Targeting SHIP1 and PI3Kγ for a synergistic inhibition of mast cell activation

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Introduction

Type I hypersensitivity reactions are immune system responses of the body triggered by the IgE/antigen-mediated activation of tissue localized mast cells but also by circulating basophils or eosinophils. Within minutes from their stimulation, mast cells release a plethora of preformed molecules whose activity induces vasodilatation and broncho-constriction and increases the vascular permeability, enhancing the recruitment of leukocytes. Such a hyperactivation of the immune system in response to a foreign molecule is commonly defined as allergic reaction. The stimulation of the high affinity IgE receptors, FcεRI, expressed on the surface of mast cells is induced by the clustering of several IgE/FcεRI complexes and promotes the formation of an intracellular signalosome that generates a cascate of signalling events. Class I phosphatydilinositol-3-kinases (PI3Ks) are activated downstream FcεRI clustering and are responsible for the generation of PtdIns(3,4,5)P_3 at the plasma membrane. On the other hand, the 5'-phosphatase SHIP1 is recruited via its SH2 domain at the plasma membrane where binds tyrosine-phosphorylated domains of several receptors and hydrolyses the PtdIns(3,4,5)P_3 at the 5' position of the inositol ring, generating PtdIns(3,4)P_2. Being recognised by PH-domain bearing proteins, both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 participates to the recruitment of a wide variety of effectors. PI3Kγ, the sole member of class IB PI3Ks, has been showed to have a pivotal role in mast cells recruitment to the tissues and degranulation as well as in systemic anaphylactic reactions; therefore PI3Kγ has been proposed as a pharmacological target for the treatment of inflammatory diseases. Interestingly, the current treatment of the allergic responses is based on the pharmacological amelioration of the symptoms and does not target the aetiology of the disease.

In the present work we demonstrate the possibility of inhibiting PI3Kγ signalling in mast cells in order to modulate hypersensitivity responses without affecting the phisiological functionality of class I PI3Ks in the other tissues.

In the first project described in the present manuscript, we show that during mast cells activation, the GPCR-mediated activation of PI3Kγ induces a signalling cascade that is not inhibited by the phosphatase activity of SHIP1 and therefore

contribute to the reinforcement of the antigen-induced mast cells activation. Moreover we demonstrate that PI3Kγ and SHIP1 are two valid targets for a combined pharmacological inhibition of mast cells activation. In the second project described in the manuscript, we demonstrate the possibility to selectively modulate PI3Kγ activation in mast cells by blocking the plasma membrane localization of the monomeric GTPase Ras using farnesyltransferase inhibitor. We demonstrate that Ras is required for the activation of PI3Kγ in cells that express p84 as adaptor subunit (p110γ/p84 heterodimer), such as mast cells but not in cells where the PI3Kγ active complex is p110γ/p101. In the final part of the manuscript we described the generation of three genetically modified mouse strains we developed: p101 knock-out, p84 knock-out and p84 knock-in. The two p84-mutant mice were designed as novel tools for the analysis of the physiological p110γ/p84 signalling *in vivo* and *ex vivo*, while the generation of the p101 mutant has been already characterised by other groups but will be further used in our research.

Allergy and allergic responses

In allergic individuals, also called atopic, the contact of the allergen with the body induces an "immediate" hypersensitivity reaction (type I reaction) that occurs within minutes from the exposure to the allergen. Indeed, IgE molecules that are already bound on the surface of mast cells or basophils due to the presence of high affinity receptors for IgE, are cross-linked by a multivalent antigen, resulting in the release of several preformed molecules and the generation of newly synthesized ones (Table 1). The release of those signalling molecules causes vasodilation, increased vascular permeability associated with oedema and acute functional changes in affected organs (such as bronchoconstriction, airway mucus secretion, urticaria, vomiting and diarrhoea)(1, 2). Allergy is currently a public concern of pandemic proportions, affecting more than 150 million people in Europe and with the increasing potential risk that within 15 years, more then half of the European population will be affected by some kind of allergic disease (data from: European Academy of Allergy and Clinical Immunology, EAACI).

Introduction to mast cell's biology

Mast cells are immune cells of hematopoietic origin, first described in 1863 by Dr. Von Recklinghausen that identified granular cells in the mesentery of the frog(3). In 1878 Paul Ehrich described a connective tissue cell population with a peculiar chemical staining phenotype that revealed the presence of big granules. Due to their peculiar phenotype, those cells appeared "well fed" (the German word is "mästung") and Ehrich named them "Mastzellen"(4). Mast cells are generally localized at the interface between the organism and the external milieu and their tissue localization makes them able to serve as immune sentinel cells but also to directly respond to external insults; at the same time mast cells modulate both innate and adaptive immune responses(5, 6) and play a protective role against pathogens(7). Mast cells are also involved in the generation of the allergic responses induced by antigen (IgE or IgG dependent) or peptide stimulation(8). The main feature that became evident as soon as mast cells were first described is the heterogeneity of such cell population. In rodents, mast cells can be divided in

two main sub-populations: connective tissue mast cells (CTMC) and mucosal mast cells (MMC) (Table 2). CTMC is the population described by P. Erhich and is localized in close proximity with venules and nerve endings and is characterised by the presence of a large reserve of heparin and histamine within the granules. MMC were identified later as a cell population localized in the gastrointestinal tract and characterised by abundant chondroitin sulphate and little histamine in their granules(9). Two main population of mast cells have been identified in human, mainly based on the protease content: tryptase containing mast cells, MCT, localized mainly in the lung and in the small intestine mucosa and mast cells containing tryptases and chymases, MCTC, localised in the skin and in the small intestine submucosa(10, 11). Interestingly both rodent MMC and human MCT show a T cell-dependent development while murine CTMC and human MCTC do not(9).

Table 1: Main mast cell mediators and their physiological function

Mediators	Function
Granule-associated	
Histamine and serotonin	Alter vascular permeability (12)
Heparin and/or chondroitin sulphate peptidoglycans	Enhance chemokine and/or cytokine functions and angiogenesis (12)
Tryptase, chymase, carboxypeptidase and other proteases	Tissue remodeling and recruitment of effector cells(13, 14)
TNF-α, VEGF and FGF2	Recruitment of effector cells and angiogenesis promotion(15–18)
Lipid-derived	
LTC4, LTB4, PGD2 and PGE2	Recruit effector cells, regulate immune responses and promote angiogenesis, oedema and bronchoconstriction (19–21)
Platelet-activating factor	Activates effector cells, enhances angiogenesis and induces physiological inflammation(21)
Cytokine	
TNF-α, IL-1α, IL-1β, IL-6, IL-18, GM-CSF, LIF, IFN-α and IFN-β	Phlogosis promoters (22, 23)
IL-3, IL-4, IL-5, IL-9, IL-13, IL-15 and IL-16	Function of Th2-type cytokines(24, 25)
IL-12 and IFN-γ	Functions of Th1-type cytokines(26, 27)
IL-10, TGF-β and VEGF	Regulate inflammation and angiogenesis (28)
Chemokine	
CCL2, CCL3, CCL4, CCL5, CCL11 and CCL20	Recruit effector cells, including dendritic cells, and regulate immune responses (29, 30)
CXCL1, CXCL2, CXCL8, CXCL9, CXCL10 and CXCL11	Recruit effector cells and regulate immune responses(30, 31)
Other	
Nitric oxide and superoxide radicals	Bactericidal (32, 33)
Antimicrobial peptides	Bactericidal (34)

Legend: CCL, CC-chemokine ligand; **CXCL**, CXC-chemokine ligand; **FGF2**, fibroblast growth factor 2; **GM-CSF**, granulocyte/macrophage colony-stimulating factor; **IFN**, interferon; **IL**, interleukin; **LIF**, leukemia inhibitory factor; **LTB4**, leukotriene B4; **LTC4**, leukotriene C4; **PGD2**, prostaglandin D2; **PGE2**, prostaglandin E2; **TGF-β**, transforming growth factor-β; **TNF-α**, tumor necrosis factor alpha; **VEGF**, vascular endothelial growth factor. Adapted from(35)

Table 2: Murine mast cells main features

Characteristic	Connective Tissue Mast cells (CTMC)	Mucosal Mast cell (MMC)
Size	10-20 (mm)	5-10 (mm)
Formaldehyde fixation	Resistent	Sensitive
Staining	Safranin	Alcian blue
T-cell depentent development	No	Yes
Protease content	Chymase: RMCP I	Chymase: RMCP II
Proteoglycans molecular mass (kDa)	Heparin 750-1000	Chondroitin sulfate 100-150
Histamine (pg/cell)	10-20	1
5-Hydroxytryptamine (pg/cell)	1-2	< 0.5
Prostaglandin D2	+	+
Leukotriene C4	-	++
Activated by		
- FceRI	Yes	Yes
- Compound 48/80	Yes	No
- Substance P	Yes	No
Inhibited by sodium cromoglycate	Yes	No

Legend:
A different sensibility to the formaldehyde fixation is one of the main features that distinguish the two cell populations. Indeed, following fixation with a formaldehyde-buffered solution, MMC do not stain, or stain poorly, with toluidine blue, whereas CTMC are resistant to this treatment and stain well. Depending on the different glycosaminoglycan content within the granules, mast cells react to Safranin or Alcian blue. **RMCP I and II:** serine proteases. Adapted from(9).

Mast cell growth and development

Mature mast cells are tissue resident cells of hematopoietic origin generated as precursor progenitors in the bone marrow. Mast cells progenitors migrate all through the body in different localizations and undergo to the final stage of differentiation upon cytokine stimulation in the tissue of destination. Although is commonly accepted that mast cells originate from a multipotent hematopoietic progenitor population, is still debated whether mast cells arise from a megakaryocyte/erythrocyte progenitor or a granulocyte/monocyte progenitor(36–38). A reasonable synthesis proposed by J.S. Dahlin and J.Hallgren is that murine committed mast cell precursors originate from a bi-potent progenitor present in the granulocyte/monocyte precursor population that is able to generate both mast cells and basophils(39). Mature mast cells are characterized by the expression of FcεRI, although has been reported that the majority of the precursor mast cell population founded in the blood of BALB/c mice is $FcεRI^+$ and $FεRI^-$ in C57BL mice, both being able to generate adult mast cells $FcεRI^+$ (40, 41). In humans, mast cells develop from a $CD13^+/CD34^+/CD117^+$ pluripotent progenitors (9, 42). A fundamental stimulus for tissue mast cells maturation is the Stem Cell Factor (SCF) that binds to c-kit (CD117), a receptor tyrosine kinase localized on mast cell's surface. SCF induces c-kit dimerization and auto-phosphorylation(43, 44).

For in vitro studies, several possibilities have been developed in order to derivate mast cells from precursors populations. One of the first attempts reported in the literature is the cultivation of mouse bone marrow cells with a conditioned medium obtained from concavalin A stimulated splenocytes(45); under these conditions is possible to generate a cell population that, by phenotype, resemble the mucosal mast cell population. Interesting, it became clear that such a cellular population could be further stimulated by the exposure to cytokines. Indeed, in presence of IL-3 containing media is possible to generate a mast cell population characterised by an increased synthesis of heparin proteoglycans, a characteristic Safranin staining and an increased histamine content that resemble the phenotype of connective tissue mast cells(9).

In the present study we generated bone marrow-derived mast cells, BMMC, by cultivating bone marrow cells in presence of stem cell factor, SCF and IL-3. The combination of the two stimuli induces the differentiation of a mast cell population that resemble the CTMC phenotype(46).

Mast cell mediators and their release

The mediators of the allergic response produced by mast cells can be grouped in three main classes: preformed granule associated molecules, newly generated lipid mediators and cytokines and chemokines(47, 48) (Table 1). The presence of several preformed granules localized in the cytoplasm is the main feature of mast cells. The granules are located in close vicinity with the plasma membrane, all around the cell perimeter and contain bioactive compounds that can rapidly be released and protect the host against diverse external insults(49). The degranulation reaction is based on multiple membrane fusion events (granule-granule or granule-membrane), all dependent on the interaction between v-SNAREs proteins (vesicle soluble N-ethylmaleimide-sensitive factor attachment protein receptors) expressed at the granule's membrane and t-SNARE present at the target membrane side(50). Although the degranulation mechanisms are not fully understood yet, it has been proposed that FcεRI activation in BMMC generates a signalling cascade that can be ideally splitted in two branches. Indeed while the activation of the Lyn tyrosine kinase promotes the downstream activation of class I PI3K and the PLCγ mediated production of Ca^{2+} that lead to the granule-membrane fusion, on the other hand, a Fyn/Gab2/RhoA mediated mechanism promotes the microtubules formation and the granule translocation in proximity of the plasma membrane, in a calcium independent mechanism(51).

FcεRI, high affinity receptor for IgE

FcεRI binds with high affinity the Fc portion of IgE molecules and is crucial in the onset of IgE-mediated allergic diseases and in the development of the most common cases of asthma, allergic rhinitis, atopic dermatitis and food or drug allergies. FcεRI exists as a tetrameric or trimeric complex. As a tetramer, FcεRI is formed by an α-chain, a β-chain and an homo-dimer of two disulphide-linked γ-chains; this structure is often indicated as $\alpha\beta\gamma_2$(52) (Figure 1). The trimeric form is defined as $\alpha\gamma_2$(52). The α-chain is a trans-membrane polypeptidic chain, member of the immunoglobulin superfamily. It shows two extracellular immunoglobulin-like domains (D1 and D2) that mediate the binding with a single IgE molecule (1:1 ratio), a trans-membrane domain with a conserved aspartic residue and a short cytoplasmic tail that apparently does not have signalling function(53). The crystal structure of the Fc region of IgE bound to FcεRIα revealed that the Cε3 domain of IgE-Fc binds two distinct sites located in the D2 domain of FcεRIα(53). The FcεRI β and γ-chains do not have any role in binding the IgE molecules and are characterized by an Immunoreceptor Tyrosine-based Activation Motif (ITAM). The ITAM consensus sequence D/E-XX-YXXL-$X_{7\text{-}11}$-L/I, is the site where the tyrosine residues are phosphorylated by protein tyrosine kinases (PTKs)(54). The β chain, with its four trans-membrane domains, is also a component of the low affinity IgG receptor (FcγRIII) in mast cells and, as well, the γ-chain is a component of the high-affinity receptor for IgG (FcγRI). The tetrameric form of the FcεRI is solely expressed in mast cells and basophils with a density of $3x10^5$ molecules per single cell in mouse(55). The trimeric form of the FcεRI, lacking the FcεRIβ, has been detected only in humans, in epidermal Langerhans cell(56, 57), monocytes(58), eosinophils(59), peripheral blood dendritic cells(60) and platelets(61). IgE have also another receptor, FcεRII (CD23) defined as IgE "low affinity" receptor. CD23 exist as a membrane bound glycoprotein or a soluble processed molecule. The membrane CD23 is a type II integral membrane protein with a C-type (calcium dependent) lectin domain at the C-terminal and is expressed as two different isoforms that differ in their cytoplasmic region. CD23a is constitutively expressed on B cells and CD23b expression is induced by IL-4 and is also found on T cells, Langerhans cells,

monocytes, macrophages, platelets, and eosinophils(62). Upon proteolysis of the CD23, several different soluble peptides are generated, all with cytokine-like activity(63). Both soluble and membrane bound CD23 isoforms are thought to have an active role in promoting allergic responses in a way that is directly stimulated by the binding with IgE(62).

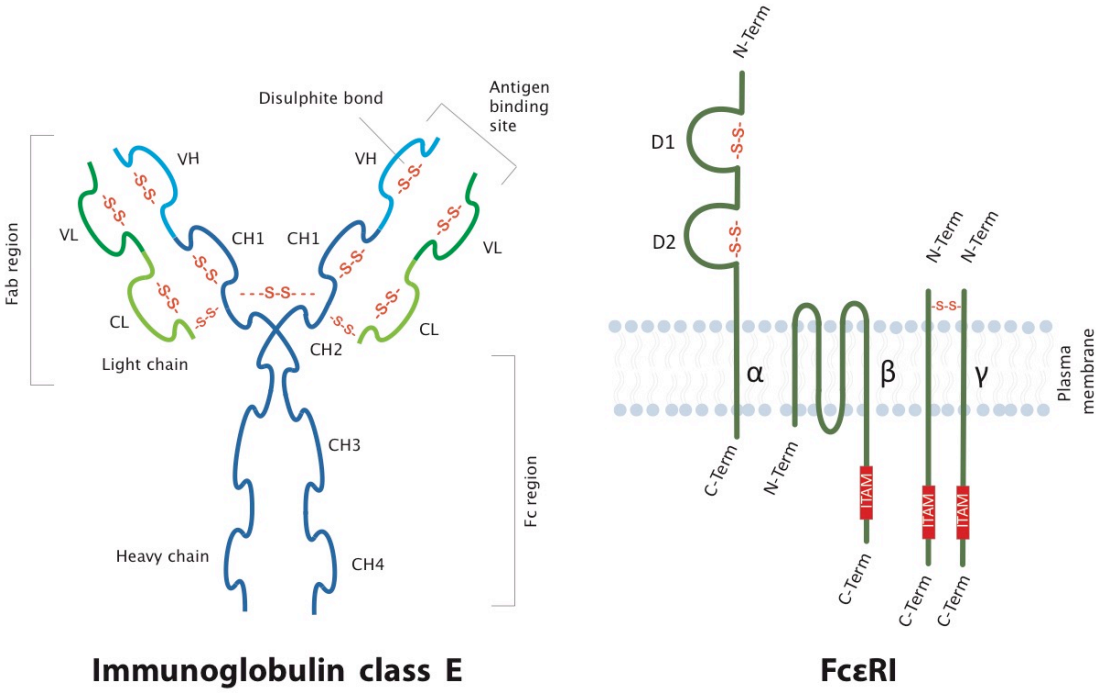

Figure 1: Structure of IgE and IgE high affinity receptor (FcεRI). A class E immunoglobulin, IgE, is composed by two isotype-specific heavy chains and two light chains (H_2L_2). The epsilon heavy chain is characterised by four Ig-like domains (CH1-CH4), three of them generating the Fc portion of the molecule. In red are represented the intra-molecular disulphide bonds. The high affinity receptor for IgE, FcεRI, consist of one α chain, one β chain and two γ chains(52, 64).

Signalling downstream of FcεRI activation

The signalling downstream of the FcεRI is initiated by the binding of a multivalent antigen able to contact several IgE molecules that are already localized on the surface of basophils or mast cells, bound to the FcεRI. Since FcεRI does not have any catalytic signalling activity per se, it recruits or activates several associated signalling proteins by conformational modification induced after the FcεRIα clustering (Figure 2). The Src family kinase Lyn is constitutively bound to the FcεRIβ chain and upon receptor clustering phosphorylates the two tyrosine residues within the ITAMs of both β and γ chain(65, 66). This allows the tyrosine kinase Syk to bind to the phosphorylated ITAMs of the γ-chain via its tandem SH2-domain, to be activated via phosphorylation and stabilized in an active conformation(67). Downstream of the activation of Syk, the linker for activation of T cell (LAT)(68, 69)and the non-T cell activation linker (NTAL)(70) are phosphorylated and act as a scaffold for multimeric signalling complexes, including Grb2, Gab2, Gads but also PLCγ1 and PLCγ2(71). The phosphorylated form of Gab2 is then able to recruit Class IA PI3K via its p85 regulatory subunits and induces the generation of $PtdIns(3,4,5)P_3$(72) by phosphorylating $PtdIns(4,5)P_2$. $PtdIns(3,4,5)P_3$ is a secondary intracellular messenger able to recruits PH domain containing proteins such as Vav, PKB, PDK1, Btk and PLCγ(73, 74) (Table 3). Active PLCγ enzymes catalyse the hydrolysis of $PtdIns(4,5)P_2$ and generate DAG and IP_3. DAG is known to promotes the catalytic activity of PKC, while IP3 binds to its own receptor on the endoplasmic reticulum and promotes intracellular Ca^{2+} liberation. The release of calcium ions from the ER induces conformational changes in the calcium-binding protein STIM1, localized in the ER membrane, that interact with the store-operated calcium channels ORAI1 in the plasma membrane inducing their opening and the extracellular calcium entry(75, 76). The complex orchestration of all those signals leads to the cellular degranulation and the “de novo” generation of signalling molecules to be released to the cellular milieu.

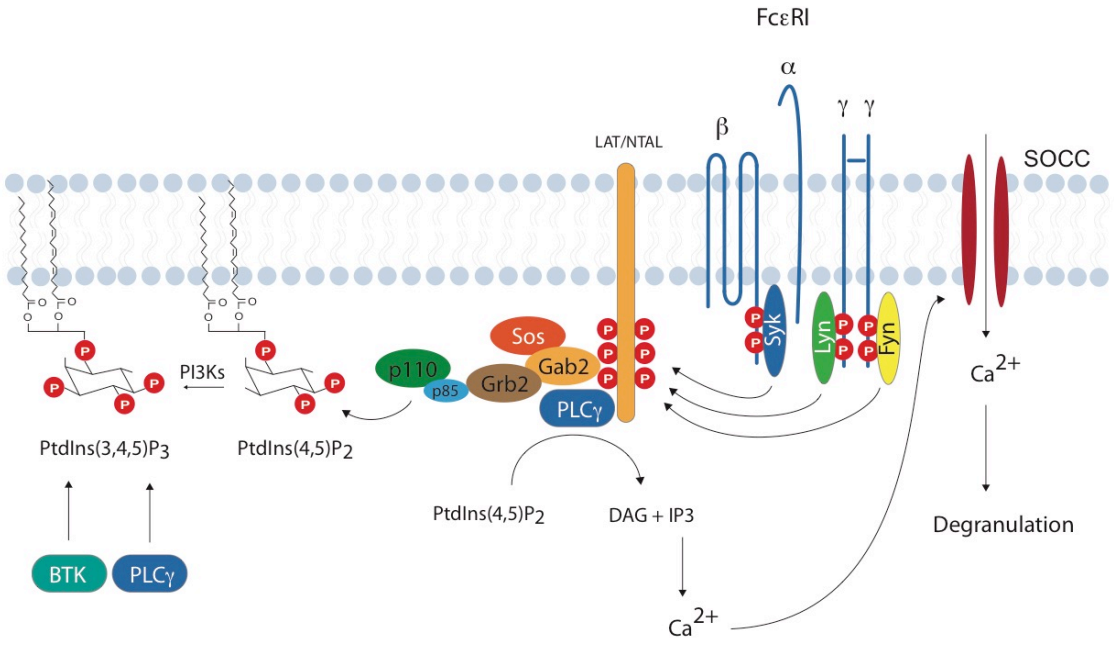

Figure 2: Signalling downstream of FcεRI. Representation of the fundamental events induced upon clustering of FcεRIα chains aggregation. Tyrosine kinases Lyn or Fyn are responsible for the phosphorylation of the ITAM motif present in FcεRIβ and FcεRIγ and for the phosphorylation of the adapter proteins Gab2, LAT or NTAL. The assembly of such a signalosome is required for the recruitment of SH2-bearing proteins at the plasma membrane. The activation of class IA PI3Ks in this representation is induced by direct interaction between the pYxxM motifs of Gab2 and the SH2 domain of p85s. PtdIns(3,4,5)P_3 is a fundamental secondary messenger able to recruit several PH-domain bearing proteins at the plasma membrane, such as Btk and PLCγ1 and to mediate their activation. Btk is a PH-domain bearing Tek family non-receptor tyrosine kinase member. Once at the plasma membrane, Btk is phosphorylated by the Lyn kinase and undergoes auto-phosphorylation as well becoming able to phosphorylate and activate PLCγ1(77).

