactivation on allergic responses in the mouse. *In vivo* anaphylaxis through the FcεRI receptor is an allergic response that is predominantly mast-cell-dependent and occurs as a result of either local or systemic exposure to allergens, which crosslink and activate antigen-specific IgE bound to the FcεRI on the mast cell surface. We assessed the capacity of wild-type and p110δ^{D910A/D910A} mice to respond to immune challenge in a passive cutaneous anaphylaxis (PCA) model¹⁹. Mice were given an intradermal injection of IgE directed against a hapten (dinitrophenyl (DNP) in this case). Between 24–48 h after this priming event the mice were challenged by systemic administration of DNP coupled to a carrier protein (HSA) together with Evan's blue dye, which binds to serum proteins. Mediators released upon mast cell activation increase vascular permeability, which allows the dye to leak from the blood vessels into the surrounding tissue, causing oedema and providing a measure of mast cell activation. We assessed the PCA response in two distinct tissue locations. In the ear of p110δ^{D910A/D910A} mice, a marked (80%) reduction in PCA was observed (Fig. 4b). This decrease is more significant than the

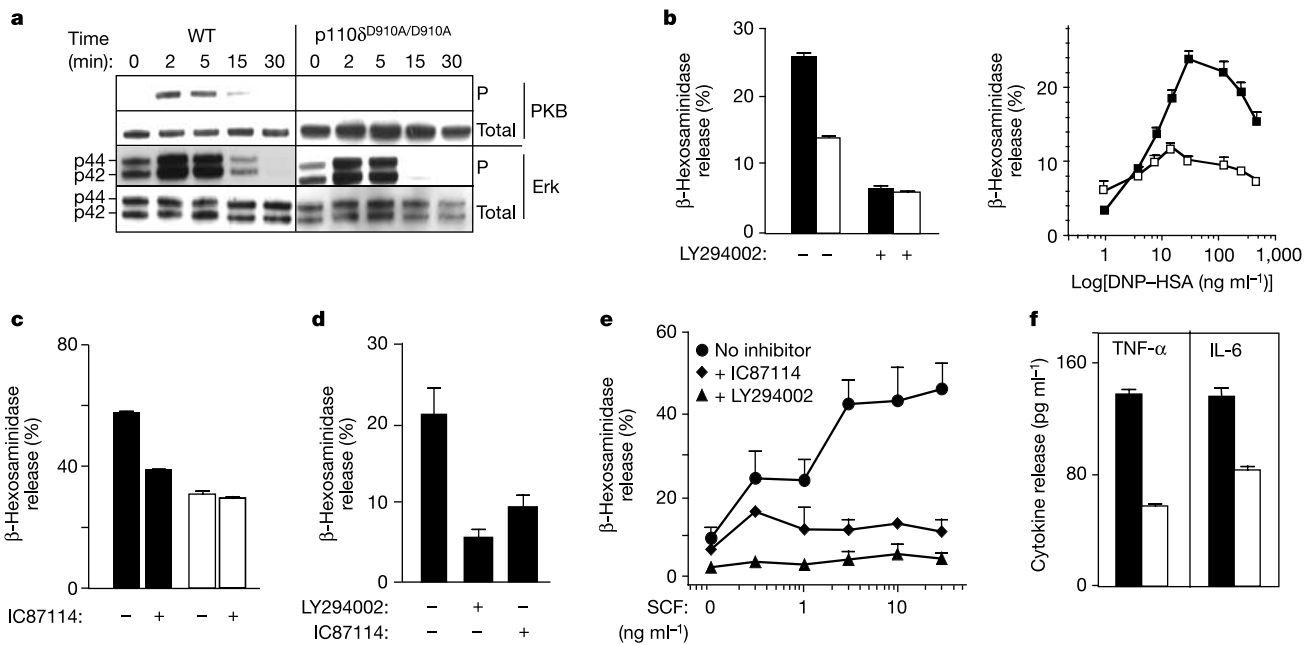


Figure 3 Effect of p110δ inactivation on FcεRI-induced BMMC responses. **a–c**, Signalling (**a**) and β-hexosaminidase release (**b**, **c**) in BMMCs stimulated with IgE–antigen. Right panel of **b**: dose titration of DNP–HSA. **d**, Effect of inhibitors on degranulation of human mast cells challenged with antigen (1 ng ml⁻¹). **e**, Effect of PI(3)K inhibitors on SCF-potentiated human mast cell degranulation (using a suboptimal antigen

dose of 0.1 ng ml⁻¹). **f**, Cytokine release from BMMCs stimulated with IgE–antigen. LY294002 and IC87114 were used at 30 and 5 μM, respectively, and added 20 min before IgE–antigen stimulation. Filled bars, wild type; open bars, p110δ^{D910A/D910A}. Data in **b–f** are expressed as mean ± s.d.