Table 3: Relevant PH domain bearing proteins in mast cell biology

PH domain bearing protein	Phosphoinositides bindinding specificity	References
Btk	PtdIns(3,4,5)P3	PH domain of Btk: Sakim, K. 1996 Btk in mast cells: Iwaki, S. 2005
Grp-1	PtdIns(3,4,5)P3	PH domain of Grp-1: Klarlund, J.K. 1998
Gab1/2	PtdIns(3,4,5)P3	Gab2 in mast cells: Nishida, K. 2002 PH domain of Gab2: Zhao, C. 1999
PDK1	PtdIns(3,4,5)P3	PDK1 mediated activation of PKB: Alessi, D. 1997 PDK1 in mast cells: Shumilina, E. 2010
PKB	PtdIns(3,4,5)P3; PtdIns(4,5)P2; PtdIns(3,4)P2	PH domain of PKB: Stocker, H. 2002; Scheid, M. 2002
PLC-γ2	PtdIns(3,4,5)P3; PtdIns(4,5)P2	PLC-g2 in mast cells: Wen, R., 2002 PH domain of PLC-g2: Falasca, M. 1998
Vav	PtdIns(3,4,5)P3; PtdIns(4,5)P2	Vav 1 and 2 in mast cells: Turner, M. 2002; Manetz, T. 2001

c-Kit, the receptor for SCF

Together with the FcεRI, the c-Kit receptor is one of the most characterised surface receptors in mast cells. Identified as CD117, c-Kit is a transmembrane tyrosine kinase receptor(78) expressed on the surface of hematopoietic stem cells and on several different non-hematopoietic tissues(79). Stem cells factor (SCF) is the ligand of c-Kit and its binding induces homo-dimerization of the receptor and auto-phosphorylation of the tyrosine residues present in the cytoplasmic portion(80). c-Kit activation induces direct binding of class IA PI3Ks adaptor subunits(81) to the phosphorylated tyrosine residues, via SH2 domain, and therefore generation of PtdIns(3,4,5)P_3. c-Kit signalling in mast cell is not only involved in cellular growth and differentiation of hematopoietic progenitors (check "Mast cell growth and development") but is also required for a full scale mast cell activation. Indeed, SCF stimulation induces a direct activation of PI3Kδ(82–85) whose product potentiate the antigen-induced BMMC degranulation but is not able to induce degranulation per se. Moreover, SCF function as chemotactic factor for mast cells(86, 87) inducing the activation of MAP kinase signalling and activation of the Src family kinase Lyn(82, 88, 89).

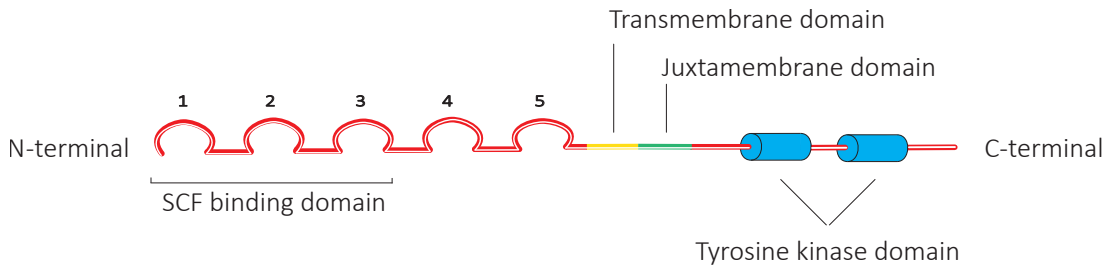

Figure 3: cKIT, the receptor for stem cell factor (SCF). cKIT is a monomeric transmembrane receptor. The N-terminal extracellular domain is formed by five Ig-like domains of witch the first three mediate the interaction with the ligand. The binding of the ligand induced the formation of a homodimer and the Ig-like domains 4 and 5 mediate the dimerization. The phosphorylation of the juxtamembrane domain, the tyrosine kinase domain and the c-terminal tail modulates the signal transduction activity(90).

ITIM containing immunoreceptors

The role of the immunoreceptor tyrosine-based inhibitory motif (ITIM) sequences was first studied on FcγRIIb expressed in B cells where they modulate the BCR mediated activation in vitro(91). The ITIM-bearing receptors belong to either the Ig Superfamily or C-type (calcium dependent) lectin superfamily and are responsible for the membrane recruitment of lipid and protein phosphatases. Those receptors can bear one or more ITIM or ITIM-like domains and most of them are still orphan for their ligands; consequently, most of the experiments performed on ITIM containing immunoreceptor were performed via chemical induced co-clustering or in genetically modified mice. For instance, the receptor gp49B1 express two Ig-like domains on the extracellular portion and presents two cytoplasmic ITIMs. Its ligand has only been described in vitro as the integrin αvβ3(92) and its activation leads to the inhibition of the FcεRI signalling in mast cells via recruitment of SH2 domain-containing tyrosine phosphatase 1 (SHP1) rather than the SH2 domain-containing lipid phosphatase SHIP1(93). It is noteworthy that gp49B1 does not require immunoglobulins, a product of adaptive immune response, to induces its inhibitory signalling. Paired Ig-like receptor B (PIR-B) has six Ig-like extracellular domains and four ITIM domains and is a ligand orphan receptor as well. It is continuously tyrosine phosphorylated and associated with SHP1 tyrosine phosphatase and it also controls mast cell activity(94). Mast Cell function-associated antigen (MAFA) is a trans-membrane receptor with a C-type lectin domain on its extracellular domain and a single ITIM domain; it is ligand-orphan and relays on SHIP1 for its inhibitory activity(95). One of the most recently discovered ITIM-bearing receptor is Allergin-1, expressed both in human (mast cells, basophils, neutrophils and DC) and mice (not expressed in basophils)(96). Indeed, murine Allergin-1 is preferentially expressed in mast cells where, due to the presence of an ITIM-like intracellular domain, it is able to recruit the tyrosine phosphatases SHP-1 and 2 as well as the 5' lipid phosphatase SHIP1 at the plasma membrane.

FcγRIIb, low affinity receptor for IgG

FcγRIIb (CD32b) is a low-affinity single chain receptor for IgG, broadly expressed in hematopoietic cells as a monomeric trans-membrane protein. FcγRIIb has two C2-type IgG-like domains on the extracellular portion of the membrane, the second of which binds the allergen-specific IgG(97), whereas the cytoplasmic portion is characterised by the presence of an ITIM domain, a landmark of the "inhibitory receptors". FcγRIIb is expressed on the surface of B cells but also in mouse mast cells and in human basophils, where it counteracts the FcεRI-induced activation(98–100). Both the FcεRI and FcγRIIb are members of the Ig superfamily and in humans, at the level of the extracellular domain, the two receptors share 38% of the primary sequence. Although both the α chain of the FcεRI (FcεRIα) and FcγRIIb share a typical conserved pattern characterized by two IgG-like globular disulphide-bonded domains of the C2 type(101), they have different affinity for Ig. FcεRIα binds IgE with high affinity ($K_a = 10^{10}$ M^{-1})(102) whereas FcγRIIb binds IgG with low affinity ($K_a = 10^6$ M^{-1})(97). Both, in mast cells and basophils, co-aggregation of FcεRI with FcγRIIb protects the cells from FcεRI-dependent activation(98, 103). At a molecular level, the co-aggregation of FcεRI and FcγRIIb induces the activation of the protein tyrosine kinase Lyn that phosphorylates the ITAM domains of FcεRI but also the ITIM domains of FcγRIIb due to the close vicinity(104). Phosphorylation of the FcγRIIb ITIM then induces the recruitment of the SH2-containing inositol phosphatase (SHIP1) (105).

The ability of antigen-specific IgG to inhibit the IgE mediated signalling is part of the mechanism behind the allergic desensitization therapy. Indeed, the repeated and prolonged allergen administration in atopic patients is, currently, the only therapy that modulates the progression of the allergies by modifying the allergen specific T cell response. Interestingly, one of the effects produced in response to the therapy is the generation of allergen specific IgG_1 and IgG_4(106, 107); although the recruitment of the inhibitory receptor FcγRIIb mediates the silencing of the IgE mediated signalling, some evidences report that the inhibitory ability of the antigen-specific IgG molecules relies on the ability to block the interaction between IgE and antigen(108).

The phosphoinositide 3-kinase (PI3K) family

The family of phosphoinositide 3-kinase enzymes unites several protein complexes capable of phosphorylating the inositol group of phosphoinositides at their 3' position. Phosphoinositides are the phosphorylated form of phosphatidylinositol, a natural component of cellular membranes and contain two non-polar fatty acids that anchor the molecule to the membrane, linked to a glycerol backbone and an inositol ring connected via a phosphate group (Figure 4)(109). The generation of different phosphoinositides activates a plethora of downstream signalling events that regulate cellular growth, proliferation but also cellular migration and endo/exocytotic events. The first reports of proteins with phosphatidylinositol kinase activity were published almost thirty years ago describing the ability of tyrosine kinase proteins involved in tumorigenesis to phosphorylate phosphatidylinositol(110–112), while some years later Cantley lab published the first work that described a novel inositol phospholipid, phosphatidylinositol-3-phosphate(113) and since then the PI3K field has been extensively investigated. The discovery of the first molecular inhibitors of PI3Ks enzymes, such as wortmannin(114, 115), enhanced the understanding of the basic mechanism regulating phospholipid biology and allowed researchers to focus on PI3K as a potential therapeutical target.

PI3Ks enzymes catalyse the transfer of the γ-phosphate group of adenosine triphosphate (ATP) to the D-3 position of the inositol ring of the phosphatidyl inositol. Enzymes belonging to the PI3K family generate different products such as PtdIns(3)P, PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 and such molecules function as docking sites for various protein effectors that interact with phospholipidic domains. Indeed, class I PI3K enzymes are able to phosphorylate the plasma membrane localized PtdIns(4,5)P_2 and generate PtdIns(3,4,5)P_3, a molecule recognized by proteins containing a pleckstrin homology (PH) domain such as Grp1 or Btk (Table 3)(116, 117). One of the most investigated targets activated downstream of PI3Ks is the Ser/Thr protein kinase B (PKB, also called Akt). PKB is recruited at the plasma membrane due to its PH domain and is phosphorylated and activated by the phosphoinositide-dependent kinase (PDK1) at the level of the Threonine 308 and by mammalian target of rapamycin complex 2 (mTORC2)

at the level of the Serine 473(118). This event is followed by a plethora of cellular responses that lead to cellular growth, cytoskeletal reorganization, changes in cellular metabolism and differentiation. The PI3K family has been divided in three classes, I, II and III, based on sequence homology, protein domain organization, substrate specificity and regulation (109, 119).

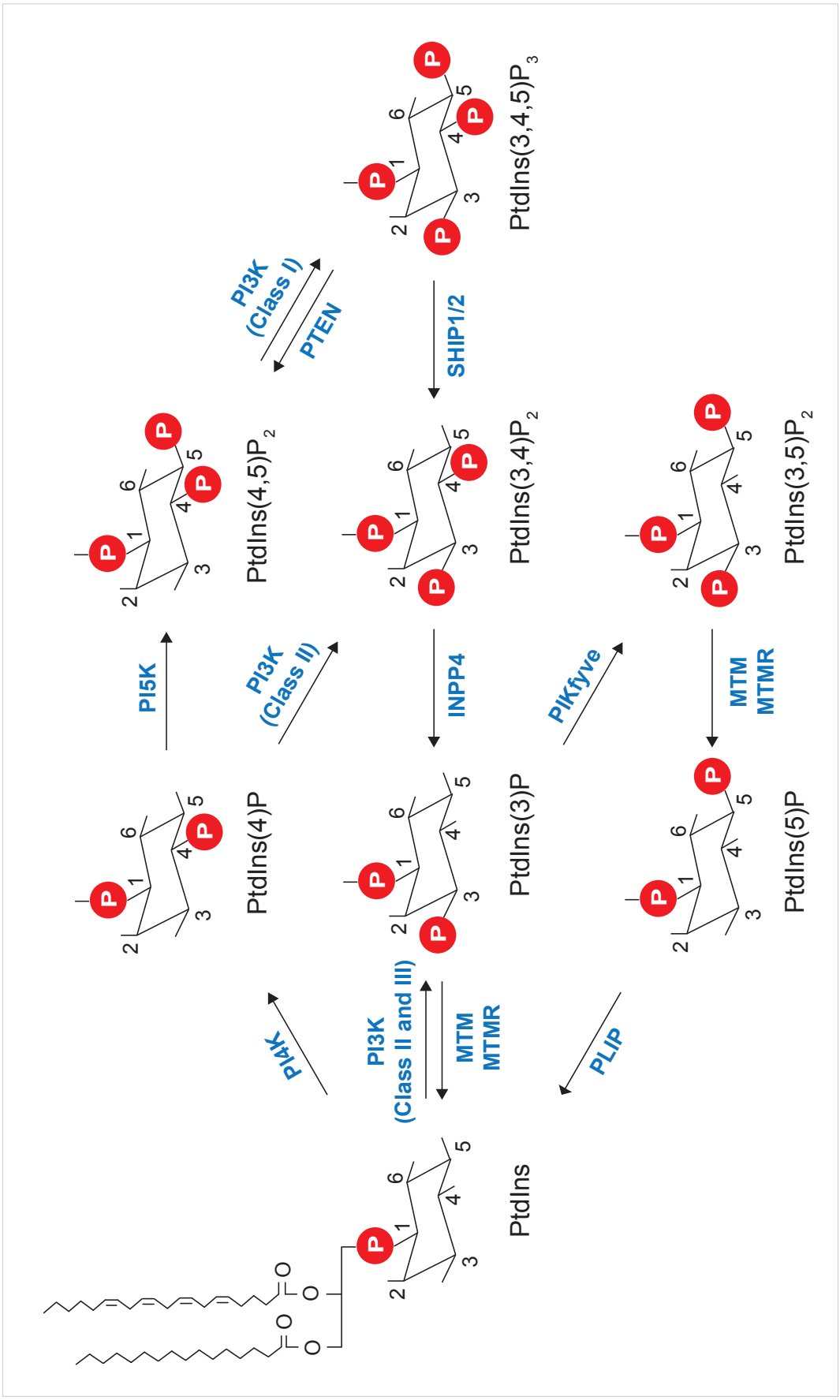
PtdIns
PtdIns(3)P
PtdIns(4)P
PtdIns(5)P
PtdIns(3,4)P2
PtdIns(4,5)P2
PtdIns(3,5)P2
PtdIns(3,4,5)P3
PI4K
PI3K (Class II and III)
MTM MTMR
PLIP
PIKfyve
PI3K (Class II)
INPP4
PI5K
PI3K (Class I)
PTEN
SHIP1/2

Figure 4

Figure 4: Phosphoinositides and phosphorylation sites. The structure of the phosphoinositide is based on a molecule of glycerol where the first and the second carbon atoms of the structure, denoted as the sn1 and sn2 position, are each attached via esterification to a fatty acid molecule and the third carbon atom, sn3 position is the phosphatidylinositol. In the present representation, position sn-1 is occupied by the palmitic acid and the position sn-2 by the arachidonic acid. PtdIns(4)P is generated by phosphatidylinositol 4-kinase, PI4K(120, 121) enzymes starting from PtdIns and is localized at the Golgi and on the endoplasmic reticulum (ER)(120). PtdIns(4)P can be phosphorylated at the 3' position by Class II PI3Ks, resulting in PtdIns(3,4)P_2 or at the 5' position by PI5Ks(122), producing PtdIns(4,5)P_2, mainly localized at the plasma membrane. Class II and III PI3Ks phosphorylate PtdIns and generate PtdIns(3)P that is mainly localized on the early endosome. PtdIns(3)P can be phosphorylated at the 5' position by PIKfyve(123) generating PtdIns(3,5)P_2 that is a substrate for the lipid phosphatases Myotubularin and Myotubularin-related(124). PtdIns(3,4)P_2 is a substrate for class I PI3Ks and can be hydrolysed by 5' phosphatases, such as SHIP1 and 2 or by the 3' phosphatases PTEN(125). Due to lack of space, here is only a representative portion of the known modulators of PtdIns. Adapted from (126)

Class I PI3Ks

Class I PI3K enzymes are heterodimeric proteins composed by a regulatory subunit and a catalytic subunit and share a common domain organization having a N-terminal adaptor-binding domain (ABD), a Ras-binding domain (RBD), a C2 domain, a helical domain and a kinase domain divided in a N- and a C-lobe (Figure 5). The region containing the helical domain and the two lobes has some similarities with the catalytic domain of protein kinases(127). The class IA regulatory subunits contain two Src homology 2 domains, nSH2 and cSH2, with an interposed coiled-coil domain called inter-SH2 (iSH2) that mediates the binding to p110. Class I PI3K enzymes are the only family members able to convert plasma membrane localized PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3 and are activated either via receptor tyrosine kinases (RTK) or G protein-coupled receptors (GPCRs) activation. Based on the activation mode, class I is further sub-divided in class IA a class IB.

Class IA PI3Ks

Class IA PI3K enzymes are heterodimeric protein complexes containing a catalytic subunit variant of p110 (one among p110α, p110β and p110δ) and one of the five adaptor subunit variants (p85α, p55α, p50α, p85β or p55γ). Three genes, *Pik3ca, Pik3cb and Pik3cd*, code for the three catalytic subunits, p110α, p110β and p110δ respectively, whereas the adaptor subunits p85α, p55α and p50α are generated by alternative splicing from the Pik3r1 gene; p85β and p55γ are encoded by Pik3r2 gene and Pik3r3 gene respectively (details about the genes encoding different PI3K subunits are present in Table 2). Not a lot of information is available about preferences or specificity of the interaction between the catalytic and the adaptor subunits, since all the three catalytic subunits can complex with the five adaptors. Class IA PI3Ks can be activated upon receptor tyrosine kinase activation or phosphorylation of adaptor proteins. The binding of a ligand to a receptor tyrosine kinase (RTK) induces dimerization of the receptor and auto-phosphorylation of its tyrosine residues that are recognized by SH2 domain-

containing molecules, for instance p85. The binding of the regulatory subunit to tyrosine-phosphorylated pYXXM motifs (Y is Tyr, X is any amino acid, M is Met) triggers the activation of the p110 catalytic subunit of PI3Ks(128). In the case of p110α, a crystal structure analysis showed that the iSH2 domain of p85α is localised in a crevasse formed by the catalytic subunit, in contact with the ABD and the C2 domain of p110(129). The binding of the regulatory subunit to the phospho-tyrosine motifs induces modifications in the inhibitory contacts between p85 and p110 that keeps the enzyme in an inactive state and leads to the activation of the lipid kinase at the plasma membrane. Class IA PI3Ks can be recruited and activated by plasma membrane localized adaptor proteins as well. For instance, PI3Ks can be activated via insulin receptor substrate 1 (IRS1) or via the growth factor receptor-bound protein 2, Grb2, which binds to the Grb2-associated binding protein, GAB; in both case p85 interacts with the phosphorylated tyrosine residues of those proteins, leading to the activation of p110. In addition there is a third possibility: it is the case of Grb2, which binds to the phosphor-YXN motif of the RTK and recruits SOS (Son of Sevenless), Ras and GAB, activating p110 independently of p85(130). Due to their involvement in the regulation of the cellular growth, motility and differentiation, aberrations of the class IA PI3K family enzymes signalling are one of the most frequent occurrences in human cancer(131, 132). The most prominent example is the case of activating somatic mutations at the level of the PIK3CA gene (p110α)(133, 134).

A lot of informations about PI3K signalling have been gained by the generation of genetically modified mice bearing either a null allele or expressing a kinase dead enzyme for the three members of class IA PI3K family. The first attempts to generate mice lacking p110α or p110β demonstrated that the two catalytic subunits of class I PI3K are indispensable for a correct development since the mice died as embryos(135, 136). However, the targeting of the two catalytic subunits has been obtained either by using a tissue specific deletion of the gene or by the overexpression of a kinase-dead isoform of the enzyme, both for p110α(137, 138) and p110β(139, 140). Mice lacking PI3Kδ are generally healthy and without an external phenotype; the generation of the first knock-in strain contributed to elucidate the role of p110δ in antigen receptor signalling in B and T cells(141).

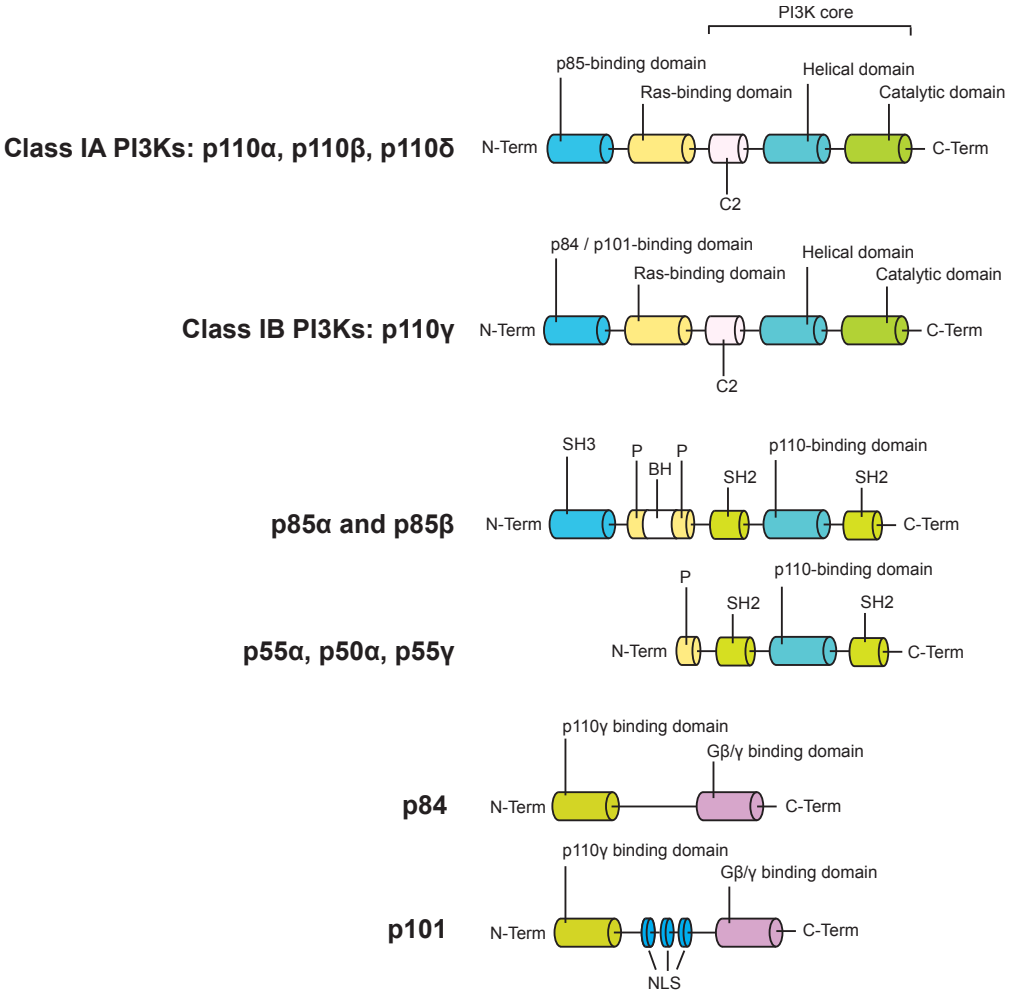

Figure 5: Class I PI3K enzymes family. The N-terminal portion of the four catalytic subunit shares a similar domain organization and the main difference is in the adaptor subunit-binding domain (p85 isoform for class IA and p84 / p101 for class IB). All the p85 adaptors have two Src homology 2 (SH2) domains that mediate the plasma membrane recruitment. In p85α and p85β there is a N-terminal SH3 domain that mediates the binding to proline rich and hydrophobic regions, two proline-rich repeats and a Breakpoint cluster BCR homology domain, BH that mediates the interaction with the Rho GTPase family members. The specificity of the single isoform of the p85 adaptors is not known. Both p84 and p101 present a N-terminal Ras binding domain and a C-terminal Gβ/γ binding domain that mediate the plasma membrane localization and activation of PI3Kγ. p101 present three nuclear localization sequences (NLS) that are not present in p84 but their functional role is currently not known. Adapted from(142).