reduction in mast cell numbers at this location (maximally 47%; Supplementary Table 1), suggesting that the remaining mutant mast cells may be defective. PCA in the back dermis—a site with unaltered mast cell numbers in p110 $\delta^{D910A/D910A}$ mice (Supplementary Table 1; Fig. 4a)—was also significantly reduced (40%; Fig. 4c). IC87114, at pharmacological doses at which it is selective for p110 δ (plasma concentration of 5 μ M from a 15 mg kg $^{-1}$ dose), reduced the allergic response by 35–40% in the back skin and ear of wild-type mice (Fig. 4d; data for the ear are not shown). At higher plasma concentrations (40 μ M from a 60 mg kg $^{-1}$ dose) IC87114 completely blocked the anaphylaxis reaction in the back dermis (Fig. 4d).

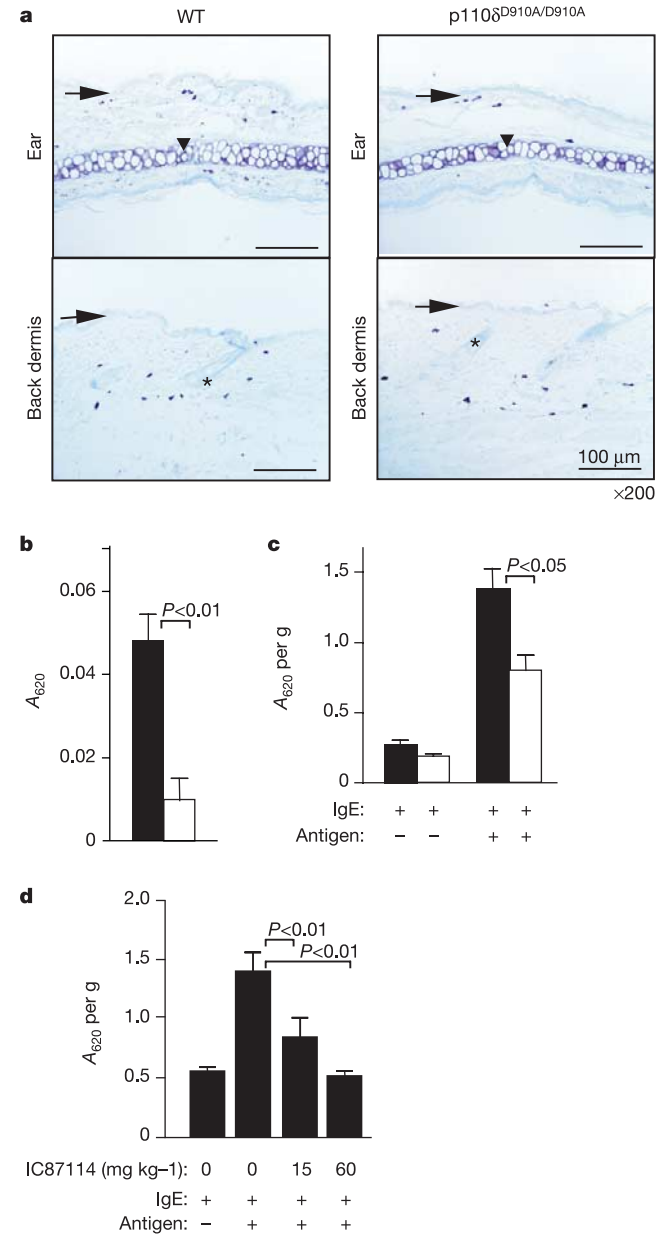


Figure 4 Effect of p110 δ inactivation *in vivo*. **a**, Reduced numbers of mast cells (stained with toluidine blue) in the ear dermis but not in the back skin. Arrows, epidermis; arrowheads, cartilage; asterisk, hair follicles with sebaceous glands. **b**, PCA response (ear) of wild-type ($n = 13$) and p110 $\delta^{D910A/D910A}$ mice ($n = 10$). **c**, PCA response (back skin) of mice injected with anti-DNP IgE, challenged with saline ($n = 4$ for wild type; $n = 5$ for p110 $\delta^{D910A/D910A}$) or DNP-HSA ($n = 5$ for wild type; $n = 6$ for p110 $\delta^{D910A/D910A}$). **d**, IC87114 attenuates the PCA response (back skin) of wild-type mice ($n = 8$ for each treatment group). Filled bars, wild type; open bars, p110 $\delta^{D910A/D910A}$.

This may be a consequence of the inhibition of other PI(3)K isoforms (such as p110 γ), which could account for the remaining PI(3)K-dependent (that is, LY294002-sensitive) degranulation seen in p110 $\delta^{D910A/D910A}$ BMMCs (Fig. 3b, left panel). These data show that this class of small molecule inhibitors of PI(3)K can completely abrogate *in vivo* cutaneous anaphylaxis.

Taken together, our data show that the p110 δ PI(3)K isoform has an important role in mast cell homeostasis and in the allergic response. Our observations are in contrast with studies in mice lacking regulatory subunits of class IA PI(3)Ks, which indicated that class IA PI(3)K enzymes are not involved in IL-3 and Fc ϵ RI-mediated allergic responses^{7,15,16,20}. The reason for this discrepancy is not clear, but may relate to the complex impact of loss of expression of class IA PI(3)K regulatory subunits on the class IA PI(3)K system, with increased