Class IB PI3Ks

PI3Kγ is the only member of class IB PI3K family identified so far and is a heterodimeric enzyme formed by a catalytic subunit, p110γ and one of the two adaptor subunits, p84 and p101. p110γ shares several features with the catalytic subunits of the class IA PI3Ks but appears to be solely expressed in mammalians and particularly in white blood cells(143–145). The two adaptor subunits, p84 and p101 are encoded by two neighbours genes, *Pik3r6* and *Pik3r5* respectively, localized on the same chromosome both in human (17p13.1) and mouse (11 band B3) (Table 4). The two adaptors present about 30% of aminoacid identity at the N- and C-terminus of the protein and differ in the core. The main difference between the two proteins is the presence of a nuclear localization sequence (NLS) in p101, but not in p84, whose physiological role is currently not known. Both p84 and p101 present a binding domain that mediates the interaction with p110γ at the N-terminus end(146, 147), whereas at the C-terminus present a Gβγ association region(146); p84 and p101 bind to p110γ in a mutual exclusive fashion(148).

PI3Kγ is mainly activated downstream G protein-coupled receptors (GPCRs). Heterotrimeric G proteins are composed by an α, a β and a γ subunit and in their inactive status are bound to guanosine diphosphate (GDP). The binding of a ligand to GPRCs induces a conformational change in the receptor, resulting in a reduced affinity of Gα subunit for GDP and increased affinity for GTP; together with the switch GDP-GTP, there is also the dissociation of Gα from the complex(149). This leads to the separation from the Gβγ dimer that now is able to diffuse at the plasma membrane and to contact potential effector proteins(150).

Currently, little information is available about the physiological role of the two adaptor subunits in the activation of PI3Kγ. Indeed, Bohnacker et al. showed that PI3Kγ uses only p84 as adaptor subunit in BMMC (p101 not being expressed) although overexpression experiments showed a partial redundancy between p84 and p101 activity in PI3Kγ mediated cellular responses(151). In the same year it was showed that the small monomeric GTPase Ras is an indispensable co-activator of the p110γ/p84 complex, but not of the p110γ/p101 heterodimer in transfected HEK293 cells and complemented BMMC. Interestingly, a recent work

published by Walser et al(152) showed that PI3Kγ can be activated without the support of the adaptor subunit p84 in BMMC. Indeed, by using Thapsigargin, a Ca^{2+}-mobilising agent, they demonstrated that PKCβ is activated upon increased level of intracellular Ca^{2+} and is able to phosphorylate the serine 582 of p110γ, promoting its lipid kinase activity. It is noteworthy that the activation of PI3Kγ by phosphorylation requires p110γ not to be in complex with p84, since the binding of p84 to p110γ masks the phosphorylation site and inhibits the access to the residue. However, the complete understanding of the dynamics and the functions of the adaptor subunits of PI3Kγ has not been achieved yet.

The role of PI3Kγ is not only restricted to the immune system. For instance, Perino A. et al.(153) showed that PI3Kγ has an important role in regulating myocardial contractility due to its ability to bind protein kinase A, PKA, and to induce the formation of a complex together with the phosphodiesterase PDE3B. Interestingly, on one hand PKA is a cAMP-dependent protein kinase that activates PDE3B, a physiological regulator of the myocardial contractility that hydrolyses cAMP to 5'-AMP. On the other hand PKA phosphorylates p110γ and inhibits its lipid kinase activity. In this context p110γ becomes the centre of a signalling complex that modulate the cAMP signalling and, consequently the myocardial contractility, but also controls the generation of plasma membrane PtdIns(3,4,5)P_3 and the activation of the PtdIns(3,4,5)P_3-dependent downstream pathways.

Another example is given by two publications from 2011 that showed a role for PI3Kγ as a promoter of obesity and insulin resistance in high fat diet fed mice. Interestingly, Kobayashi et al.(154) demonstrate that mice lacking PI3Kγ are protected from high-fat diet induced insulin resistance and elevated hepatic content of triglyceride (steatosis) and show a reduction of macrophage infiltration in adipose tissue, a recognised key feature of metabolic syndrome. Interestingly the same phenotype was reproduced by knocking out PI3Kγ in mice that develop obesity as a consequence of their genetic background, demonstrating that PI3Kγ exert its protective role by influencing the generation of a systemic inflammation, independently from the body weight status.

Few months later Becattini et al. (155) described PI3Kγ as a negative regulator of the diet-induced thermogenesis and as a promoter of obesity and insulin

resistance. Furthermore, by performing bone marrow reconstitution experiments, they demonstrated that PI3Kγ activity on diet-induced obese mice depends on its lipid kinase activity within a non-hematopoietic compartment, not identified yet. This represent an extremely interesting finding since it allowed us to postulate a more convoluted involvement of the class IB PI3K member outside of its traditional role in leukocytes/GPCR-associated responses and designate PI3Kγ as a potential pharmaceutical target as a metabolic regulator.

Table 4: Class I PI3K family members and chromosomal location

Subunit	Human		Mouse		
	Gene	Chr	Gene	Chr	
p110 α	PIK3CA	3q26.3	Pik3ca	3 B	Class IA
p110 β	PIK3CB	3q22.3	Pik3cb	9 E4	
p110 δ	PIK3CD	1p36.2	Pik3cd	4 E2	
p85 α	PIK3R1	5q13.1	PIK3r1	13 D1	
p55 α					
p50 α					
p85 β	PIK3R2	19q13.2	PIK3r2	8 B3.3	
p55 γ	PIK3R3	1p34.1	PIK3r3	4 D1	
p110 γ	PIK3CG	7q22.3	Pik3cg	12 B	Class IB
p84	PIK3R5	17p13.1	PIK3r5	11 B3	
p101	PIK3R6	17p13.1	PIK3r6	11 B3	

Role of class I PI3K in lymphocytes biology

Three classes of receptors mainly regulate lymphocyte's development and functionality: cytokines receptors superfamily, B and T cell antigen specific receptors and co-stimulatory receptors. While the stimulation of cytokines receptors superfamily mainly induces the activation of the JAK protein kinases family(156), antigen-specific and co-stimulatory receptors stimulation mainly promotes the activation of Src, Tec and Zap70/Syk tyrosine kinases. Although some evidences correlate the activation of cytokines receptors/JAK pathway to PI3K signalling(157, 158), the canonical activation of class IA PI3K members is based on the interaction between phosphorylated tyrosine residues and the Src Homology 2 (SH2) domains of p85 adaptor subunits. Is noteworthy to mention that chemokines, a family of signalling proteins involved in chemotaxis (chemotactic cytokines), due to their ability to interact with GPCRs, induce the activation of class IB PI3K, PI3Kγ, although there are evidences that also PI3Kβ might be activated by chemokine receptors(139, 159).

The TCR-mediated recognition of the antigen-MHC complex in naïve T cells, a process described as immunological synapsis, is characterised by a plethora of cytoplasmic modifications that lead to the formation of a signalosome. The recruitment of p85 adaptor subunits at the plasma membrane leads to a rise in PtdIns(3,4,5)P_3 production and a rapid increase of cytoplasmic calcium ions due to the activation of PLCγ1 and to the following intracellular calcium stores opening(160–162).

Evidences that PI3Ks play a role in lymphocytes biology came from experiments performed in presence of pan-PI3K inhibitors. Wortmannin and LY294002 were showed to be able to block both antigen-induced IL-2 production and $CD4^+$ differentiation but also co-receptor (CD3) induced IL-2 production and $CD8^+$ proliferation in T cells(163, 164). The secretion of IL2 and the expression of IL-2 receptors produces an autocrine stimulation that lead to a clonal expansion, resulting in the creation of a pool of antigen-specific cells ready to respond to the stimulation.

Interestingly, mice lacking p110γ show a diminished proliferative response to antibody-mediates stimulation of CD3, the TCR co-stimulatory receptor whose

canonical activation promotes Lck and ZAP-70 tyrosine kinases activation and recruitment of class IA PI3Ks. Interestingly, this inhibition is partially rescued by anti-CD28 or IL2 stimulation(165). In the same study, the authors demonstrated that PI3Kγ is activated upon TCR ligation, it associates with Lck, Zap70 and $G\alpha_{q/11}$ and contributes to the generation of a pool of PIP_3 that localizes at the immunological synapsis formed during the APC-T cells interaction.

Mice lacking p110γ also show a reduced thymocyte populations that reflect defects during the maturation process with an increase of $CD4^-$ $CD8^-$ double negative T cells compared to wild type littermates(166). Interestingly, PI3Kγ seems not to play a key role in B cells(167).

The complete ablation of p110δ generate mice that do not show any significant difference in the morphology and composition of the peripheral blood or the bone marrow and do not show any significant defect in stimulation-induced responses in T cells in comparison with wild type mice; on the other hand those mice show both a defective B1 and marginal zone B-cell development, a general decreased expression of serum immunoglobulin in unstimulated cells (especially IgM and IgG_1) and a defective humoral response(168, 169). Interestingly, as result of the targeting, those mice showed a reduced expression of the class IA PI3K adaptor subunits. Interestingly, mice expressing a catalytically inactive isoform of p110δ (p110δ knock-in) show a reduced antigen receptor signalling in B and T cells(141), an increased IgE production and a tendency to develop autoimmunity(170–172). More over it has been show that mice lacking p110δ, fail to down-regulate the RAG genes expression in immature B-cells, a process that is essential for the receptor editing and the progression to the mature B cells stage(173). Interestingly this phenomenon was already reported in experiment performed using Wortmannin, a pan-PI3K inhibitor(174, 175).

Ras small GTPases and Class I PI3K

Ras is one of the most extensively investigated families of small monomeric GTPases and class I PI3Ks are well-known effectors of Ras. Ras family members are ubiquitously expressed 21kDa proteins, whose identification is due to the pioneering studies performed on the tumorigenic ability of unidentified virus preparations in rodents that revealed the existence of mutated gene sequences able to promote cancerous growth of the tissues(176, 177). Indeed, Ras plays a key regulatory role in cellular proliferation, differentiation and apoptosis(178) by modulating the activation of several intracellular pathways such as MAPK (Raf-MEK-ERK) or PI3K itself. Members of Ras protein family share more then 80% of sequence homology and generally differ in their C-terminal sequence, the hypervariable domain(179). Nevertheless, all Ras isoform present a four aminoacid sequence at the C-terminus, named CAAX box, where C is a cysteine, A is an aliphatic amino acid and X is a variable aminoacid, that upon post-translational modifications allows Ras localization at the plasma membrane. After protein translation, the first modification of the CAAX box is the isoprenylation of Ras, namely the attachment of a 15-carbon isoprenyl group farnesyl to the sulfhydryl group of the cysteine(180). The same cysteine residue can alternatively be modified with the adding of a 20-carbon geranylgeranyl isoprenyl unit(181). Two different enzymes have been identified in eukaryotes: Farnesyltransferase (FTase) and Geranylgeranyltransferase-I (GGTase-I)(180).

After farnesylation of the cysteine residue of the CAAX, the last three amino acids of the CAAX box are cleaved by RAS-converting enzyme-1 (RCE-1) and the carboxy terminal cysteine is methylated(179, 182, 183); those modifications allow Ras to localize at the plasma membrane and to be able to participate to the signalling.

Once at the plasma membrane, Ras proteins are founded in their active state, bound to guanosine triphosphate (GTP) or in a inactive form, bound to guanosine diphosphate (GDP)(184) and by virtue of their dual ontology, Ras proteins function as a molecular switch within the cellular systems.

Indeed, the exchange of GDP for GTP induces a conformational status change that influences the binding of Ras to their effectors, mainly by affecting two motile

regions of the proteins, the switch I (residue 32-40) and switch II (residue60-76) regions(185).

Both the rate of GTP hydrolysis and the nucleotide exchange in Ras proteins are enhanced and regulated by accessory proteins such as guanine nucleotide exchange factors, GEFs(186), or GTPase activating proteins, GAPs(187). While the GEFs promote the formation of active - GTP-associated - Ras, the GAPs promote the Ras hydrolytic activity and the transition to an inactive status. Interestingly, germline deletions or loss of function mutations in one of the GAP of Ras, NF1, result in a deregulation of Ras signalling that leads to elevated levels of Ras-GTP and is associated with a predisposition to develop a variety of benign and malign tumors(188).

All Ras effectors contain a Ras binding domain (RBD) that in class I PI3K enzymes is localized at the N-terminus, just after the adaptor subunit binding domain and whose tertiary structure consist of 5 β-sheets flanked by 2 α-helices (189). A recent work describes 7 members of Ras family being able to interact with the p110-RBD: H-Ras, K-Ras4A, K-Ras4B, N-Ras, R-Ras, R-Ras2, R-Ras3(190).

The result of the crystal structure analysis of the p110γ/Ras complex(191), published in 2000, provided several indication about the molecular interaction between the two molecules. Indeed, X-ray crystallography analysis showed a critical loop in the p110γ RBD that, differently from other Ras downstream effectors, interacts with both switch I and switch II regions of GTP-bound Ras and indicate a possible allosteric modulation of Ras-mediated p110γ activation. Furthermore, the same work indicated that the substitution of five residues in the p110γ RBD (T232D, K251A, K254S, K255A and K256A, "DASAA" mutant) generates a Ras-binding deficient PI3Kγ. The first "in vivo" report showing the interaction between Ras and PI3K describes a reduced PtdIns(3,4,5)P_3 generation in neutrophils isolated from genetically modified p110γ^{DASAA} mice(192) and indicates the requirement of Ras for PI3Kγ activation.

The "Project II" part, contained in this manuscript, describes how we explored the possibility of inhibiting Ras farnesylation in order to selectively inhibit PI3Kγ activation in cell that express PI3Kγ as a p110γ/p84 heterodimer, without effecting the PI3Kγ activity in cells where PI3Kγ is expressed as p110γ/p101.

Class II and Class III PI3K

Class II PI3Ks enzymes are high molecular weight monomers identified for the first time in Drosophila M.(193) In mammals are present three isoforms of class II PI3Ks: PI3KC2α, PI3KC2β and PI3KC2γ(194) that are ubiquitously expressed in human. They all present a Ras-binding domain, a C2-like domain and a core kinase domain but they lack of a binding domain for adaptor subunits. Being presently the least class of PI3K studied, there are still divergent data about their substrate specificity. Undoubtedly, such a lack of information about Class II PI3Ks enzymes is due to the absence of specific inhibitory molecules that in the case of class I PI3Ks have had a pivotal role in understanding their cellular and molecular dynamics.

The sole member of class III PI3K enzymes is VPS34 (Vacuolar Sorting Protein 34), discovered and cloned in 1990 in S. Cerevisiae(198). As member of the family, VPS34 posses PI3-kinase activity and homologous proteins have been isolated in Drosophila M., C. Elegans and in mammals as well, where the protein is ubiquitously expressed(199–201). PtdIns is the molecular substrate of VPS34 and its catalytic product, PtdIns(3)P, is a molecule recognized by FYVE and PX domain-containing proteins. VPS34 is associated to VPS15, which posses an intrinsic serine/threonine kinase activity and is considered its regulatory subunit(202). Due to their cellular localization and physiological role, VPS34 and PtdIns(3)P have been often associated with the activation of mTORC1(203, 204), an atypical serine/threonine kinase multiprotein complex modulated by the cellular energy status and in particular by amino acids sensing. Unfortunately, due to the lack of specific molecular inhibitors and to the fact that PtdIns(3)P is not solely produced by class III PI3K but also by class II, the possibility of a cross-regulation/interaction need to still be investigated.

Pharmacological inhibition of PI3K's activity

As already mentioned in this manuscript, the development of molecular inhibitors of lipid kinase enzymes improved significantly the understanding of the role of the different lipid kinase family members. The first two molecular inhibitors of PI3K used in research were wortmannin(205, 206) and LY294002(207). Wortmannin, isolated from the filamentous fungus *Penicillium funiculosum*(208), does not show any antibacterial property but is able to inhibit PI3K activity with an in vitro IC_{50} in the nanomolar range(205). Wortmannin binds into the ATP-binding pocket of PI3K to a lysine residue (Lys802 in p110α, Lys833 in p110γ(209–211)). LY294002, a molecule derived by the flavonoid quercetin, is a less active compound than wortmannin with an IC_{50} value of around of 4μM(205, 212). Ly294002 targets the ATP-binding pocked of PI3K as well, without forming a covalent bond. Due to their molecular conformation, the targets of both compounds are not only PI3Ks family enzymes but also phosphatidylinositol 3-kinase related kinases (PIKKs) such as DNA-PKcs, mTOR, ATM and ATR(213–215).

In the last years a particular interest has been focused on the generation of isoform selective inhibitors (Figure 6). One example is the development of BYL719(216), a selective inhibitor of PI3Kα, whose gene sequence is mutated in many human cancers and generates an over-activation of the kinase activity. BYL719 is currently in clinical trial for the treatment of adult patients with advanced solid tumors and is also going to be studied in combination with other anti-neoplastic drugs for a broad range of cancer diseases. Another interesting example is the case of TGX221(217), an isoform selective PI3Kβ inhibitor, developed starting from the core structure of LY294002 and ameliorated to gain selectivity towards the p110β catalytic subunit. Indeed the development of TGX221 complements the reduced amount of information available about this isoform due to the lethal phenotype of PI3Kβ knock out animals. IC87114 is the first developed selective PI3Kδ inhibitor with an IC_{50} = 0.5 μM(218) and a 58-fold higher selectivity relative to PI3Kγ. IC87114 was first used to investigate the role of PI3Kδ in neutrophils activation(218). In the present manuscript we used NVS PI3-4, a selective PI3Kγ inhibitor(219, 220) for our experiments.

NVS-PI3-4 binds into the ATP-binding pocket of PI3Kγ and inhibits the fMLP-induced respiratory burst assay in human neutrophil with an IC_{50} = 0.22 μM; moreover the molecule shows a minimal inhibitory activity against protein kinases. Nevertheless, if the isoform selective PI3K inhibitors contributed to the understanding of the single molecular pathways, the use of the pan-PI3Ks inhibitors is gaining an increasing interest in clinical applications.

For instance, PX866 is an analog of wortmannin with irreversible PI3K inhibitor activity that is currently in clinical phase 2 for the treatment of different types of solid cancer (ClinicalTrial.gov). SF1126 is a pan-PI3K inhibitor, analog of LY294002 and is currently undergoing clinical phase I test for the treatment of advanced or metastatic solid tumors and relapsed or refractory neuroblastoma. NVP-BKM120(221) is a pan-class I PI3K inhibitor, currently in clinical trials, alone or in combination with other compounds, for the treatment of advanced solid tumors (ClinicalTrial.gov). In table 5 are indexed all the class I PI3K/mTOR inhibitors that are currently in clinical trials (November 2015, ClinicalTrial.gov).

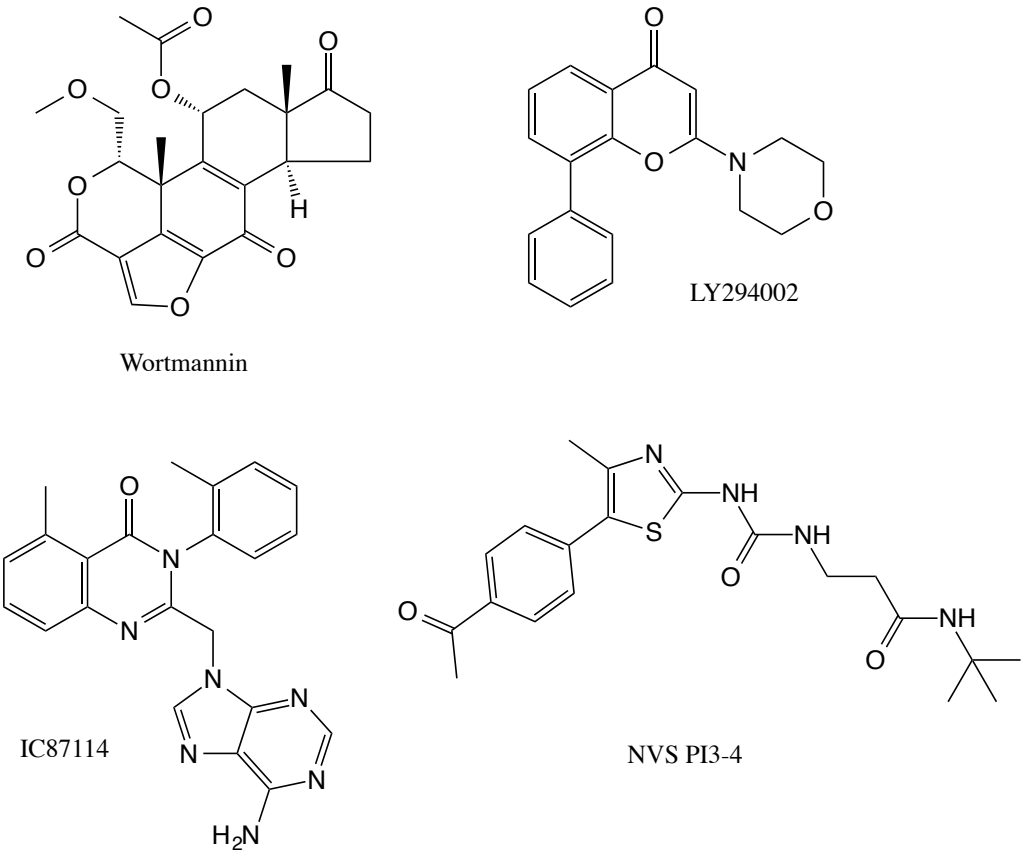

Figure 6: Molecular structures of the cited PI3K inhibit

Table 5. Class I PI3K/mTOR inhibitors in clinical trials

Target	Molecule	ClinicalTrials.gov Identifier	Stage phase	Indication	References
p110 α	BYL719	NCT02145312	Phase I	Advances solid tumors with PI3KCA mutations	Furet, P. 2013
	GDC-0032	NCT02285179	Phase I	Breast cancer	Wallin, J.J. 2013
	MLN1117	NCT01899053	Phase I	Advances solid tumors with PI3KCA mutations	So, L. 2013
p110 β	AZD6482	NCT00853450	Phase I	Antitrombotic therapy	Nylander, S. 2012
p110 δ	CAL-101, Idelalisib	FDA and EMA approved, 2014		Chronic lymphocytic leukaemia	Herman S., 2010; Lannutti, B. 2011
	GSK2269557	NCT02294734	Phase II	Respiratory disease	Down, K. 2015
	AMG 319	NCT01300026	Phase I	Lymphoid malignancy	Cushing, T.D., 2015
	INCB040093	NCT02456675	Phase II	Hodgkin lymphoma	www.incyte.com
	TGR-1202	NCT01767766	Phase I	Relapsed or refractory hematologic malignancies	www.tgtherapeutics.com
	GS-9901	NCT02258555	Phase I	Lymphoid malignancy	www.gilead.com
	PWT-143	NCT02521389	Phase I	Hematologic cancers	MEI pharma
	CDZ173	NCT02435173	Phase I	Genetic PI3K delta mutation	Novartis Pharmaceuticals
	UCB-5857	NCT02303509	Phase I	Psoriasis	www.ucb.com
p110 α / δ	AZD8835	NCT02260661	Phase I	Advanced solid malignancies	Barlaam, B. 2015
	BAY80-6946	NCT01660451	Phase II	Non-hodgkin's lymphoma	Glauer, J. 2013
	GDC-0941	NCT01437566	Phase II	Breast cancer	Shi, L. 2015
p110 β / δ	AZD8186	NCT01884285	Phase I	Prostate, lung and breast cancer	Barlaam, B. 2015
	GS-9820	NCT01705847	Phase I	Lymphoid malignancy	Shugg, R.P. 2013
p110 δ / γ	IPI-145	NCT01882803	Phase II	Non-hodgkin's lymphoma	Winkler, D.G. 2013
		NCT02004522	Phase III	Small lymphocytic lymphoma; CLL	
	RP-6530	NCT02017613	Phase I	Haematologic malignancies	www.Rhizen.com
	RV1729	NCT01813084	Phase I	Asthma/COPD	Norman, P. 2014

Pan-class I	BKM120	NCT01633060	Phase III	Metastatic breast cancer	Maira, S.M. 2012
	XL-147	NCT01587040	Phase I	Malignant neoplasm	Foster, P. 2015
	ZSTK474	NCT01682473	Phase I	Advanced solid malignancies	Yaguchi, S. 2006
Pan-class I/mTOR	BEZ235	NCT01453595	Phase I / II	Renal cancer	Maira, S.M. 2008
	BGT226	NCT00600275	Phase I / II	Solid tumors, breast cancer	Chang, K. 2011
	GSK2126458	NCT00972686	Phase I	Solid tumors	Knight, S.D. 2010
		NCT01725139	Phase I	Pulmunary fibrosis	
	VS-5584	NCT01991938	Phase I	Non hematologic cancers; metastatic cancer; lymphoma	Hart, S. 2013
	PF-04691502	NCT00927823	Phase I	Solid tumors	Yuan, J. 2011
	PF-05212384	NCT01420081	Phase II	Endometrial cancer	Tabernero, J. 2015
	PQR309	NCT02483858	Phase I	Advanced solid tumors	www.piqur.com
	DS-7423	NCT01364844	Phase I	Advanced solid tumors	Kashiyama, T. 2014
	GDC-0980	NCT01455493	Phase II	Endometrial carcinoma	Wallin, J.J. 2011
	XL-765	NCT01587040	Phase II	Malignant neoplasm	Papadopoulos, K.P. 2014
Pan PI3K	PX866	NCT01331083	Phase II	Metastatic prostate cancer	Koul, D. 2007
	CLR457	NCT02189174	Phase II	Advanced solid tumors	Novartis Pharmaceuticals
	SF1126	NCT02337309	Phase I	Neuroblastoma	Garlich, J.R. 2008

Phosphoinositide phosphatases

The phosphatidylinositol signalling regulates several aspects of the cellular homeostasis and is constantly modulated by two classes of enzymes: lipid kinases and phosphatases. The product of PI3K class I enzymes, PtdIns(3,4,5)P_3, is a fundamental intracellular second messenger and can be hydrolyzed by three inositol polyphosphate phosphatases: PTEN, SHIP1 and SHIP2. PTEN is a 3' poly-phosphatase that generates PtdIns(4,5)P_2, while SHIP1 and SHIP2 (Figure 7) are 5' poly-phosphatases and produce PtdIns(3,4)P_2.

SH2-containing inositol-5'-phosphatase 1 (SHIP1) is best known as the effector of the inhibitory signalling by FcγRIIb and is able to negatively regulates BCR(222), FcεRI(223) and TCR(224) signalling. The INPP5D gene, that produces SHIP1, was independently cloned in 1996 by three groups and the product of this gene was first described as a protein that becomes tyrosine phosphorylated and associates with the Shc protein upon cytokine or grow factors stimulation(225–227).

Presently, two mechanisms are known to be responsible for SHIP1 dependent inhibitory activity. On one hand, being a lipid phosphatase, SHIP1 is recruited via its SH2 domain at the plasma membrane where binds tyrosine-phosphorylated domains of several receptors(228) and hydrolyses the PtdIns(3,4,5)P_3 at the 5' position of the inositol ring, generating PtdIns(3,4)P_2; consequently, elimination of the plasma membrane PtdIns(3,4,5)P_3 prevents the recruitment and activation of several Pleckstrin Homology (PH) containing molecules such as phosphoinositide dependent kinase 1 (PDK1), Bruton's Tyrosine Kinase (Btk) and Phospholipase C-γ (PLCγ)(229). Interestingly, due to the different ability of PH-domain bearing proteins to bind both PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2, the phosphatase activity of SHIP1 can be considered a modulation of the PI3K signalling more then a termination. On the other hand, SHIP1 works as a docking unit and upon membrane localization recruits the adaptor molecule p26Dok1 that in turn recruits a not yet identified Ras GTPase activating protein (RasGAP) and leads to the inhibition of Ras and all the downstream signalling cascades including the Erk1/2 MAPK pathway(230, 231). As for PI3K family enzymes or PTEN, mutations in SHIP1 were detected in patients affected by acute

lymphoblastic leukemia and acute myeloid leukemia(232) and there are also evidences for development of erytroleukemia promoted by loss of SHIP1(233). Genetically modified mice lacking SHIP1(234) were vital but suffered from progressive splenomegaly, massive myeloid infiltration of the lung, wasting and, in some cases, a shortened lifespan. Nevertheless the generation of SHIP1 knock out mice gave a fundamental contribution to asses the importance of plasma membrane localised PtdIns(3,4,5)P_3 and its modulation (Table 4). Studies performed in BMMC lacking SHIP1 contributed to name this lipid phosphatase as the "gatekeeper of mast cells degranulation"(223). On the other hand, SHIP1 is dispensable for the generation of BMMC(223). Indeed, SHIP1 knock out BMMC are phenotypically indistinguishable at light microscopy observation from wild type BMMC and exhibit increased degranulation and cytokine secretion in response to IgE / FcεRI cross-linking compared with wild type cells (223). Moreover SHIP1 knock out BMMC response to SCF or monomeric IgE stimulation with aberrant degranulation unlike wild type cells(235–237).

Interestingly, mice lacking SHIP1 revealed also an immunoregulatory role for the 5' lipid phosphatase; an interesting study demonstrated that in mice lacking SHIP1 there is a significant expansion of a myeloid suppressor cell population in peripheral lymphoid tissues that protects mice against graft versus host disease (GVHD) induced by allogenic bone marrow transplantation(238). Indeed, transient silencing of SHIP1 expression in adult transgenic mice induces the expansion of a $Mac1^+Gr1^+$ cell population in the spleen and in the mesenteric lymphonodes that is able to suppress an allogenic T cell response following bone marrow transplantation and moreover increases the donor B lymphoid repopulation in the recipient. This result clearly shows that SHIP1 does not merely exert a "gatekeeper" activity within the cells, limiting the presence of PtdIns(3,4,5)P_3 at the plasma membrane, but, moreover, either by generating PtdIns(3,4)P_2 or by virtue of its docking activity, it regulates several, yet to be discovered, cellular pathways.

SHIP2 (Figure 6) is widely expressed in nonhematopoietic cells and is implicated in insulin signalling and control of obesity(239–241). It is encoded by a separate gene but still retains 65% homology with SHIP1 within the catalytic domain. Interestingly, BMMCs lacking SHIP2 show an enhanced antigen induced

degranulation and cytokine release in comparison with wild type cells but do not show any corresponding calcium influx or tyrosine phosphorylation of MAPKs although the antigen induced phosphorylation of PKB at serine 473 is enhanced(242).

The phosphatase and tensin homolog deleted on chromosome 10, PTEN lipid phosphatase is considered a physiological inhibitor of PI3K signalling. Characterised almost 20 years ago as a tumor suppressor gene(243–245), PTEN is a 403 amino acid protein that is structurally divided in 5 domains: a PIP2 binding domain, PDB, a phosphatase domain, a C2 domain, a carboxy-terminal tail and a PDZ-binding domain. PTEN hydrolyses the 3' positioned phosphate from $PI(3,4,5)P_3$ to generate $PI(4,5)P_2$(246) and is considered the most commonly mutated gene in human tumors. Indeed, loss of PTEN function leads to increased levels of PIP3 and following activation of PDK1 and PKB, promoting an uncontrolled cell growth and survival(247). Generation of PTEN knock out mice lacking only one allele provided evidences of its tumor suppressor ability(248) although homozygous knock out mice are embryonic lethal(249). Interestingly, in a mouse model with inducible deletion of PTEN, the absence of the phosphatase generates mast cells hyperplasia and increased the mast cells number in all the tissues as well as increased stimulus hypersensitivity and vascular permeability(250).

Table 6: Impact of SHIP1 gene targeting on leukocytes. Adapted from(251).

Cell type	Phenotype of SHIP1 KO
Basophils	SHIP1 KO mice show increased Th2 skewing due to increased IL-4 secretion from basophils (Kuroda et al. 2011)
B cells	Increased BTK membrane association; hyper-responsive to cross-linking of BCR; Loss of anergy, production of auto-antibodies (Bolland et al.1998; Helgason et al. 2000; O'Neil et al. 2011)
Dendritic cells	Enhanced survival and proliferation but impaired maturation; Reduced nitric oxide production; DC lacking SHIP1 suppress T cell proliferation; (Antignano et al. 2010)
Mast cells	Enhanced degranulation of BMMC, CTMC, and MMC; enhanced TLR expression; enhanced cytokine production and release in BMMC; (Huber et al. 1998; Kalesnikoff et al. 2002; Ruschmann et al. 2012)
Myeloid cells	Increased myeloid suppressor cell number; increased M2 macrophage skewing (indirect mechanism via increasedIL-4 secretion from basophils); Increased ratio of PtdIns(3,4,5)P3 to PtdIns(3,4)P2 on phagosomal membrane; decreased early NADPH oxidative activity in phagosomes; (Ghanash et al. 2004; Kuroda et al. 2009)
Natural killer cells	Deficient receptor repertoire; defective INFγ secretion; increase in periferal number; defective cytolytic function; (Fortenbery et al., 2010)
T cells	Increased regulatory T cell differentiation, decreased Th17development; enhanced Th1 differentiation and CD8 cytotoxic activity; decreased Th2 differentiation; (Tarasenkoet al., 2007)

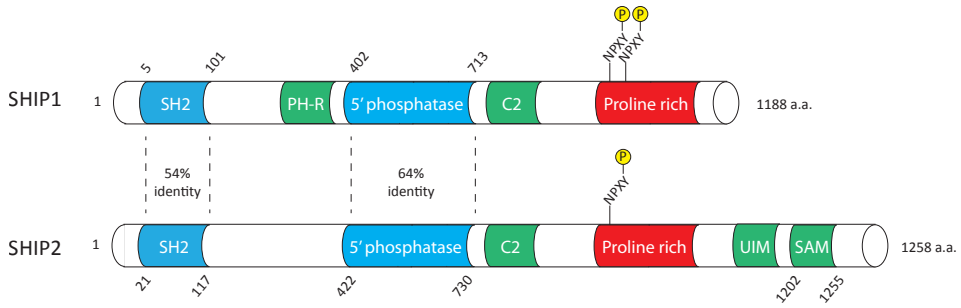

Figure 7: Domain organization of SHIP1 and SHIP2. SHIP1 presents an amino-terminal Src Homology 2 (SH2) domain(228) followed by a newly identified pleckstrin homology-related (PH-R) domain(252), a centrally located 5'-phosphatase domain, a C2 domain that binds PtdIns(4,5)P_2 and enhances SHIP1 catalytic activity(253). Two domains localized at the proline rich C-terminal region bind adaptor proteins such as Shc, Dok1(254) and Dok2(255) and are essential for SHIP1 functioning in BMMC and B cells(256, 257).

Lipid phosphatase activators and inhibitors

Due to their active role in modulating PI3K signalling, lipid phosphatases such as SHIP1, SHIP2 or PTEN have become an interesting target for pharmacological modulation of their enzymatic activity. Having a peculiar expression restricted to the hematopoietic lineage, SHIP1 has been identified as a potential pharmacological target for the treatment of diseases characterised by an over-active PI3K signalling in immune cells. The first molecule able to promote SHIP1 phosphatase activity was isolated by a marine invertebrate, *Dactylospongia elegans* collected in Papua New Guinea and was described in 2005 as "pelorol". In an *in vitro* assay, pelorol was able to enhance the SHIP1 dependent conversion of inositol-1,3,4,5-tetrakisphosphate (IP_4) to inositol-1,3,4-trisphosphate (IP_3)(258). Synthetic analogue of perol, AQX-MN100 and AQX-016A were then generated by Aquinox Pharmaceutical, Inc. and were characterised by the ability to bind the C2 domain of SHIP1(259). The compound AQX-151, used as well in the present manuscript, was further developed starting from the core of AQX-MN100 as a 500,000-fold more water-soluble molecule. AQX-151 inhibits the phosphorylation of PKB in stimulated MOLT-4 cells and has a dose dependent protecting effect on mouse passive cutaneous anaphylaxis (PCA) model(260). Currently, AQX-1125, developed by Aquinox Pharmaceutical, Inc., is in phase II clinical trial for the treatment of chronic obstructing pulmonary disease (COPD), bladder pain syndrome/interstitial cystitis and atopic dermatitis (Clinicaltrial.gov).

On the other hand, several attempts have been performed in order to inhibit the lipid phosphatase activity of SHIP1. The 3 alpha-aminocholestane (3AC) is currently the only known selective SHIP1 phosphatase inhibitor, having an IC50 of 10 μM and a high degree of selectivity over SHIP2 and PTEN lipid phosphatases(261) even though there are no details about the mechanism of action. In vivo testing of 3AC showed its ability to protect transplanted mice against allogenic GVHD but also to induce apoptosis in SHIP1 expressing leukemia and myeloma cell lines, suggesting that inhibiting SHIP1 phosphatase activity could either enhances allogenic transplantation or improve the treatment of hematologic tumors(261, 262). Interesting, three molecules, "1PIE",

"2PIQ" and "6PTQ", have been identified as potential pan-SHIP inhibitors (SHIP1 and SHIP2) and showed to be able to reduce the cell viability of SHIP2 expressing breast cancer cell lines, opening a novel interesting alternative for the treatment of breast cancer(263).

Together with the protecting role exercited by the SHIP1 and SHIP2 inhibitors, a similar result has been obtained by isolating molecules able to inhibit the PTEN phosphatase activity. Interestingly, since long time is known that vanadium compounds, such as orthovanadate, are able to inhibit a broad range of phosphatase enzymes(264), nevertheless the first compounds with a recognised specificity for PTEN were isolated in 2004(265). Among them, VO-OHpic, was the most potent and specific compound. In vivo and in vitro experiments performed in presence of VO-OHpic showed that PTEN inhibition induces a cellular senescence response without inducing any DNA damage and inhibits tumorigenesis in vivo, in a human xenograft model of prostate cancer(266) but also induces cardioprotection against ischemia-reperfusion injury(267) in mice and overcome the inhibitory effect of IC87114, a selective PI3Kδ inhibitor in a breast cancer cell lines(268).

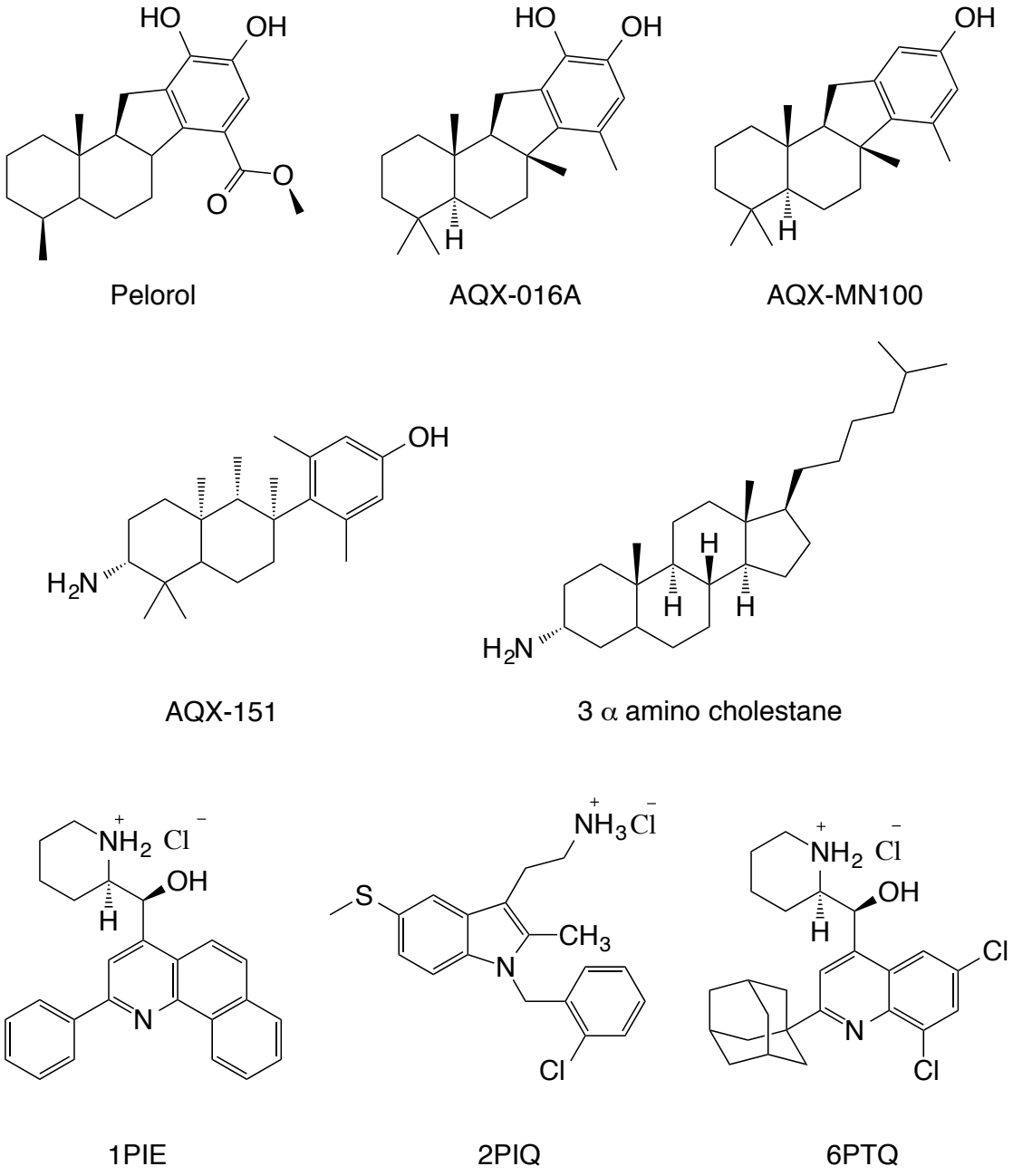

Figure 8: Molecular structure of the main lipid phosphatase modulators.

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Project I:

Dual targeting of SHIP1 and PI3Kγ for the inhibition of mast cell activation

Manuscript in preparation

Dual targeting of SHIP1 and PI3Kγ for the inhibition of mast cells activation

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Abstract

Background IgE induced activation of tissue-localized mast cells and circulating basophils is the triggering event generating type I hypersensitivity reactions. A fundamental step in IgE-mediated activation of mast cells is the recruitment of class I PI3Ks enzymes at the plasma membrane where they phosphorylate PtdIns(4,5)P_2 and generate PtdIns(3,4,5)P_3, an intracellular second messenger with fundamental signalling functions. On the other hand, the levels of PtdIns(3,4,5)P_3 are tightly modulated by the activity of lipid phosphatases such as SH2-containing inositol-5'-phosphatase-1, SHIP1, that hydrolyses PtdIns(3,4,5)P_3 and generates PtdIns(3,4)P_2, a molecule that is no longer a target for PI3K family enzymes.

Objective In the present work we tested two hypotheses: (i) PI3Kγ, a member of class IB PI3Ks activated upon GPCR and IgE / FcεRI stimulation, generates a pool of PtdIns(3,4,5)P_3 in bone marrow derived mast cells (BMMC) that is not directly hydrolysed by the 5'-phosphatase SHIP1. (ii) Dual pharmacological modulation of SHIP1 and PI3Kγ produces a synergistic inhibition of mast cells activation and therefore represents a promising strategy for the treatment of allergic diseases.

Results We have shown that AQX-151, an allosteric activator of SHIP1, inhibits the IgE-induced activation of BMMC but does not block the GPCR-mediated PI3Kγ activation induced by adenosine. The same result was obtained by stimulating SHIP1 phosphatase activity through co-clustering of the FcεRI and FcγRIIb in BMMC. Furthermore the treatment of BMMC with AQX-151 in combination with NVS PI3-4, a selective PI3Kγ inhibitor, blocks the IgE induced BMMC activation in a synergistic manner.

Conclusion With the present work we demonstrate that PI3Kγ signalling is able to bypass SHIP1 phosphatase activity and therefore reinforces the IgE-induced signalling in BMMC. By demonstrating the synergistic activity of AQX-151 and NVS PI3-4 in inhibiting BMMC activation we provide a proof of concept for a novel pharmacological combination for the treatment of allergic disease.

Introduction

Type I hypersensitivity reactions are initiated by the clustering of high affinity Immunoglobulin class E (IgE) receptors (FcεRI) expressed on the surface of mast cells or basophils and are characterized by an immediate cellular activation and a rapid tissue specific response(1). The FcεRI is a trans-membrane signalling complex composed of an α-chain that binds a single IgE molecule but does not have any signalling ability, two homologous disulphide-linked γ-chains, each containing one immunoreceptor tyrosine-based activation motif (ITAM) and one membrane-tetraspanning β chain with intracellular signalling function that presents an ITAM motif as well(2). Clustering of several α chains leads to the activation of the FcεRI and brings the Src family kinase Lyn, constitutively bound to the FcεRIβ, in close vicinity to the ITAMs of both β and γ-chains where it phosphorylates them(3, 4). The presence of phosphorylated ITAM domains allows the recruitment of SH2 bearing proteins at the plasma membrane, such as the protein tyrosine kinase Syk but also the recruitment of class IA PI3K adaptor subunits(5).

Plasma membrane localized class I PI3K enzymes phosphorylate PtdIns(4,5)P_2, a minor component of the phospholipidic membrane, at the 3' position and generate PtdIns(3,4,5)P_3. The presence of PIP_2 and PIP_3 at the plasma membrane modulates several cellular events such as gene expression, rearrangement of cytoskeletal structures, cell motility and endo- or exocytosis(5). Plasma membrane localized PtdIns(3,4,5)P_3 in turn recruits and promotes the activation of pleckstrin homology (PH) domain-containing proteins such as Phosphoinositide dependent kinase 1 (PDK1), Protein Kinase B (PKB), Bruton tyrosine kinase (BTK) and phospholipase Cγ (PLCγ). Active PLCγ mediates the cleavage of PtdIns(4,5)P_2 into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), the latter contributing to the generation of a massive Ca^{2+} influx that leads to the granule's content release in mast cells(6).