expression of the remaining regulatory subunits and reduced expression of all p110 isoforms, and even increased PI(3)K signalling under certain circumstances^{21–25}. This contrasts with the present study, in which p110 δ was selectively inactivated by introduction of a point mutation in the ATP-binding site, mimicking the effect of a systemically administered ATP-competitive p110 δ inhibitor. Indeed, *in vivo* administration of a p110 δ -selective inhibitor reproduced the therapeutic effect of the D910A mutation in the context of anaphylaxis. Interference with PI(3)K signalling pathways is being explored as a means of therapeutic intervention in many different pathological conditions²⁶. Our work suggests a promising therapeutic potential for p110 δ -selective compounds to treat allergy and other pathological conditions in which mast cells have a central role. □

Methods

Mice

p110 $\delta^{D910A/D910A}$ mice have been described previously¹¹ and were backcrossed on a C57BL/6 or BALB/c background for 6–10 generations. Age-matched, 6–10-week-old littermate mice were used for all experiments.

Mast cell cultures

Total bone marrow samples from the femurs of 3–5 mice were cultured in RPMI medium supplemented with 10% ultra-low IgG FBS (Gibco), 10 ng ml $^{-1}$ IL-3 (Tebu-bio), 20 ng ml $^{-1}$ SCF (Tebu-bio), 2 mM L-glutamine, 100 U ml $^{-1}$ penicillin and streptomycin (Gibco) in a 5% CO $_2$ atmosphere. After a 4-week maturation period, BMMCs were collected and tested for c-kit receptor and Fc ϵ RI expression by flow cytometry using phycoerythrin-labelled rat anti-c-kit (Pharmingen) or anti-DNP IgE (SPE-7; Sigma) and monoclonal fluorescein isothiocyanate-anti-mouse IgE (Molecular Probes) antibodies, respectively. BMMCs used for this study were cultured with IL-3 and SCF, unless otherwise indicated, with culture times not exceeding 6 weeks.

Cytokine stimulation, lysis and immunoblotting

Cells were starved for 24 h in normal culture medium lacking IL-3 and SCF. After stimulation with 20 ng ml $^{-1}$ IL-3 and/or 20 ng ml $^{-1}$ SCF, the cells were washed twice with ice-cold PBS, lysed and processed for immunoblotting and kinase assays as described¹¹.

Lipid kinase assay

In vitro lipid kinase assays were carried out as previously described¹¹ (see Supplementary Information for further details). *In vivo* levels of PIP $_3$ were determined by a time-resolved fluorescence resonance energy transfer ligand displacement assay, as described²⁷.