PI3Kγ is the sole class IB PI3K member and is mainly activated downstream G protein coupled receptors (GPCRs) stimulation(7),(8). Being a heterodimeric enzyme, PI3Kγ is composed by a catalytic subunit, p110γ and an adaptor subunit, p84 in BMMC. The p110γ/p84 complex has been described in the contest of

adenosine-mediated autocrine/paracrine reinforcement of antigen-induced BMMC activation(8); nevertheless there are evidences showing impaired antigen induced FcεRI signalling in absence of PI3Kγ. For instance, mice lacking p110γ are protected from IgE/antigen induced systemic and passive cutaneous anaphylaxis and show a reduced antigen-induced tissue-resident mast cells recruitment(9). Interestingly, it has been recently shown that PI3Kγ can also be activated in a GPRC-independent and adaptor subunit-free manner upon antigen induced BMMC stimulation(10). In this contest, FcεRI activation induces a Ca^{2+} influx that activates PKCβ which competes with p84 for the binding to p110γ and phosphorylates it at the Ser582, promoting its lipid kinase activity.

In the present work we demonstrate that GPCR-mediated activation of PI3Kγ induces a stimulus in BMMC that is not directly controlled by the SH2-containing inositol-5'-phosphatase-1, SHIP1, that is considered a physiological inhibitor of the PI3Ks activity. We tested our hypothesis by stimulating the phosphatase activity of SHIP1 in cells with an allosteric activator, AQX-151, and also by stimulating SHIP1 membrane recruitment via co-clustering of the FcεRI and FcγRIIb receptors. Both mechanism are able to promote SHIP1 activity and inhibit the IgE induced activation of mast cells but do not block the GPCR-mediated activation of PI3Kγ. Moreover, we tested the combination of the pharmacological SHIP1 activation and the inhibition of PI3Kγ in order to prevent the IgE mediated activation of BMMC; indeed, the combination of AQX-151 and NVS PI3-4, a selective PI3Kγ inhibitor, inhibits BMMC activation in a synergistic manner. In our opinion, the demonstration that PI3Kγ signalling is not directly influenced by the inhibitory modulation of SHIP1, suggest a potential cross-talk mechanism between lipid kinases and lipid phosphatases activity in immune cells. Moreover, the therapeutical combination of a molecular activator of the SHIP1 phosphatase activity and a selective inhibition of the PI3Kγ kinase activity is a promising pharmacological alternative for the treatment of IgE-mediated allergic disease.

Results

GPCR dependent PI3Kγ signalling is not inhibited by AQX-151 mediated activation of SHIP1.

The 5' phosphatase SHIP1 is a key regulator of the PtdIns(3,4,5)P_3 levels in mast cells and is considered a promising pharmacological target for the development of anti-inflammatory drugs due to its sole expression within the hematopoietic cell lineage(11). In the present work we test AQX-151 (Fig.S1A), a pelorol-derived compound able to promote SHIP1 catalytic activity(12) and we show its ability to inhibit bone marrow derived mast cells (BMMC) activation. The measurement of the release of β-hexosaminidase in BMMC is commonly considered as readout of the stimulus induced degranulation reaction, since β-hexosaminidase is contained within the same granules where histamine is stored(13, 14). Pre-incubation of IgE anti-DNP sensitized BMMC with AQX-151 inhibits, in a dose dependent manner, the release of β-hexosaminidase induced by the stimulation of the cells with an experimental antigen, DNP-HSA. AQX-151 does not have any evident off-target effect in BMMC lacking SHIP1(15) (Fig.1A). SHIP1 knock-out BMMC are characterised by a higher level of background β-hexosaminidase release in unstimulated cells compared to wild type BMMC and an hypersensitive degranulation response upon antigen stimulation (Fig.1B). IgE-induced BMMC activation can be, both *in vivo* and *in vitro*, reinforced by co-stimulatory molecules that activate different receptors on the cellular surface and promote PtdIns(3,4,5)P_3 production. Indeed, Stem Cells Factor (SCF), the ligand of cKIT, is known to directly promote PI3Kδ activation(16) whereas adenosine preferentially binds the A3 Adenosine Receptor (A3AR) and induces PI3Kγ activation in mast cells(8). We pre-incubated IgE-sensitized BMMC with AQX-151, stimulated them with DNP-HSA + SCF or Adenosine and measured mast cell degranulation. AQX-151 is able to inhibit the degranulation induced by IgE+Ag and IgE+Ag+SCF but does not block the adenosine-induced reinforcement of the degranulation response (Fig.1C). AQX-151 inhibits also the antigen-induced phosphorylation of PKB in IgE sensitised BMMC without having any inhibitory

effect on SHIP1 KO BMMC (Fig.1D, Fig.S1B and C). As a direct effect of SHIP1 activation, AQX-151 also inhibits the IgE-induced activation of the MAPK, most probably by recruiting DOK1 and a not yet identified RasGAPs(17). (Fig.1D). Both, adenosine and SCF stimulation of BMMC promote the activation and phosphorylation of PKB at the serine 473 by direct stimulation of PI3Kγ and PI3Kδ respectively and without the need of IgE mediated activation. In order to investigate the role of SHIP1 in such a process, IL3- and serum-starved WT BMMC were incubated with AQX-151 and stimulated with adenosine or SCF. Although AQX-151 inhibits the SCF induced activation of PKB, adenosine / PI3Kγ signalling is not affected by the activation of SHIP1 (Fig.1E). This demonstrates that SHIP1 is able to inhibit IgE and/or SCF induced activation of BMMC but does not affect the GPCR mediated / PI3Kγ mediated mast cell activation.

Figure 1

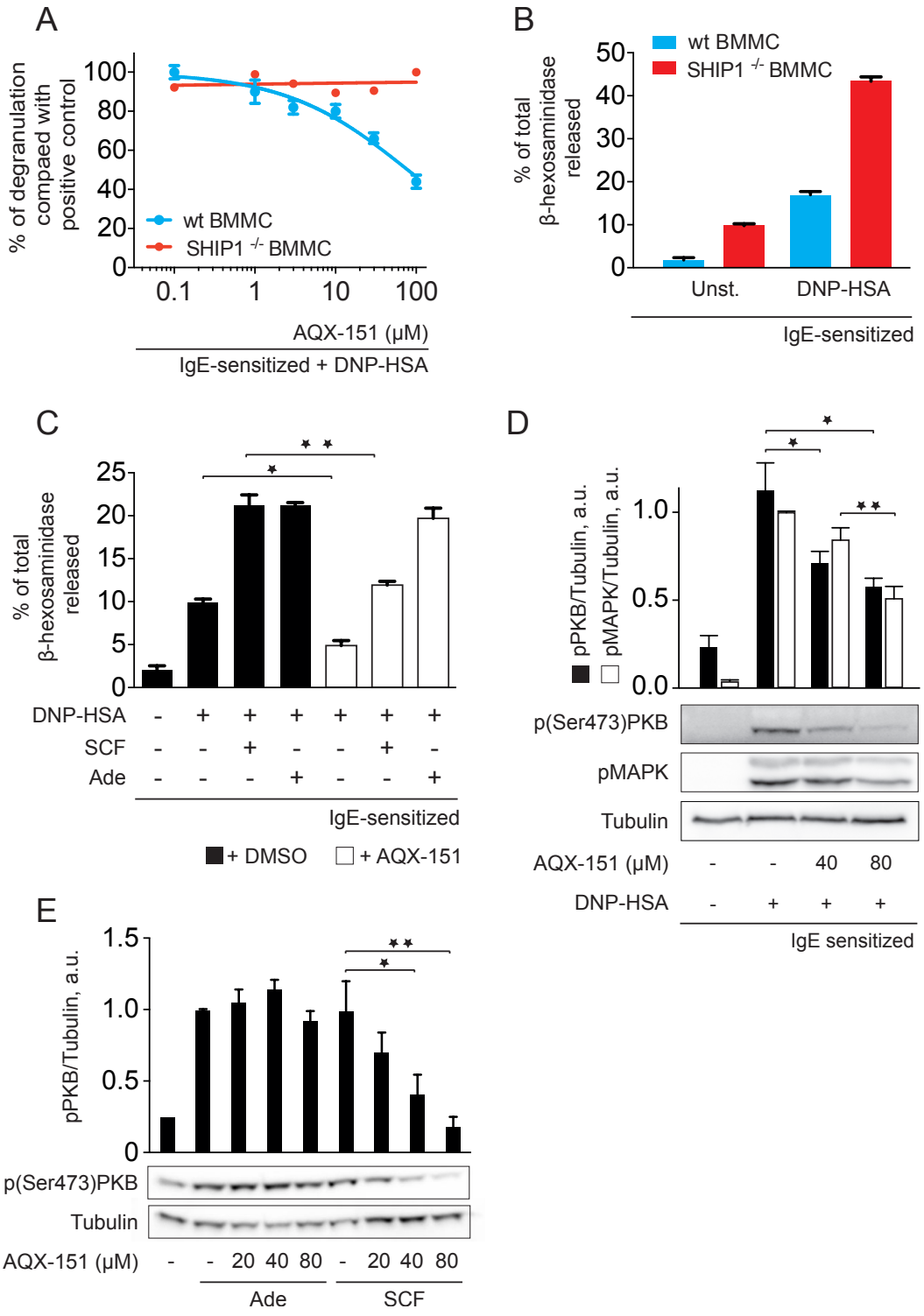
A
% of degranulation compaed with positive control
100
80
60
40
20
0
wt BMMC
SHIP1 -/- BMMC
0.1
1
10
100
AQX-151 (µM)
IgE-sensitized + DNP-HSA
B
wt BMMC
SHIP1 -/- BMMC
% of total β-hexosaminidase released
50
40
30
20
10
0
Unst.
DNP-HSA
IgE-sensitized
C
% of total β-hexosaminidase released
25
20
15
10
5
0
DNP-HSA - + + + + + +
SCF - - + - - + -
Ade - - - + - - +
IgE-sensitized
+ DMSO
+ AQX-151
D
pPKB/Tubulin, a.u.
pMAPK/Tubulin, a.u.
1.0
0.5
0.0
p(Ser473)PKB
pMAPK
Tubulin
AQX-151 (µM) - - 40 80
DNP-HSA - + + +
IgE sensitized
E
pPKB/Tubulin, a.u.
1.5
1.0
0.5
0
p(Ser473)PKB
Tubulin
AQX-151 (µM) - - 20 40 80 - 20 40 80
Ade
SCF

Figure 1: AQX-151 inhibits IgE-induced BMMC activation: (A) WT and SHIP1KO BMMC were sensitized overnight with IgE anti DNP-HSA (clone SPE-7, 0.1µg/ml) and incubated with different doses of AQX-151 for 1h before stimulating the cells with 10 ng/ml DNP-HSA (multivalent antigen) for 20 minutes. The degranulation reaction was calculated by detecting the β-hexosaminidase activity in the cell supernatant. Normalized analysis of the degranulation response for each genotype (B) WT and SHIP1 KO BMMC prepared like in (A). Not normalised analysis of the results (C) WT BMMC were prepared like in (A) and treated with 40 µM AQX-151 for 1hour; either 10 ng/ml SCF or 1 µM adenosine were added as co-stimulatory molecules immediately after DNP-HSA (n=3, mean ± SEM, ✻P < .05 and ✻✻P < .001). (D) IgE-sensitized BMMC were IL-3 starved in medium containing 2% FCS for 3h, then treated with the indicated doses of AQX-151 and stimulated for 5 minutes with 10 ng/ml DNP-HSA. Phosphorylation levels of PKB and MAPK were detected by western blotting and quantified using ImageJ. (n=2, mean ± SEM, ✻P < .05 and ✻✻P < .001). (E) WT BMMC were starved in medium containing 2% FCS for 3h then treated with 40 µM AQX-151 for 1h and stimulated with 10 ng/ml SCF or 1 µM Adenosine. Phosphorylation levels of PKB and MAPK were detected by western blotting and quantified using ImageJ (n=2, mean ± SEM, ✻P < .05 and ✻✻P < .001).

Anti Immunoglobulin-dependent SHIP1 recruitment inhibits IgE mediated BMMC activation but does not interfere with GPCR mediated PI3Kγ signalling.

Co-clustering of FcεRI and FcγRIIb in BMMC induces an inhibitory stimulation that physiologically prevents mast cells activation with a mechanism that involves SHIP1 recruitment at the plasma membrane and hydrolysis of PtdIns(3,4,5)P_3(18). The co-clustering of the two receptors can be induced by incubating IgE-sensitized BMMC with a polyclonal anti-immunoglobulin IgG antibody (Rabbit-Anti-Mouse IgG, RAM IgG, Fig.2A) and results in the activation of SHIP1(19). Such an effect is due to the presence of the Fc fragment of the IgG molecule that bind the FcγRIIb expressed of BMMC; indeed a truncated version of the same antibody lacking the Fc portion [F(ab')2 fragment, Fig.2A] induces clustering of the FcεRI without interacting with the FcγRIIb, and promotes a dose-dependent cell degranulation (Fig.2B, blue line).

Indeed, incubation of IgE sensitized BMMC with RAM IgG yielded no significant granule release over a broad range of concentrations in wild type cells (Fig.2B, blue dashed line) whereas SHIP1 knock-out BMMC readily degranulated in a concentration-dependent manner (Fig.2B, red line). Hence, incubation of IgE sensitized BMMC with the F(ab')2 fragment represent a stimulatory signal being able to induce the clustering of the IgE/FcεRI and leads to a dose dependent cellular degranulation. Incubation of IgE sensitized SHIP1 KO BMMC with either RAM IgG or the F(ab')2 fragment resulted in almost identical degranulation response (Fig.2B, red line and red dashed line). Pre-incubation of IgE sensitized wild type BMMC with RAM IgG reduced the BMMC degranulation promoted by the F(ab')2 fragment and similarly reduces the co-stimulatory signalling induced by SCF whereas adenosine stimulation was unperturbed in presence of RAM IgG (Fig.2C). Being a downstream target of PI3K activity, the phosphorylation of PKB is considered an indirect readout of the plasma membrane levels of PtdIns(3,4,5)P_3. The clustering of the FcεRI but also adenosine or SCF stimulations promote the activation of different receptors and different class I PI3Ks but all induce the activation and phosphorylation of PKB. Here we show that pre-incubation of IgE sensitized BMMC with a polyclonal Rabbit-Anti-Mouse

anti-immunoglobulin antibody (RAM IgG) reduces the F(ab')2 fragment induced phosphorylation of the serine 473 of PKB and the reduction persists by co-stimulating the cells with F(ab')2 + SCF whereas the stimulation of the cells with F(ab')2 + Ade is not inhibited by pre-incubation with RAM IgG (Fig.2D). Here we confirm our hypothesis that PI3Kγ signalling is not controlled by the SHIP1 phosphatase activity in BMMC.

Figure 2

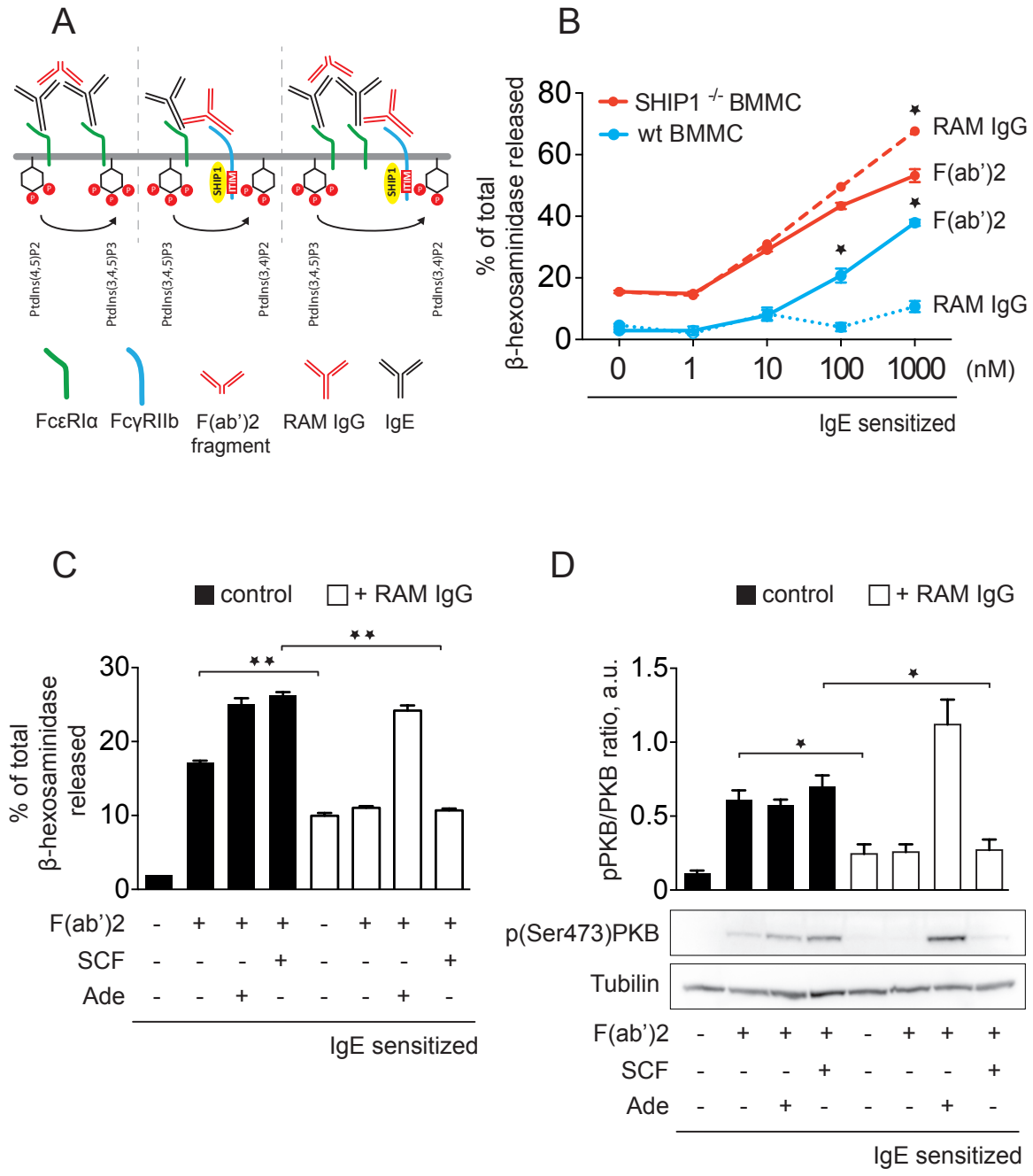
A
PtdIns(4,5)P2
PtdIns(3,4,5)P3
PtdIns(3,4,5)P3
PtdIns(3,4)P2
PtdIns(3,4,5)P3
PtdIns(3,4)P2
SHIP1
FcεRIα
FcγRIIb
F(ab')2 fragment
RAM IgG
IgE
B
% of total β-hexosaminidase released
SHIP1 -/- BMMC
wt BMMC
RAM IgG
F(ab')2
F(ab')2
RAM IgG
(nM)
IgE sensitized
C
control
+ RAM IgG
% of total β-hexosaminidase released
F(ab')2
SCF
Ade
IgE sensitized
D
control
+ RAM IgG
pPKB/PKB ratio, a.u.
p(Ser473)PKB
Tubilin
F(ab')2
SCF
Ade
IgE sensitized

Figure 2: Incubation of IgE-sensitized BMMC with anti Ig antibodies induces SHIP1 activation: (A) Schematic representation of F(ab')2 fragment and RAM IgG and their mode of action. FcεRIα is represented in green and FcγRIIb in blue. (B) WT and SHIP1KO BMMC were sensitized overnight with IgE anti DNP-HSA (clone SPE-7, 0.1µg/ml) and stimulated with equimolar doses of RAM IgG or F(ab')2 fragment for 20 minutes. The degranulation reaction was calculated from the β-hexosaminidase activity in the cell supernatant. A one-way anova test was performed within the wild type and within the SHIP1$^{-/-}$ sub-group, F(ab')2 vs. RAM IgG (n=2, mean ± SEM, ✻P < .05 and ✻✻P < .001). (C) WT BMMC were sensitized overnight with IgE anti DNP-HSA (clone SPE-7, 0.1µg/ml) and stimulated with equimolar doses (100 nM) of RAM IgG or F(ab')2 fragment for 20 minutes +/- SCF, 10 ng/ml or Adenosine, 1 µM (n=2, mean ± SEM, ✻P < .05 and ✻✻P < .001). (D) IgE-sensitized BMMC were incubated with 100 nM IgG for 10 minutes and then stimulated with 100 nM F(ab')2 fragment, SCF (10 ng/ml) or Adenosine (1 µM) for 5 minutes. Phosphorylation levels of PKB were detected by western blotting and quantified using ImageJ (n=2, mean ± SEM, ✻P < .05 and ✻✻P < .001).

Anti Ig-dependent SHIP1 recruitment protects BMMC from antigen-induced activation.

After we observed that incubation of IgE sensitized BMMC with RAM IgG reduces F(ab')2 fragment mediated BMMC activation (Fig.2B, C, D), we were wondering whether pre-incubation with RAM IgG also protects the cells from antigen stimulation. IgE-sensitized wild type BMMC were exposed to different concentrations of RAM IgG and stimulated with DNP-HSA, a multivalent antigen. Incubation with RAM IgG protects BMMC from antigen-induced degranulation (Fig.3A), limits the phosphorylation of PKB and MAPKs (Fig.3B) and moreover inhibits the *de novo* synthesis and release of pro-inflammatory cytokines such as IL-4, IL-6 and TNF-α (Fig.3C and Fig.S2B). Here we show that RAM IgG/ FcγRIIb-mediated recruitment of SHIP1 prevents the antigen-induced activation of BMMC and thus blocks the release of the granules content and inhibits the synthesis and the release of cytokines.