DNA synthesis

After a 24-h starvation period in culture medium without IL-3 and SCF, 10 5 cells in triplicate were incubated with 20 ng ml $^{-1}$ IL-3 and/or 20 ng ml $^{-1}$ SCF, together with [3 H]-thymidine and collected 24–72 h later.

Degranulation and cytokine secretion

Degranulation experiments were carried out in triplicate in 96-well plates. Exponentially growing cells were stimulated with IgE-antigen complex for 60 min. Degranulation is expressed as a percentage of total β -hexosaminidase activity in the input cells (see Supplementary Information for detailed methods). Cytokine released into the supernatants was assessed using Quantikine kits (R&D Systems).

Human mast cell studies

Human mast cells were obtained by culture of peripheral blood CD34 $^+$ cells in Stem Pro 34 media (Invitrogen) containing recombinant human IL-3 (30 ng ml $^{-1}$), IL-6 (100 ng ml $^{-1}$) and SCF (100 ng ml $^{-1}$) (Pepro Tech) for 1 week, followed by culture without IL-3 (but with IL-6 and SCF as above) for a further 6–7 weeks as described²⁸ (see Supplementary Information for detailed methods).

Cell adhesion and migration

For adhesion assays, cells starved of cytokines for 24 h were washed and re-suspended in culture medium without cytokines. Cells were plated at 5×10^4 per well in a 50- μ l volume in 96-well plates pre-coated overnight with $5 \mu\text{g ml}^{-1}$ human plasma fibronectin (Gibco). After 30 min of stimulation with or without SCF (in 100 μ l final volume), non-adherent cells were removed by inverting plates for 15 min. The adherent cells were lysed using HTAB and β -hexosaminidase activity measured to assess the fraction of adherent cells. Adhesion is expressed as percentage of input. For migration assays, exponentially growing cells were washed and re-suspended in Tyrodes buffer. 10^5 cells were seeded in 100 μ l in the upper chamber of 8- μ m-pore-diameter transwells (BD Biosciences) with or without 100 ng ml^{-1} SCF in Tyrodes buffer in the lower chamber. After a 3–4-h incubation at 37°C and 5% CO_2 , the cells in the lower chamber were lysed using HTAB and β -hexosaminidase activity measured to assess the fraction of migrated cells.

Passive cutaneous anaphylaxis

The PCA protocols were adapted from refs 29, 30 (see Supplementary Information for detailed protocol).

Statistical analysis

All *in vitro* data shown are representative experiments (mean \pm s.d.) from different BMMC cultures (established from at least 3–5 littermate mice each). Data for *in vitro* experiments were statistically analysed using a *t*-test and differences between wild-type and p110^{D910A/D910A} BMBCs were statistically significant ($P < 0.05$) unless otherwise stated. Results from *in vivo* experiments (mean \pm s.e.m.) were assessed using a Mann–Whitney *U*-test with results of analysis and animal numbers presented in the relevant figure legends. GraphPad Prism software was used for all statistical analyses.

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DNA end resection, homologous recombination and DNA damage checkpoint activation require CDK1

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A single double-strand break (DSB) induced by HO endonuclease triggers both repair by homologous recombination and activation of the Mec1-dependent DNA damage checkpoint in budding yeast^{1–6}. Here we report that DNA damage checkpoint activation by a DSB requires the cyclin-dependent kinase CDK1 (Cdc28) in budding yeast. CDK1 is also required for DSB-induced homologous recombination at any cell cycle stage. Inhibition of homologous recombination by using an analogue-sensitive CDK1 protein^{7,8} results in a compensatory increase in non-homologous end joining. CDK1 is required for efficient 5' to 3' resection of DSB ends and for the recruitment of both the single-stranded DNA-binding complex, RPA, and the Rad51 recombination protein. In contrast, Mre11 protein, part of the MRX complex, accumulates at unresected DSB ends. CDK1 is not required when the DNA damage checkpoint is initiated by lesions that are processed by nucleotide excision repair. Maintenance of the DSB-induced checkpoint requires continuing CDK1 activity that ensures continuing end resection. CDK1 is also important for a later step in homologous recombination, after strand invasion and before the initiation of new DNA synthesis.

In budding yeast, a chromosomal DSB created by HO endonuclease has been used both to study the kinetics and efficiency of DSB