Figure 3

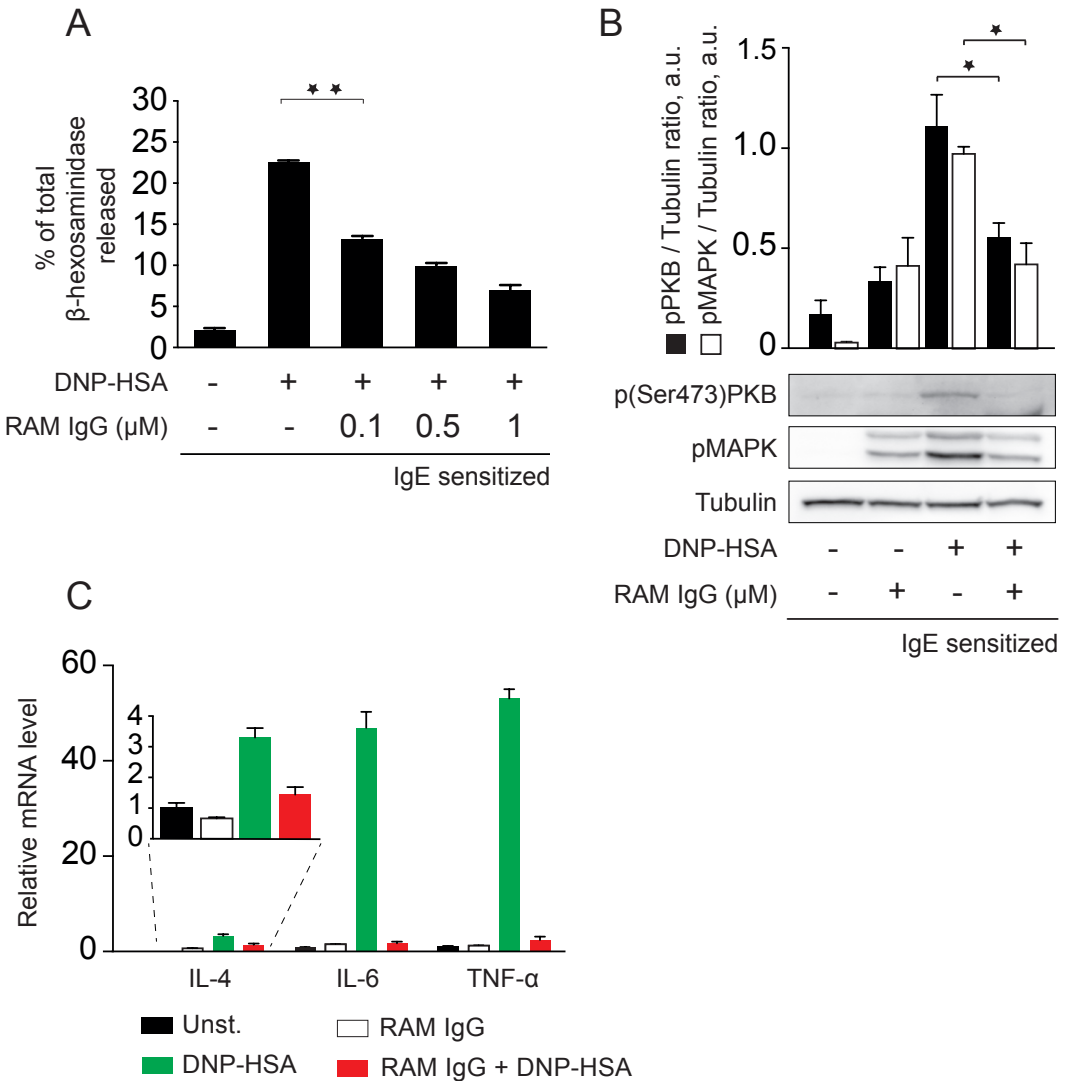
A
% of total β-hexosaminidase released
30
25
20
15
10
5
0
DNP-HSA - + + + +
RAM IgG (μM) - - 0.1 0.5 1
IgE sensitized
B
pPKB / Tubulin ratio, a.u.
pMAPK / Tubulin ratio, a.u.
1.5
1.0
0.5
0
p(Ser473)PKB
pMAPK
Tubulin
DNP-HSA - - + +
RAM IgG (μM) - + - +
IgE sensitized
C
Relative mRNA level
60
40
20
0
4
3
2
1
0
IL-4
IL-6
TNF-α
Unst.
DNP-HSA
RAM IgG
RAM IgG + DNP-HSA

Figure 3: Adenosine-induced BMMC activation is not counteracted by anti Ig antibodies-induced SHIP1 activation: (A) Overnight IgE-sensitized BMMC were incubated with the indicated dose of RAM IgG for 10 minutes and then stimulated with 10 ng/ml DNP-HSA for 20 minutes. The degranulation reaction was calculated by detecting the β-hexosaminidase activity in the cell supernatant (n=2, mean ± SEM, ❋P < .05 and ❋❋P < .001). (B) WT BMMC were sensitized overnight with IgE anti DNP-HSA (clone SPE-7, 0.1µg/ml), then incubated with 100 nM of RAM IgG and stimulated with 10 ng/ml DNP-HSA for 5 minutes. Phosphorylation levels of PKB were detected by western blotting and quantified using ImageJ (n=2, mean ± SEM, ❋P < .05 and ❋❋P < .001). (C) IgE-sensitized BMMC were incubated with 100 nM RAM IgG and then stimulated with 10 ng/ml DNP-HSA for 1 hour. The cells were lysed, RNA extracted followed by cDNA synthesis. The mRNA levels of IL4, IL6 and TNF-α were determined by qPCR; 18s mRNA served as an internal standard to generate calibration curves (n=3, mean ± SEM).

PI3Kγ is indispensable for IgE / F(ab')2 induced activation of BMMC.

PI3Kγ has a key role in the generation of PtdIns(3,4,5)P_3 downstream of IgE mediated stimulation(8, 9). In the present work we wanted to confirm the observation made in PI3Kγ knock out mice by inducing IgE sensitized BMMC activation with the F(ab')2 fragment of the anti Ig antibody. Wild type and PI3Kγ knockout BMMC, sensitized with monoclonal anti-DNP IgE, were incubated with different concentrations of polyclonal F(ab')2 fragment or complete RAM IgG and degranulation activity was monitored. In BMMC lacking p110γ, F(ab')2 fragment induced degranulation was reduced at all the range of concentrations compared to WT, with a statistically significant difference above stimulation with 10 nM (Fig. 4A). It is noteworthy that in PI3Kγ knockout BMMC no significant difference between the stimulation induced by the F(ab')2 fragment or by the RAM IgG was observed. The phosphorylation and activation of PKB induced by F(ab')2 fragment was compared and quantified in WT and PI3Kγ knockout BMMC. Here we show that in IgE sensitized BMMC the F(ab')2 fragment induced phosphorylation of serine 473 of PKB is significantly reduced in PI3Kγ knockout BMMC over a 2log concentration range of the F(ab')2 fragment in comparison with WT BMMC (Fig. 4C and E). The F(ab')2 induced cellular responses in IgE-sensitized WT BMMC were sensitive to a selective PI3Kγ inhibitor, NVS PI3-4 (Fig.S2C), with an IC_{50} of 0.95 μM for degranulation (Fig.4B) and an IC_{50} of 0.91 μM for the phosphorylation of PKB at serine 473 (Fig. 4D and F). This confirms previous data of our group and demonstrates that PI3Kγ has a central role in BMMC activation induced via antibody mediated clustering.

Figure 4

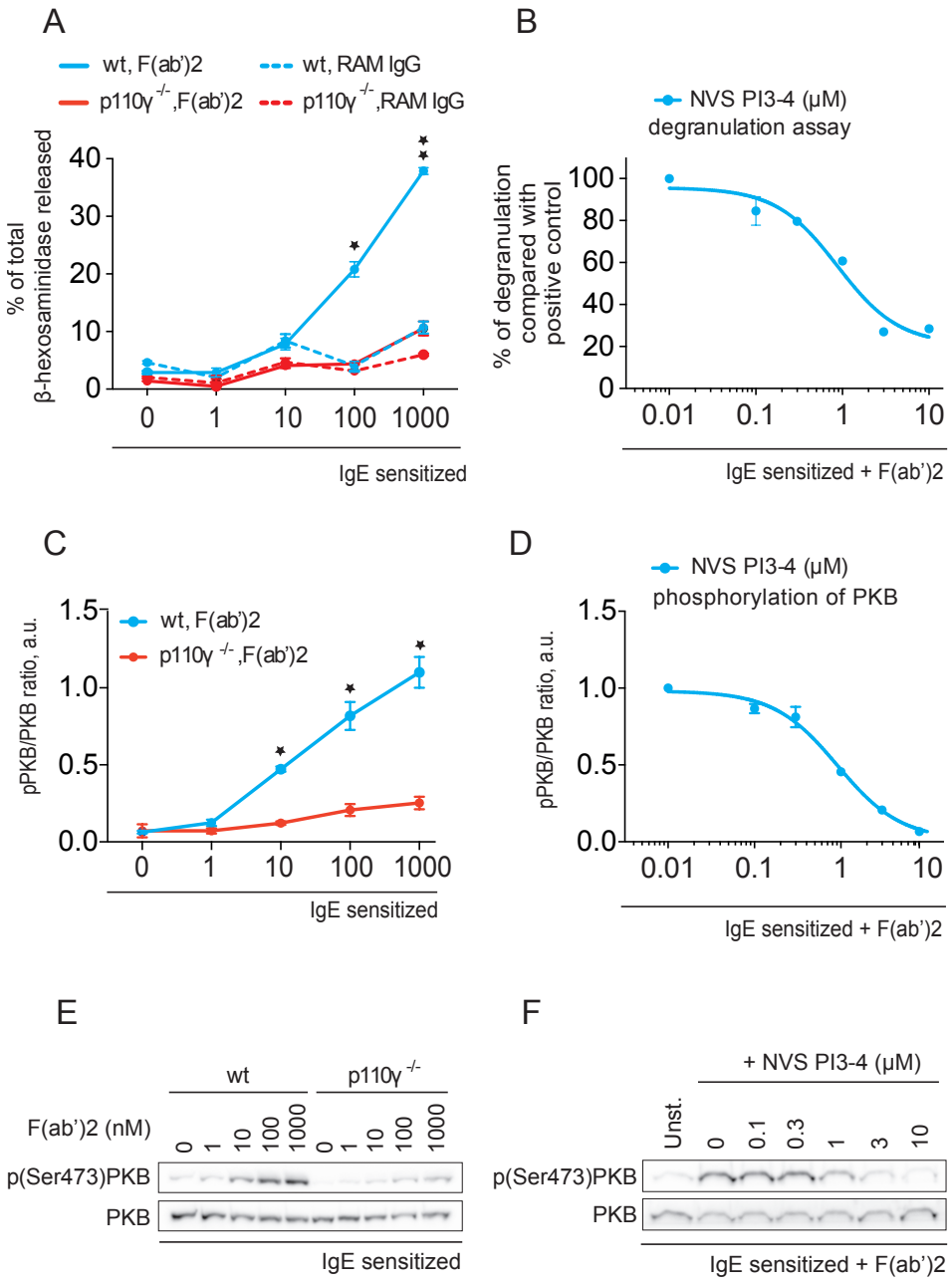
A
wt, F(ab')2
wt, RAM IgG
p110γ -/-,F(ab')2
p110γ -/-,RAM IgG
% of total β-hexosaminidase released
IgE sensitized
B
NVS PI3-4 (μM)
degranulation assay
% of degranulation compared with positive control
IgE sensitized + F(ab')2
C
wt, F(ab')2
p110γ -/-,F(ab')2
pPKB/PKB ratio, a.u.
IgE sensitized
D
NVS PI3-4 (μM)
phosphorylation of PKB
pPKB/PKB ratio, a.u.
IgE sensitized + F(ab')2
E
wt
p110γ -/-
F(ab')2 (nM)
p(Ser473)PKB
PKB
IgE sensitized
F
+ NVS PI3-4 (μM)
Unst.
p(Ser473)PKB
PKB
IgE sensitized + F(ab')2

Figure 4: PI3Kγ is indispensable for IgE / F(ab')2 fragment induced activation of BMMC: (A) WT and PI3Kγ KO BMMC were sensitized overnight with IgE anti DNP-HSA (clone SPE-7, 0.1µg/ml) and stimulated with equimolar doses of RAM IgG or F(ab')2 fragment for 20 minutes. Degranulation was followed by β-hexosaminidase activity. A one-way anova test was performed, F(ab')2 fragment stimulated wild type vs. F(ab')2 fragment stimulated p110γ knock out (n=2, mean ± SEM, ✻P < .05 and ✻✻P < .001). (B) Dose response curve for NVS PI3-4, degranulation reaction; WT BMMC were sensitized overnight with IgE anti DNP-HSA (clone SPE-7, 0.1µg/ml), incubated with different doses of NVS PI3-4 and stimulated with F(ab')2 fragment (100nM) for 20 minutes. Degranulation was followed by β-hexosaminidase activity. (C) IgE sensitized WT BMMC and PI3Kγ KO BMMC were IL-3 starved in medium containing 2% FCS for 3h and then stimulated with different concentrations of F(ab')2 fragment. Phosphorylation status of PKB was detected. The ratio pPKB/PKB has been quantified using ImageJ A one-way anova test was performed, F(ab')2 fragment stimulated wild type vs. F(ab')2 fragment stimulated p110γ knock out (n=2, mean ± SEM, ✻P < .05 and ✻✻P < .001). (D) Dose response curve for NVS PI3-4, PKB phosphorylation; WT BMMC were sensitized overnight with IgE anti DNP-HSA (clone SPE-7, 0.1µg/ml), were IL-3 starved in medium containing 2% FCS for 3h and incubated with different doses of NVS PI3-4. Phosphorylation status of PKB was detected. The ratio pPKB/PKB has been quantified using ImageJ (n=3, mean ± SEM). (E) Western blot figure showing one of the experiments quantified in figure C. (F) Western blot figure showing one of the experiments quantified in figure D.

Pharmacological combination of AQX-151 and NVS PI3-4 synergistically inhibits IgE/Ag induced BMMC activation

Both AQX-151 (activating SHIP1) and NVS PI3-4 (inhibiting PI3Kγ) modulate PtdIns(3,4,5)P_3 homeostasis and regulate the IgE mediated activation of BMMC. The autocrine/paracrine signalling via GPCR/PI3Kγ is however insensitive to AQX-151. Thus we tested the efficacy of the combination of the two drugs in order to assess a possible drug synergism for IgE / antigen induced degranulation or phosphorylation of PKB. In the degranulation assay, AQX-151 showed an IC_{50} value 12 times higher then NVS PI3-4 (11.1 μM versus 0.947 μM respectively) and 20 times higher for the phosphorylation of PKB (20 μM versus 1μM) (Table1). Hence we designed a constant ratio experiment using a 12/1 ratio of AQX-151 and NVS PI3-4 for the degranulation assay and 20/1 for the phosphorylation of PKB. The experimental data were then tested for drug synergy by calculating the Chou-Talalay Combinatorial Index (C.I.) with dedicated software that provides a quantitative assessment of the synergy. The CI depends on the fraction affected considered (e.g. Fa = 0.75 indicates 75% of inhibition) and determines whether the drug combination is synergistic (CI <1), additive (CI = 1), or antagonistic (CI >1). AQX-151 and NVS PI3-4 show synergism in inhibiting BMMC degranulation at low doses and the combination becomes not synergistic anymore at the higher doses used in the study (Fig.5A). For instance we can observe that for a Fa value of 0.49 (indicates 49% of inhibition), the CI value is 0.52, indicating a good level of synergism (CI<1 indicate synergism). The activation of PKB was synergistically inhibited at all drug combinations investigated (CI always lower then 1, Fig. 5B), with a tendency of having a highest synergistic effect at higher doses (Fa = 0.9, Fig. 5B and C). In addition, a Dose Reduction Index, DRI value, was calculated (as described in the Material and Methods section). DRI values >1 indicate a favourable combination of the two drugs whereas a DRI value <1 would be interpreted as an antagonistic combination. To achieve 50% of degranulation inhibition (Fa= 0.5), the combination of the two drugs required 2.32 times less PI3Kγ inhibitor and 19.3 times less SHIP1 activator in comparison to the single agent inhibition. Similar results were obtained for the inhibition of the phosphorylation of PKB (Table 2A and 2B). Here we show that the

pharmacological targeting of two different modulators of the PtdIns(3,4,5)P_3 homeostasis leads to a synergistic inhibition of IgE mediated BMMC activation.

Figure 5

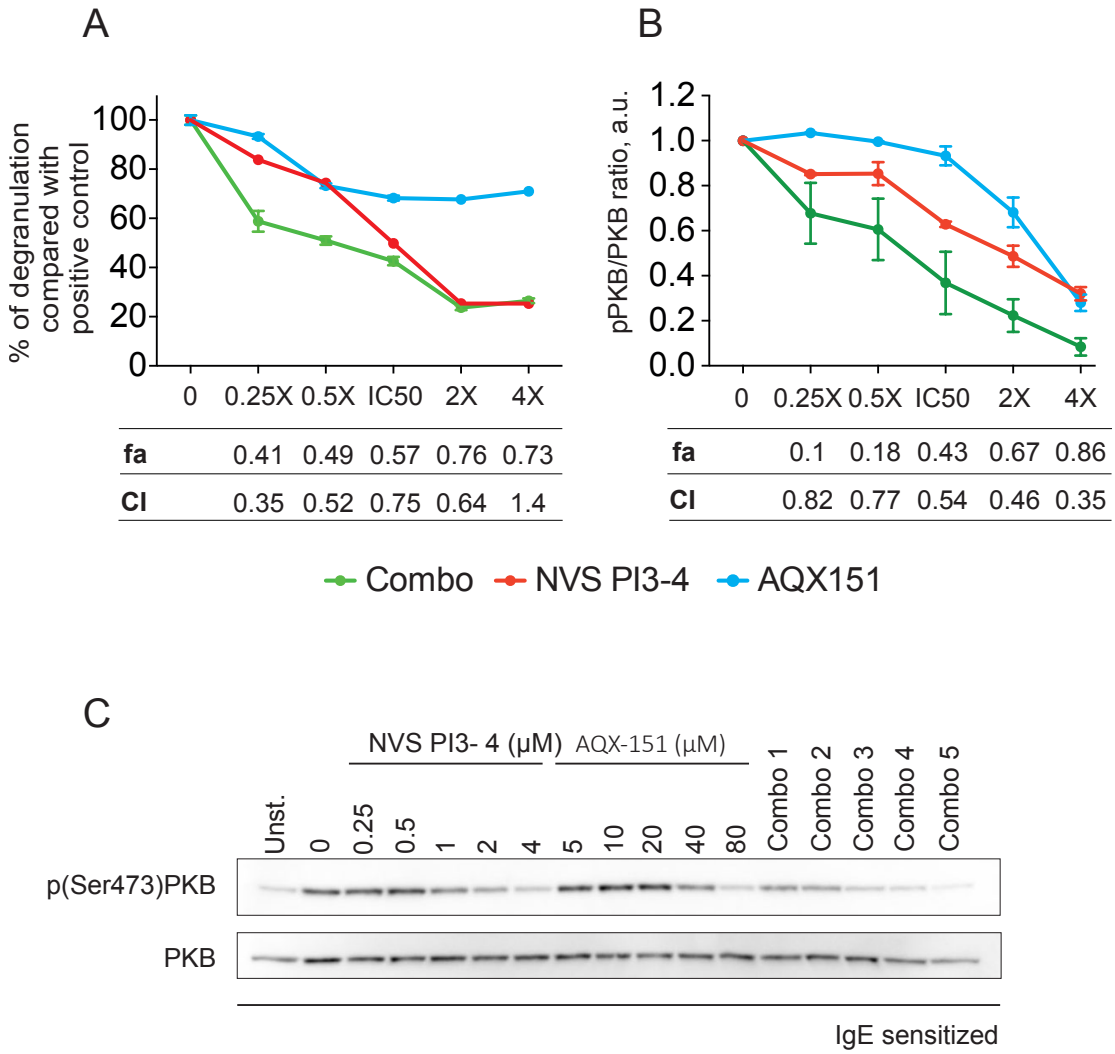
A
% of degranulation compared with positive control
100
80
60
40
20
0
0 0.25X 0.5X IC50 2X 4X
fa 0.41 0.49 0.57 0.76 0.73
CI 0.35 0.52 0.75 0.64 1.4
B
pPKB/PKB ratio, a.u.
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 0.25X 0.5X IC50 2X 4X
fa 0.1 0.18 0.43 0.67 0.86
CI 0.82 0.77 0.54 0.46 0.35
Combo
NVS PI3-4
AQX151
C
NVS PI3- 4 (μM)
AQX-151 (μM)
Unst.
0
0.25
0.5
1
2
4
5
10
20
40
80
Combo 1
Combo 2
Combo 3
Combo 4
Combo 5
p(Ser473)PKB
PKB
IgE sensitized

Figure 5: Combination of NVS PI3-4 and AQX-151 for the inhibition of IgE-induced BMMC activation: (A) WT BMMC were sensitized overnight with IgE anti DNP-HSA (clone SPE-7, 0.1µg/ml), incubated with the indicated concentration of drug and stimulated with 10ng/ml DNP-HSA for 20 minutes. Degranulation was followed by β-hexosaminidase activity. (B) WT BMMC were IL-3 starved in medium containing 2% FCS for 3h, then incubated with the indicated concentration of drug during last hour of starvation and then stimulated for 5 minutes with 10 ng/ml DNP-HSA. The ratio pPKB/PKB was quantified using ImageJ (n=2, mean ± SEM). (C) Example of one experiment quantified in (B). Fa is the fraction affected by the single doses of drug. Fa = 0.3, indicates that the given concentration of drug has an inhibitory effect of the 30%. CI, combinatorial index, indicates the synergistic effect of the combination of two drugs and is calculated at a given Fa value. CI = 1 indicates an additive effect; CI > 1, indicates antagonism; CI<1, indicates synergism.

Discussion

In this study we have shown that the GPCR-mediated activation of PI3Kγ induces a stimulation that is not directly inhibited by SHIP1 or by its plasma membrane recruitment and therefore can reinforces the IgE-mediated BMMC activation. Indeed, the recruitment of the SH2-containing 5'-lipid phosphatase (SHIP1) at the plasma membrane is one of the most characterised mechanisms that regulate the PtdIns(3,4,5)P_3 homeostasis(18, 20, 21) and, interestingly, there are several key receptors within the immune system that are known to recruit or to be regulated by SHIP1(22). Our results demonstrate for the first time that the GPCR-mediated activation of PI3Kγ generates a pool of PtdIns(3,4,5)P_3 that is not controlled by the SHIP1 inhibitory activity and thus can be considered a "long lasting stimulus" for the cells while the activation of PI3Kδ generates a signal that is readily hydrolysed by SHIP1(20). While one of the mechanisms that lead to the FcεRI-mediated recruitment of class IA PI3K members is clearly connected to the presence of a SH2 domain expressed in the p85 adaptor subunits(23–25), the direct activation of PI3Kγ downstream of FceRI has not been clearly demonstrated yet. Nevertheless, PI3Kγ signalling activity has been extensively studied since the identification of p110γ as a lipid kinase able to link the stimulation of GPCRs to the generation of PtdIns(3,4,5)P_3 in mammalian cells(26) and it has been recently reported that p110γ is activated upon antigen/IgE mediated stimulation, in a GPCR-independent manner, by PKCβ and does not associate with the adaptor subunit p84 since PKCβ compete with it for the binding with the catalytic subunit(10). In the present work we postulate an ideal separation between the signalling mediated by the activation of RTKs or TK-adaptor proteins, where the activation of class IA PI3Ks is balanced by SHIP1 plasma membrane recruitment and the signalling downstream of GPRCs that leads to the activation of PI3Kγ and the generation of a "wave" of PtdIns(3,4,5)P_3 that bypasses the "gatekeeper" ability of SHIP1. This ideal separation between RTK and GPCR signalling is of course useful for the investigation of the single pathways involved in mast cells activation but is certainly far from the physiological regulation of the events.

In the present work we used an experimental chemical activator of the SHIP1 phosphatase activity, AQX-151, a molecule developed by the group of R.J. Andersen(12), that allowed us to induce SHIP1 activation in BMMC without the need of any receptor-mediated stimulation. Of note, the present molecule has never been tested before in BMMC thus we are the first testing AQX-151 as an inhibitor of BMMC activation and degranulation.

The results obtained here demonstrate that upon antigen/IgE-mediated stimulation, the pool of PtdIns(3,4,5)P_3 generated by BMMC is sensitive to the SHIP1 phosphatase activity and thus readily hydrolysed upon pre-incubation of the cells with AQX-151. In our opinion this result does not indicate that PI3Kγ is not recruited upon FceRI activation but rather that, as already described(8), the GPCR-mediated activation of PI3Kγ is an autocrine and paracrine event that relays on a RTK-mediated generation of PtdIns(3,4,5)P_3 that is readily inhibited by the recruitment of SHIP1.

The pharmacological targeting of SHIP1, both by activating or inhibiting its catalytic activity is an interesting and promising strategy for the treatment of several immune system-related disorders such as asthma, allergic rhinitis or atopic dermatitis(27, 28) but could also lead to novel approaches for the treatment of haematological tumors or for the facilitation of allogenic transplantation(29, 30). Currently, AQX-1125 is the most promising allosteric SHIP1 activator developed by Aquinox pharmaceutics and is in clinical trial phase 2 for the treatment of Chronic Obstructive pulmonary disease (COPD) and bladder pain syndrome.

In the present work we also describe another way to promote a “physiological” activation of SHIP1 by inducing the co-clustering of the FcεRI and the FcγRIIb(31). This is a known inhibitory mechanism discovered in BMMC and B cells and is based on the ability of the FcγRIIb, the low affinity receptor for IgG, to recruit SHIP1 at the plasma membrane. In order to reproduce this mechanism *in vitro* we incubated IgE sensitized BMMC with a rabbit-anti-mouse anti immunoglobulin antibody (RAM-IgG) and we demonstrated that although this mechanism leads to the inhibition of the IgE/antigen mediated activation of BMMC, is not able to block the signalling induced by the GPCR mediated activation of PI3Kγ. Interestingly, a therapeutical approach based on the co-clustering of FcεRI and FcγRIIb has been

proposed by the group of A. Saxon that generated a polypeptide (GE2 peptide) able to induce the co-clustering of the two receptors and demonstrated its ability to inhibit IgE mediated reactivity in human cell lines and in mice(32). During the realization of the present project we were also interested in generating such a polypeptide in order to test our hypothesis but unfortunately due to the relevant size of the protein (60Kd) and to the difficulties of producing a secreted polypeptide without the presence of any immunoglobulin contaminant in the cellular medium, we did not succeed in our goal.

Here we also confirm the key role for PI3Kγ as a master regulator of BMMC activation. Indeed we show that BMMC activation can be induced by stimulating IgE-sensitized cells with an anti-Ig antibody fragment, F(ab')2, that cluster the IgE / FcεRI complexes without the need of an antigen. Even in the case of the F(ab')2 fragment stimulation, we observed a defective degranulation in BMMC lacking PI3Kγ and a strong reduction in the F(ab')2 fragment / IgE induced phosphorylation of the serine 473 of PKB. The same results could be further reproduced in wild type BMMC incubated with a selective PI3Kγ inhibitor.

Finally, after we proved that the phosphatase activity of SHIP1 is not able to block the GPCR mediated PI3Kγ signalling and we confirmed the key role of PI3Kγ in modulating BMMC activation, we tested AQX-151, the allosteric SHIP1 activator, in combination with NVS PI3-4, a selective PI3Kγ inhibitor for their ability to inhibit IgE mediated BMMC activation. Indeed we observed a synergistic inhibition of the BMMC activation both in terms of reduction of the antigen-induced cellular degranulation and the phosphorylation of PKB. To our knowledge this is the first report describing the use of a dual approach based on the molecular targeting of PI3Kγ and SHIP1 for the modulation of the IgE mediated mast cells activation. The achievement of a synergistic inhibitory effect obtained by the combination of the two compounds is extremely interesting for a future clinical application because allows a potential reduction of the doses of the single molecule employed in the therapy and reduces the risk of high dose dependent side effects.

In our opinion, the ability of different members of class I PI3K to generate different pools of PtdIns(3,4,5)P_3 that can be controlled or not by SHIP1 could be determined by two factors: either the different pools of PtdIns(3,4,5)P_3 are

generated in topological distinct regions of the plasma membrane where SHIP1 does not have access or there is a cross-regulation whose protagonists are the GPCR or some GPCR effector and SHIP1. An interesting view about the cross regulation of PI3Kγ activity and SHIP1 could be deduced by the work of Zhang et al(33), that demonstrated that SHIP1 can be phosphorylated by the cyclic AMP-dependent protein kinase (PKA) at the Serine 440, resulting in an increase of its catalytic activity. Here we could speculate that being the A3 adenosine receptor, A3AR, associated with a trimeric G protein (Gi/beta/gamma) known to inhibit cAMP generation(34), this could result in a lack of SHIP1activation of in presence of adenosine stimulation. Indeed, among the identified adenosine receptors (AR), A1, A2a, A2b and A3, all expressed in murine BMMC, A3AR seems to be the one involved in the adenosine-dependent activation of PI3Kγ(8).

Interestingly we could also speculate about a spatial topological separation between RTKs and GPCRs plasma membrane localization that could hamper the access of SHIP1 to the pool of PtdIns(3,4,5)P_3, since it has been demonstrated that PI3Kγ itself is able to generate two distinct pools of PtdIns(3,4,5)P_3 depending on whether p110γ is in complex with p84 or p101.

Conclusion: In the present work we demonstrate that the signalling induced by the GPCR mediated activation of PI3Kγ is not directly controlled by the phosphatase activity of SHIP1 and is able to reinforce the IgE mediated activation of BMMC. Although the therapeutical activation of the SHIP1 phosphatase activity is a valid approach and is currently under clinical phase testing, in our experiments we provide a proof of concept that the dual pharmacological targeting of SHIP1 and PI3Kγ could generate a novel powerful approach for the treatment of the IgE mediated allergic diseases.

Experimental procedures

Cell culture and mice

Bone marrow–derived mast cells (BMMCs) were differentiated from bone marrow of 8-12 weeks old C57BL/6J wild-type (wt) mice, p110$\gamma^{-/-}$ mice(35) and SHIP1 $^{-/-}$ mice(36) in IMDM^{comp} medium (+5 ng/mL murine stem cell factor [SCF] for 4 days) at 37°C in a 5% CO_2 atmosphere and subsequently cultured as described by Laffargue et al(8). PI3Kγ knock-out and SHIP1 knock-out mice were in the same genetic background of wild type mice and were used age matched from 8 to 12 weeks.

Antibodies and reagents:

Affinity-purified intact IgG and F(ab')2 fragment of polyclonal Rabbit anti Mouse Immunoglobulin (H+L) (RAM) from Jackson ImmunoResearch (West Grove, PA). Anti phospho-PKB antibody made in mouse was gently provided by Dr. S. Korur. Anti SHIP1 (P1C1) is a mouse monoclonal IgG_1 (Santa Cruz Biotechnology). Mouse monoclonal anti-activated MAP Kinase (MAPK-YT) antibodies were from Sigma-Aldrich. Adenosine, Dinitrophenyl-human serum albumin (DNP), mouse anti-DNP IgE (clone SPE-7) if not stated differently were from Sigma-Aldrich. Murine stem cell factor (mSCF) and murine IL-3 were from Peprotech. NVS-PI3-4 was obtained from Novartis Institutes for BioMedical research (Horsham, UK). AQX-151 was kindly provided by Prof. Raymond Andersen, University of British Columbia.

Bone marrow derived mast cells generation:

Bone marrow (BM) was obtained from decapitated femurs by centrifugation, and cells were re-suspended in IMDM containing 10% HIFCS, 2 mM L-Gln, 10 µg/ml PEST, 50 µM β-mercaptoethanol, additionally supplemented to 2 ng/ml recombinant murine IL-3 and 5 ng/ml murine SCF (only added once). Cells were cultured at 37°C, 5% CO_2 with re-addition of 2 ng/ml IL3 every second day. After 4 weeks, BMMC differentiation was monitored by the expression of c-Kit and FcεRI using flow cytometry. Subsequently, bone marrow-derived mast cells (BMMCs) were diluted weekly to 0.5×10^6 cells/ml maintaining 20% recycled

medium mixed with 80% fresh medium. After 4 weeks, BMMC differentiation was monitored by the expression of c-Kit and FcεRI using flow cytometry.

Beta-hexosaminidase release assay:

BMMCs were sensitized overnight with 100 ng/ml IgE (SPE-7). Concentration of 100ng/ml resemble the physiological levels of antigen specific IgE present in mouse and human sera after the exposure to parasites and allergens(37). Cells were washed twice and re-suspended at $2x10^6$ cells/ml in modified Tyrodes buffer. Where indicated, the cells were pre-incubated with RAM IgG for 10 minutes and degranulation was induced by the indicated DNP-HSA concentrations or by F(ab')2 fragment of anti Ig antibody. The reaction was stopped after 20 min by addition of 0.1 M $Na_2CO_3/NaHCO_3$. Plates were centrifuged at 4°C for 5 min and 100 µl aliquots (triplicate) of supernatant were transferred to 96 well plates. Beta-hexosaminidase activity in supernatants was assessed with *p*-nitrophenyl N-acetyl-β-D-glucosaminide (*p*-NAG) as a substrate, incubated 1h at 37°C and the reaction was stopped with 100 µl of 0.15 $Na_2CO_3/NaHCO_3$. The amount of p-nitrophenol generated was assessed by measuring the absorbance at 410 nm on a spectrophotometer (Spectramax 340). The amount of beta-hexosaminidase released was calculated by using the formula:

Release (R) [%] = $(A_{\text{each sample}} - A_{\text{background}}) / (A_{\text{total lysate}}) \times 100$

PKB phosphorylation assay:

BMMCs were IL-3 starved in IMDM growth medium containing 2% FCS for 3 hours in a 24-well plate and then stimulated for the indicated time. The cells were then moved on a Eppendorf tube and the stimulation was stopped on ice. Where indicated the cells were pre-incubated with RAM IgG for 10 minutes or with the indicated inhibitor for 1 hour and then stimulated. Stimulations were done at 37°C for the indicated times. The cells were immediately collected by centrifugation, spun for 1 min at 2000 g and lysed in 2x Laemmli sample buffer. Protein samples were then heated to 95°C for 8 minute, centrifuged, separated by SDS-PAGE and transferred to polyvinylidene fluorid (PVDF)-membranes (Millipore) by semi-dry transfer. Membranes were blocked with 5% (w/v) milk

powder (Migros) in TBS-T buffer [20 mM Tris/HCl, pH 7.6, 137 mM NaCl, 0.1% (v/v) Tween 20 (Fluka)]. Blots were incubated overnight at 4°C with the primary antibody, followed by incubation with horseradish peroxidase (HRP)-coupled secondary goat anti- mouse or anti-rabbit antibodies (Sigma). Protein bands were visualized by enhanced chemiluminescence (Millipore). Images were quantified with ImageJ 1.48v software (NIH) using the rectangle tool. Band pixel values were background corrected using an area above or below the selected band as background. Levels of protein phosphorylation were normalized to the total level of the protein.

Cytokine secretion

BMMCs were preloaded overnight with 100 ng/ml IgE, washed, re-suspended at $2x10^6$ cells/ml and stimulated for 1h at 37°C. Where indicated, WT BMMCs were incubated for 1 h with inhibitors or 0.1% DMSO before stimulations. The cells were harvested and the amount of cytokines in the cellular supernatant was measured by specific ELISA kits from R&D Systems according to the manufacturer's protocol.

Isolation of total RNA, RT-PCR, and qPCR

Total RNA was isolated from BMMC with RNeasy mini kit (Qiagen, cat. no. 74104) according to the manufacturer's protocol. cDNA was generated with 1 mg of total RNA with M-MLV reverse transcriptase (RT buffer and protocol, Invitrogen) and RNAsin (Promega) and stored at –80°C until ready for use. An ABPrism 7000 (Applied Biosystems, Foster City, CA) and SYBR GREEN PCR Master Mix (Applied Biosystems) were used for qPCR analysis of TNF-α, IL-6 and IL-4. 18s mRNA served as an internal standard to generate calibration curves.

PCR assays were performed in triplicate and fold induction was calculated against control-treated samples using the comparative Ct method (ΔΔCt).

Primers sequences used:

IL4 (mus musculus)

Forward 5'-TGTGCCAAACGTCCTCACA-3'

Reverse 5'-GCACCTTGGAAGCCCTACAG-3'

IL6 (mus musculus)

Forward 5'-ACAACCACGGCCTTCCCTACTT-3'

Reverse 5'-CACGATTTCCCAGAGAACATGTG-3'

TNF-alpha (mus musculus)

Forward 5'-ATCCGCGACGTGGAACTG-3'

Reverse 5'-CGAAGTTCAGTAGACAGAAGA-3'

Statistical Analysis

Numeric results were tested for significance using a two-tailed Student's t test, (paired or unpaired, as imposed by datasets). * and ** refer to p-values $p < .05$ and $p < .001$ respectively. Calculations were carried out using Graph Pad Prism 6 software.

Drug interaction analysis

Synergism between AQX-151 and NVS PI3-4 was determined by analysis of Combinatorial Index (CI) adapted from the median-principle method of Chou and Talalay(38, 39). Calcusyn 2.0 software (Biosoft, Ferguson, MO) was used for CI analysis(40)

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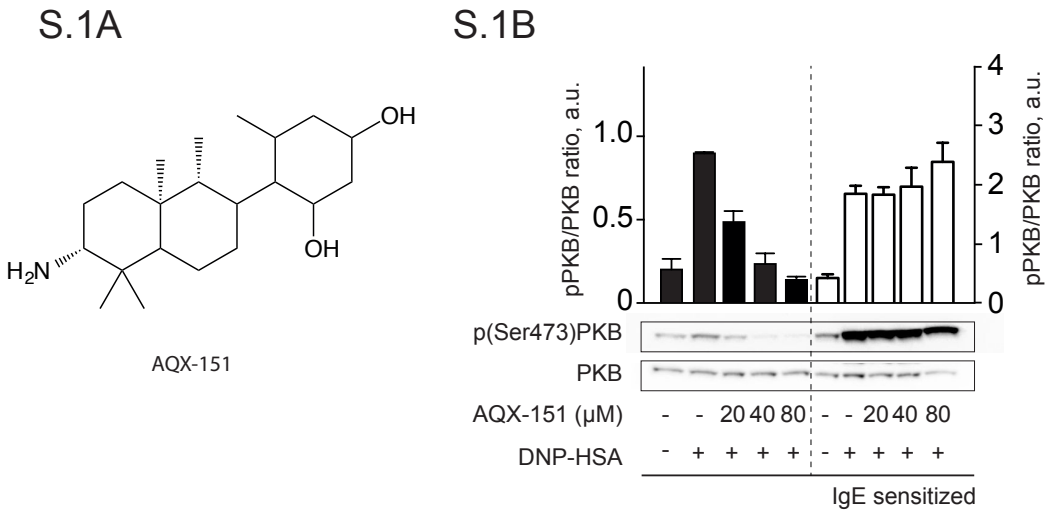
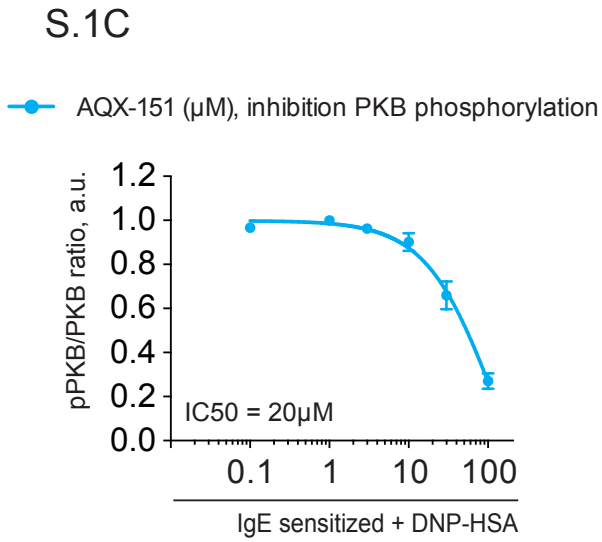
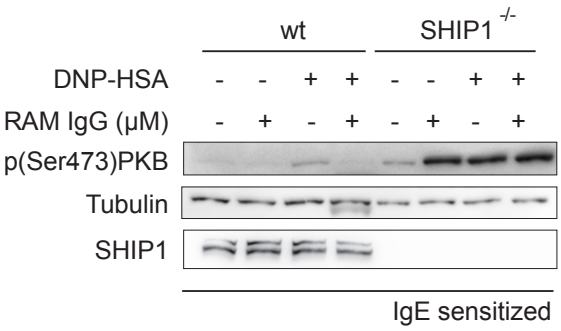
Supplementary figures





S1A: Molecular structure of AQX-151. S1B: IgE-sensitized WT and SHIP1 KO BMMC were IL-3 starved in medium containing 2% FCS for 3h, then treated with the indicated doses of AQX-151 and stimulated for 5 minutes with 10 ng/ml DNP-HSA. Phosphorylation status of PKB was detected by western blotting. The ratio pPKB/PKB was quantified using ImageJ (n=2, mean ± SEM). S1C: Dose response curve for AQX-151, PKB phosphorylation: WT BMMC were sensitized overnight with IgE anti DNP-HSA (clone SPE-7, 0.1µg/ml), were IL-3 starved in medium containing 2% FCS for 3h and incubated with different doses of AQX-151. The Phosphorylation status of PKB was detected by western blotting; the ratio pPKB/PKB was quantified using ImageJ (n=3, mean ± SEM).

S.2A



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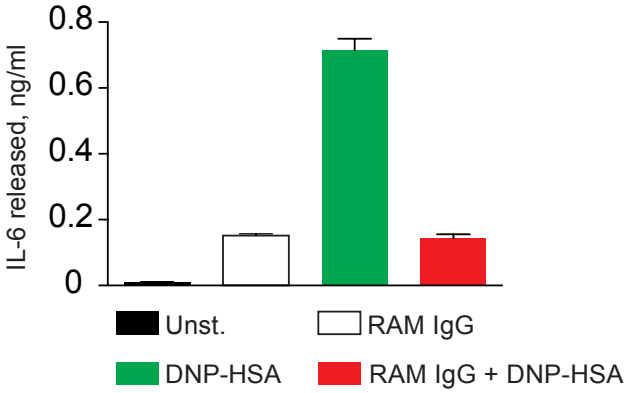
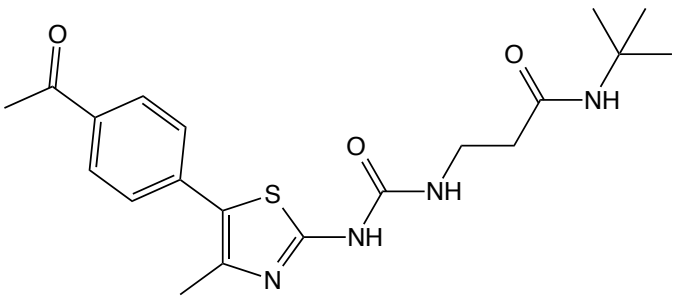


S.2C



NVS PI3-4

S2A: WT and SHIP1 KO BMMC were IL-3 starved in medium containing 2% FCS for 3h then incubated with 1µM RAM IgG for 10 minutes and then stimulated for 5 minutes with 10 ng/ml DNP-HSA. The ratio pPKB/PKB was quantified using ImageJ (n=2, mean ± SEM). **S2B:** Overnight IgE-sensitized BMMC were incubated with 1 µM RAM IgG for 10 minutes and then stimulated with 10 ng/ml DNP-HSA for 1 hour. The cell supernatant was then analysed by ELISA for the detection of the IL6 released. **S2C:** Molecular structure of NVS PI3-4.

Drugs concentration used for the calculation of the Combinatorial Index (CI)

	0.25X	0.5X	IC50	2X	4X	
NVS PI3-4 (μM)	0.23	0.47	0.95	1.9	3.8	Degranulation assay
AQX-151 (μM)	2.77	5.54	11.1	22.2	44.4	
NVS PI3-4 (μM)	0.25	0.5	1	2	4	Phosphorylation of PKB
AQX-151 (μM)	5	10	20	40	80	

Table 1. Concentration of the single compound used for the calculation of the Combinatorial Index. Degranulation assay and detection of the phosphorylation status of PKB were performed in presence of five different concentrations of the single compound and in combination. It is calculated as CI = $D_1/(Dx)_1 + D_2/(Dx)_2$, where $(Dx)_1$ is the dose that give an "X" effect when is in combination and D_1 is the dose of the single drug needed for obtaining the same effect. CI = 1 indicates an additive effect; CI > 1, indicates antagonism; CI<1, indicates synergism.

A) Dose reduction index (DRI), degranulation assay

Fa	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
NVS PI3-4	14.906	7.503	4.755	3.271	2.321	1.647	1.133	0.718	0.361
AQX-151	16.082	17.206	17.996	18.671	19.313	19.976	20.725	21.677	23.192

B) Dose reduction index (DRI), phosphorylation of PKB

Fa	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
NVS PI3-4	1.7	1.88	2.02	2.14	2.25	2.37	2.51	2.69	2.99
AQX-151	2.95	5.04	7.2	9.63	12.59	16.46	22.04	31.46	53.75

Table 2. Values obtained by computational calculation of the DRI, dose reduction index, for the degranulation assay and for the phosphorylation of PKB. DRI is the dose reduction possible per each drug to obtain a given effect in a synergistic combination. It is calculated as DRI = $(Dx)_1 / D_1$, where $(Dx)_1$ is the dose that give an "X" effect when is in combination and D_1 is the dose of the single drug needed for obtaining the same effect. If DRI >1, it allows a dose-reduction.

Project II:

Blocking mast cell activation with Ras inhibitors – moving toward cell-specific PI3Kγ targeting

Manuscript in preparation

Blocking mast cell activation with Ras inhibitors – moving toward cell-specific PI3Kγ targeting.

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Abstract

Phosphoinositide 3-kinase gamma (PI3Kγ) plays a central role in cellular mechanisms underlying inflammation, and, therefore, is regarded as an attractive drug target for treating inflammatory disorders. However, strategies that allow cell-type specific modulation of PI3Kγ activity are still missing. PI3Kγ is a heterodimer composed of p110γ catalytic subunit and one of the two possible adaptor proteins – p84 or p101. p84 and p101 are differentially expressed in various cell types and have partially non-redundant functions. They promote the generation of phosphatidylinositol (3,4,5)-trisphosphate at spatially distinct membrane domains, thereby eliciting specific cell responses. Furthermore, small GTPase Ras is indispensable for activation of p84/p110γ, but not p101/p110γ complexes. Here we present a proof of concept approach for mast-cell specific PI3Kγ targeting using Ras inhibitors. Mast cells are primary effector cells in inflammation and allergy. Targeting mast cell activation is therefore a highly efficient strategy to attenuate the progression of these diseases. We demonstrate here that Ras inhibition affects PI3Kγ-dependent functions of mast cells, such as the activation of protein kinase B (PKB/Akt), migration, cytokines expression, and the release of histamine containing granules. PI3Kγ-dependent macrophage activation and migration was, however, maintained under these conditions. The observed differential dependence on Ras was shown to be mainly determined by the p110γ adaptor subunit. Altogether, our results support non-redundant physiological roles of p110γ adaptors, demonstrate the importance of Ras in mast cell activation, and show that the modulation of Ras-PI3Kγ interactions opens novel routes for treatment of allergic and inflammatory diseases.

Introduction

Leukocytes chemotaxis, release of inflammatory mediators during mast cells degranulation or reactive oxygen species (ROS) formation by neutrophils represent some of the most important defence mechanisms of the immune system that are tightly controlled by the activation of phosphoinositide-3 kinase gamma (PI3Kγ) (1-3). Being a central enzyme in innate and adaptive immune responses, PI3Kγ is also implicated in various inflammatory disorders. Mice lacking PI3Kγ activity are resistant in models of rheumatoid arthritis (4), allergic asthma (5), glomerulonephritis and systemic lupus (6) as well as anaphylaxis (2, 7), cardiovascular disease (8), and diet-induced obesity (9). PI3Kγ therefore represents a highly potential therapeutic target in inflammatory disease, cardiovascular disorders and obesity. PI3Kγ is highly expressed in hematopoietic tissues and cells (10-13), but is also present in other cells, including cardiomyocytes, vascular smooth muscle and endothelia (14, 15). Broad PI3Kγ inhibition could therefore potentially be associated with various adverse effects including decreased host defence (16, 17). The possibility of cell type-specific PI3Kγ targeting, that will allow alleviation of pathological condition without general suppression of host immune system, is therefore of great value.

PI3Kγ is the only member of Class IB PI3Ks. It is generally considered to be activated downstream of G protein-coupled receptor (GPCR) (12, 13, 18), although a GPCR-independent PKCβ-mediated PI3Kγ activation has recently been described (19). PI3Kγ acts as a heterodimer between a catalytic p110γ subunit and one of two possible adaptor proteins – p84 (also called $p87^{PIKAP}$) or p101 (12, 13, 18). We previously showed that adaptor proteins are essential to transduce signals from GPCRs to PI3Kγ (10). Some of the functions of adaptor subunits are overlapping, as both proteins were able to promote PKB/Akt phosphorylation and migration of mast cells. However, p101 and p84 also possess adaptor-specific physiologic functions. We identified distinct pools of PtdIns(3,4,5)P_3 at the plasma membrane emerging from the two PI3Kγ/adaptor subunit complexes. These pools display a differential sensitivity to cholesterol depletion, and their capacity to promote release of mast cell granules (10). Adaptor-specific responses were also described in neutrophils (20, 21), where p101 played a key role in cell

migration, while p84 was essential for ROS production upon chemoattractant stimulation. Moreover, adapter proteins are not equally distributed among hematopoietic cells. While lymphocytes, neutrophils and macrophages possess mainly p101 protein, p84 is the only adapter subunit in mast cells (10, 12, 13). Finally, a further distinction between adaptor subunits was revealed by the analysis of the role of Ras in the activation of the PI3Kg complexes (22). Whereas p101/p110γ is recruited and stimulated by Gβγ subunit of GPCRs and does not require Ras to be operational, Ras is indispensable for membrane recruitment and activation of the lipid kinase in the p84/p110γ complex. Differential involvement of Ras opens new opportunities for targeted regulation of two PI3Kγ complexes that could have a great impact on deciphering novel ways to specifically control distinct cell responses.

In the presented study we exploited Ras-PI3Kγ interactions as a proof-of-concept approach for a cell-specific regulation of PI3Kg activity. We show that in cells with an abundance of the p101 adaptor protein, such as monocytes/macrophages, PI3Kγ-triggered outputs are resistant to Ras inactivation. On the other hand, in cells with a predominant expression of p84 adaptor subunit, like mast cells, PI3Kγ-dependent responses are susceptible to modulation of Ras signalling. All main steps of mast cell activation, including migration, cytokines production and degranulation are affected by inhibition of Ras. Mast cells are primary effector cells in allergic response. Our results, therefore, suggest that Ras inhibition might represent a novel strategy for treating allergic disease without compromising host defence mechanisms. N- and H-Ras isoforms are activated downstream of GPCR, and therefore both could be implicated in PI3Kγ signalling in mast cells.

Results

FTI-277 affects PI3Kγ-dependent signalling in mast cells, but not in macrophages

To evaluate the possibility of differential cell-dependent Ras involvement in the PI3Kγ signalling, we decided to block Ras activation by using farnesyltransferase inhibitors FTI-277. Farnesyltransferase inhibitors (FTIs) were originally developed as anti-Ras compounds for cancer therapy. They block Ras farnesylation that is required for its translocation to the plasma membrane, thus inhibiting proper function of Ras (23-25). FTI-277 indeed caused relocalization of GFP-tagged H-, N-, and K-Ras from plasma membrane to the cytosol in mast cells (fig. S1A). The effect was more profound in case of H-Ras isoform. The remaining low-level plasma membrane localization of N- and K-Ras could be explained by the possibility of alternative geranylgeranylation of these isoforms (26).

We compared the action of FTI-277 on PI3Kγ activation in bone marrow-derived mast cells (BMMC) and bone marrow-derived macrophages (BMMØ). Both cell types were treated with 5μM FTI-277 for 72 hours, and different PI3Kγ-dependent responses were analysed. We first measured the effect of FTI-277 on the activation of the main PI3K downstream target - protein kinase B (PKB/Akt). To distinguish between PI3Kγ-dependent and non-PI3Kγ-dependent responses cells were stimulated with either GPCR ligands (adenosine (Ade) in case of mast cells and complement component 5a (C5a) in case of macrophages) or with receptor tyrosine kinase (RTK) ligands (stem cell factor (SCF) for mast cells and macrophage colony-stimulating factor (M-CSF) for macrophages). In mast cells FTI-277 led to a significant decrease in PKB phosphorylation at Ser473 upon activation with Ade, but had no effect on PKB activation upon stimulation with SCF, demonstrating that inhibitory FTI-277 action is specific for PI3Kγ (Fig. 1A). The FTI-277 effect on mast cells was time-dependent, with increased inhibitory response upon longer incubation with the compound (fig. S1B). In contrast to mast cells, Ras inhibition did not affect neither C5a-, nor M-CSF- induced activation of PKB in macrophages (Fig. 1B).

Leukocytes migration toward the site of injury or infection represents a key step in the innate immune response, as well as in the progress of acute and chronic

inflammation. Chemoattractant-mediated leukocyte recruitment has been shown to be largely dependent on the presence of functional PI3Kγ (1, 7). We therefore next assessed the effect of Ras inhibition on the ability of mast cells and macrophages to migrate *in vitro* (Fig. 1C,D). In line with the results for PKB phosphorylation, only Ade-stimulated migration of mast cells was significantly impaired by FTI-277 (Fig. 1C). Macrophages migration toward GPCR agonists C5a or Rantes, as well as RTK ligand M-CSF remained intact upon treatment with the inhibitor (Fig. 1D and fig. S2). To verify that the absence of FTI effect on macrophages is not explained by the inability of the inhibitor to enter the cell or stay inside the cell, we checked accumulation of unfarnesylated prelamin A, one of the markers for farnesyltransferase inhibition (27). BMMC and macrophages demonstrated similar increase in the level of prelamin A, showing that under conditions used FTI-277 efficiently blocks protein farnesylation in both cell types (fig. S3A). Finally, we analysed whether Ras inhibition affects the ability of mast cells to produce cytokines and to release inflammatory mediators during degranulation. FTI-277 significantly decreased expression of TNFa and IL6 in mast cells stimulated with IgE/antigen and Ade (Fig. 1E) and attenuated degranulation response in IgE/antigen- activated cells upon co-stimulation with Ade, but not with SCF (Fig. 1F).

Gamma subunit of heterotrimeric G-protein can be isoprenylated with either farnesyl- or geranylgeranyl- moiety (28). To exclude the possibility that the observed inhibitory action of FTI-277 on PI3Kγ signalling is a result of impaired Gγ processing and functioning, we stimulated mast cells with another GPCR ligand - platelet activating factor (PAF). PAF acts through Gaq subunit and triggers cyclic AMP-responsive element-binding protein (CREB) phosphorylation. Treatment with FTI-277 had no effect on CREB phosphorylation in mast cells and macrophages (fig. S3B), showing that G-protein activity remains intact.

We therefore have shown that although FTI-277 efficiently blocks protein farnesylation in mast cells and macrophages, it impairs PI3Kγ signalling in mast cells only. All main steps of PI3Kγ-dependent mast cell activation – phosphorylation of PKB, migration, cytokines production and degranulation – were affected by Ras inhibition. Macrophages responses, however, remained intact upon treatment with FTI-277.

Figure 1

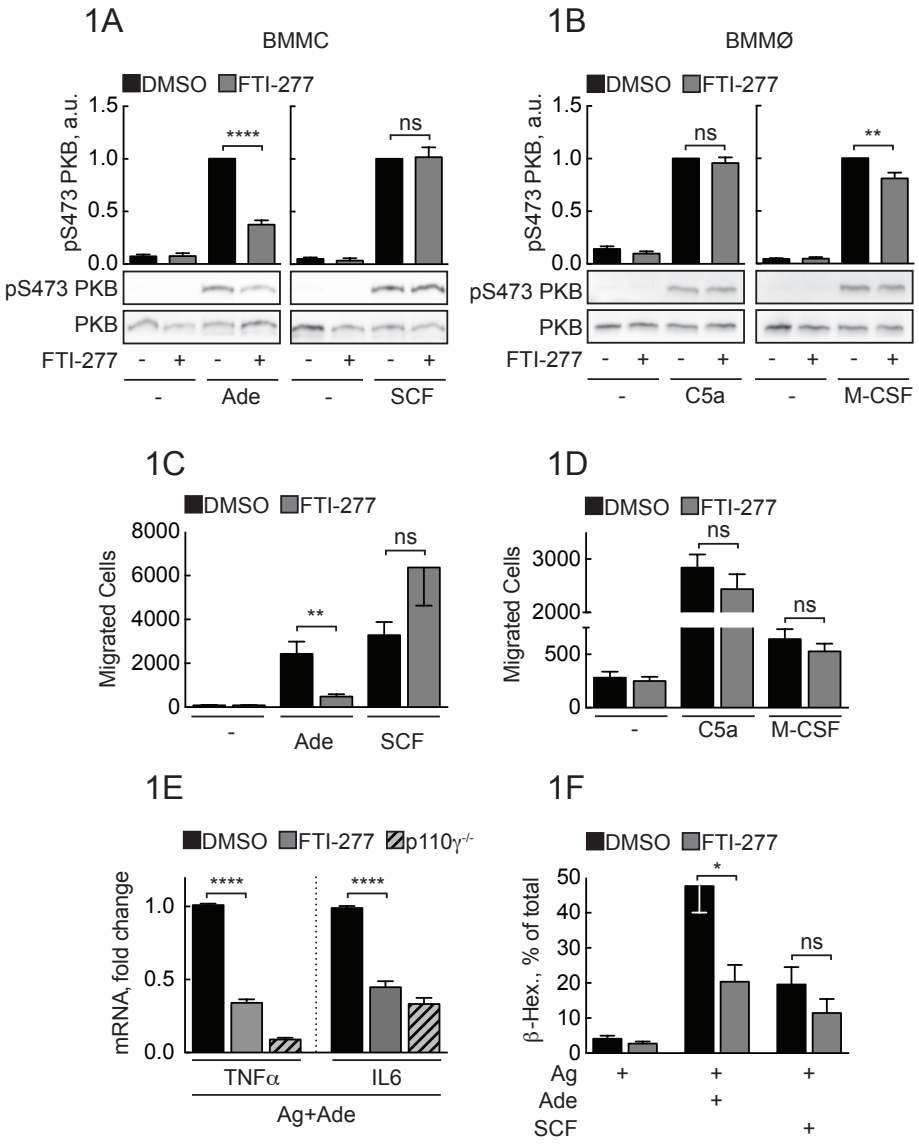

Fig. 1. Mast cells but not macrophages activation is affected by Ras inhibition with FTI-277. (A and B) BMMCs (A) and BMMØ (B) were treated with either DMSO or 5μM FTI-277 for 72 hours, starved for 4 hours and activated with 2μM Ade, 10ng/ml SCF, 10nM C5a or 30ng/ml M-CSF for 2 min at 37°C. Phosphorylation of PKB at Ser473 was determined by Western blot analysis of cell lysates with anti-PKB-Ser473 antibodies and normalized to the total level of PKB. (C and D) Migration of BMMC (C) and BMMØ (D) was assessed in Transwell chambers for 6h at 37°C with indicated stimuli in the lower well, followed by quantification of migrated cells. (E) DMSO- or FTI-277-treated WT-BMMCs as well as p110$\gamma^{-/-}$ BMMCs were exposed overnight to anti-DNP IgE (100ng/ml) followed by stimulation with DNP-HSA (Ag, 2ng/ml) together with 1μM Ade for 1 hour. TNFa and IL6 expression was determined by qPCR and normalized to the level of GAPDH expression. Fold change of expression in FTI-277 treated or p110$\gamma^{-/-}$ cells was quantified relative to DMSO-treated control. (F) DMSO- or FTI-277-treated WT-BMMCs were loaded overnight with anti-DNP IgE (100ng/ml) followed by stimulation with DNP-HSA (Ag, 2ng/ml) alone or in combination with 2μM Ade or 10ng/ml SCF. Release of b-Hexosaminidase was quantified 20 min after stimulation. All data is presented as mean ± standard error of mean (SEM) (n>3; p-values are *: p<0.05, **: p<0.01, ****: p<0.0001).

Differential sensitivity to Ras inhibition is determined by PI3Kγ adaptor subunit.

PI3Kγ adaptor subunits p84 and p101 are differentially expressed in various cell types (10-13). Mast cells have only p84 adaptor protein, while macrophages express both subunits (10). To evaluate and compare the abundance of p84, p101 and p110γ in these cells we first measured the level of their messenger RNAs (mRNA) by quantitative polymerase chain reaction (qPCR) assay (Fig. 2A). According to qPCR data, while mast cells express only p84 subunit, p101 is the dominating adaptor protein in macrophages, with its expression level being 10 times higher that that of p84. Next we used recombinant p84/p110γ and p101/p110γ complexes kindly provided by R. Williams to quantify the absolute amount of corresponding proteins (Fig. 2B). BMMCs possess similar amount of p84 and p110γ (≈39000 and 34000 molecules/cell, correspondingly), and do not express any p101. The amount of p110γ in macrophages is comparable with that in mast cells (≈30000 molecules/cell), while p84 is 2 times less abundant (≈19000 molecules/cell). In accordance with qPCR data the number of p101 molecules per cell is 10x higher than p84 (≈174000), making p101 the dominant adaptor protein in macrophages. It was suggested previously that free monomeric p101 is unstable and undergoes cytosolic degradation (29). Therefore, we were surprised to detect almost 6 times higher abundance of p101 compared to p110γ. It is possible, that the excess of the p101 is localized in the nucleus, as was shown in p110γ/p101 overexpression experiments (29, 30).

It was previously reported that Ras is indispensible for membrane recruitment and activation of p84/p110γ, but not p101/p110γ complexes (22). To assess if the observed difference in sensitivity to FTI-277 between mast cells and macrophages could be explained by the difference in the adaptor subunit content of these cells, we used mast cells complementation technique described in (10). p110γ $^{-/-}$ BMMCs lack both PI3Kγ catalytic and adaptor subunits. These cells therefore could be used as a model system, where after introduction of either p84/p110γ or p101/p110γ differences in the signalling outputs of the two complexes can be analysed in the same cellular context. We reconstituted p110γ $^{-/-}$ BMMCs with p84/p110γ or p101/p110γ complexes, treated them with FTI-277

and analysed Ade-induced PKB phosphorylation at Thr308 (Fig. 2C) and Ser473 (fig. S4). Only cells that had p84 as an adaptor subunit exhibited significant reduction in the level of phosphorylated PKB upon FTI-277 treatment. On the other hand, PI3Kγ activation in cells expressing p101 adaptor protein was insensitive to FTI-277 action. It is important to mention, that in both cases FTI-277 caused significant decrease in the level of phosphorylated mitogen-activated protein kinase (MAPK), demonstrating efficient Ras inhibition in both cell types. As a complementary independent approach for Ras inhibition we overexpressed the GTPase-activating protein (GAP) domain of neurofibromin 1 (NF1) together with p84/p110γ or p101/p110γ in p110γ $^{-/-}$ BMMCs. The ability of cells to migrate towards Ade was then tested *in vitro* in a Transwell chamber migration assay (Fig. 2D). Similar to the effect of FTI-277 on PKB phosphorylation, only p84/p110γ containing cells lost their migratory potential after NF1 overexpression, while migration of cells expressing p101 was insensitive to Ras inhibition. Our results demonstrate that PI3Kγ adaptor subunit indeed determines whether cells will be sensitive to Ras inactivation or not, and that the observed difference between mast cells and macrophages might be explained by the fact that p84 is the only adaptor subunit in BMMCs, while p101 is the dominant adaptor protein in macrophages.

Another possible reason for distinct FTI action on mast cells and macrophages could be the difference in the expression of protein prenyltransferases. Higher level of farnesyltransferase (FTase) expression might require higher concentration of the inhibitor. Alternatively, higher abundance of geranylgeranyltransferase (GGTase) might result in a more efficient process of alternative prenylation. Both cases could lead to decreased sensitivity to FTIs. We assessed the level of FTase and GGTase-I expression in mast cells and macrophages (fig. S5). Both enzymes act as heterodimers. They share the same alpha subunit encoded by the *FNTA* gene; beta subunits are encoded by the genes *FNTB* (FTase) or *PGGT1B* (GGTase-1) (31, 32). Contrary to our hypothesis, expression level of FTase and GGTase-1 was approx. 2 times lower in macrophages compared to mast cells (S5A). Moreover, treatment of cells with FTI-277 did not cause significant difference in the expression of

prenyltransferases (fig. S5B, C). We therefore concluded that FTase and GGTase abundance is not the reason for differential FTI-277 effect.

Figure 2

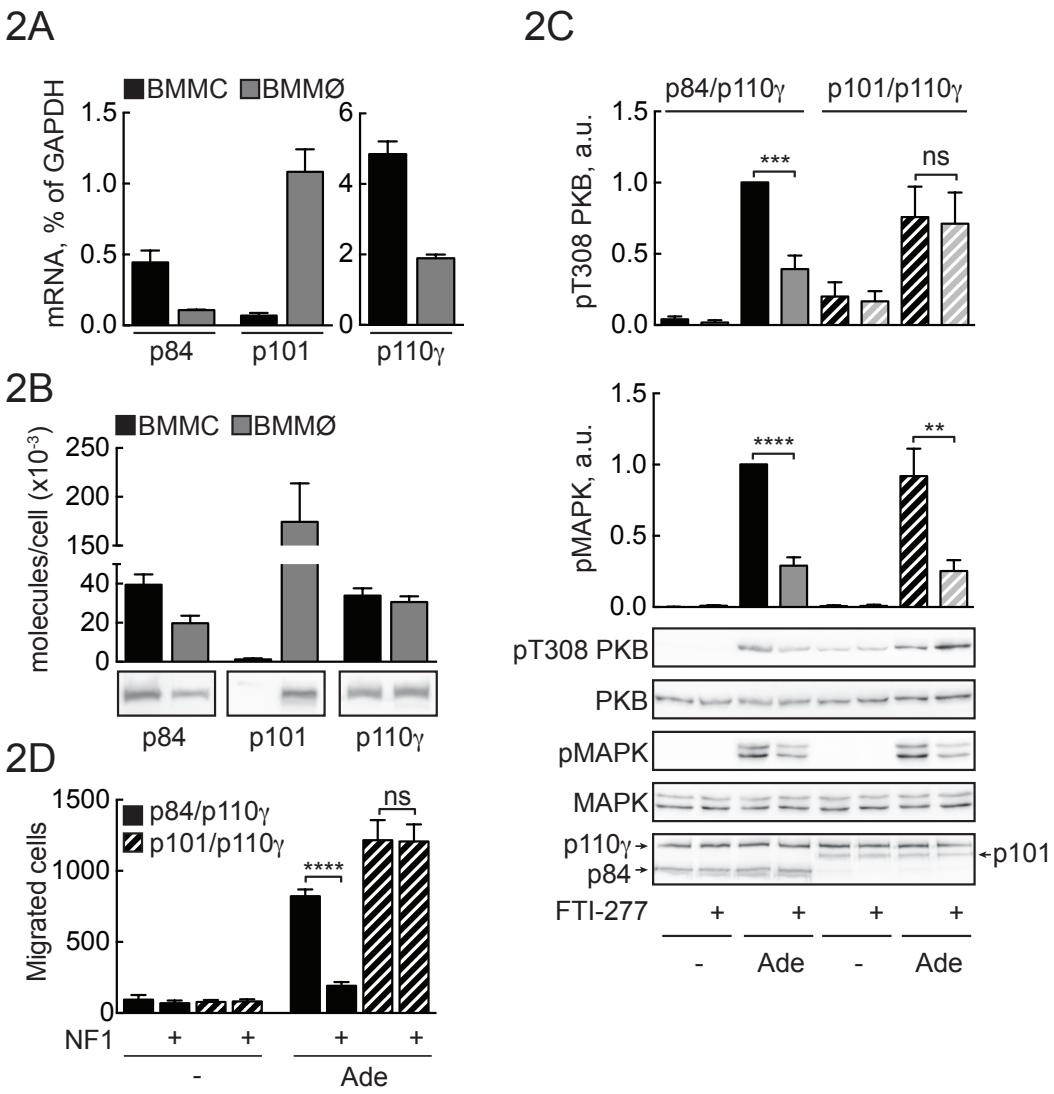

Fig. 2. Sensitivity to Ras inhibition is determined by PI3Kg adaptor subunit. (A) Expression of p110γ, p84 and p101 in BMMCs and BMMØ was assessed by qPCR and normalized to the corresponding level of GAPDH. (B) p84, p101 and p110γ proteins were detected in BMMCs and BMMØ lysates by Western blotting and quantified using recombinant p84/p110g and p101/p110g complexes with known protein concentration. (C) p110γ$^{-/-}$ BMMCs were transfected with plasmids encoding functional p110g and either HA-tagged p84 or p101. 5 hours after transfection cells were put in a fresh medium containing DMSO or 5μM FTI-277. Next day cells were starved in IL3-free medium containing 2% FCS and stimulated with 2mM Ade for 2 min at 37°C. Phosphorylation of PKB at Thr308 and pMAPK was determined by Western blotting and normalized to the total PKB or MAPK levels, correspondingly. The abundance of p110γ was assessed with an anti-p110g, whereas p84 and p101 were detected with anti-HA antibodies. (D) p110γ and HA-p84 or HA-p101 were coexpressed with or without Flag-NF1 in p110γ$^{-/-}$ BMMCs; additionally, GFP-expressing plasmid was used to select for transfected cells. Migration of GFP-positive BMMCs was assayed in Transwell chambers for 6 hours in the presence of 2μM Ade in the lower well. Subsequently, GFP-positive cells were quantified. All data is presented as mean ± standard error of mean (SEM) (n>3; p-values are **: p<0.01, ***: p<0.001, ****: p<0.0001).

N- and H-Ras are activated downstream of GPCRs in mast cells.

As a next step we tried to find out which of the Ras isoforms is involved in the PI3Kγ signalling in mast cells. Seven members of Ras subfamily (H-Ras, N-Ras, K-Ras4A, K-Ras 4B, R-Ras, R-Ras2 and R-Ras3) function as upstream activators of PI3K by directly binding to its catalytic subunit p110 (33). Among these seven isoforms four (N-Ras, K-Ras4B, H-Ras and R-Ras) are similarly expressed at the mRNA and protein level in both cell types (Fig. 3A, B). In order to estimate the relative ratio between these isoforms, we expressed 3x-HA tagged N-, K-, and H-Ras in HEK293 cells and used the lysates as standards for quantification (fig. S6). In BMMCs K-Ras appeared to be the most abundant isoform, with expression level 2 times higher than N-Ras and 4 times higher than H-Ras. In macrophages N- and K-Ras are equally present, and are 4 times more abundant than H-Ras.

To determine which Ras protein is activated downstream of GPCR we performed GTP-Ras pulldown assay. All three isoforms were switched on after macrophages stimulation with C5a (Fig. 3D). In mast cells, however, stimulation with Ade led to the activation of N- and H-Ras only (Fig. 3C). In spite of the higher amount of K-Ras protein in BMMCs, it was not activated downstream of Ade signalling, suggesting that K-Ras is not involved in PI3Kγ activation in mast cells. As expected, treatment with FTI-277 resulted in significant decrease in the amount of activated Ras in mast cells (Fig. 3E). However in macrophages FTI-277 did not lead to the reduction in GTP-loaded N-Ras or K-Ras (Fig. 3F). This represents another level of difference in FTI-277 action in mast cells and macrophages, and shows that it is probably not only the adaptor subunit that determines if PI3Kγ signalling in the cell is sensitive to Ras inhibitors or not. A possible explanation for the lack of FTI-277 action on Ras activation in macrophages could be increased level of alternative geranylgeranylation or increased Ras half-life in these cells.

Figure 3

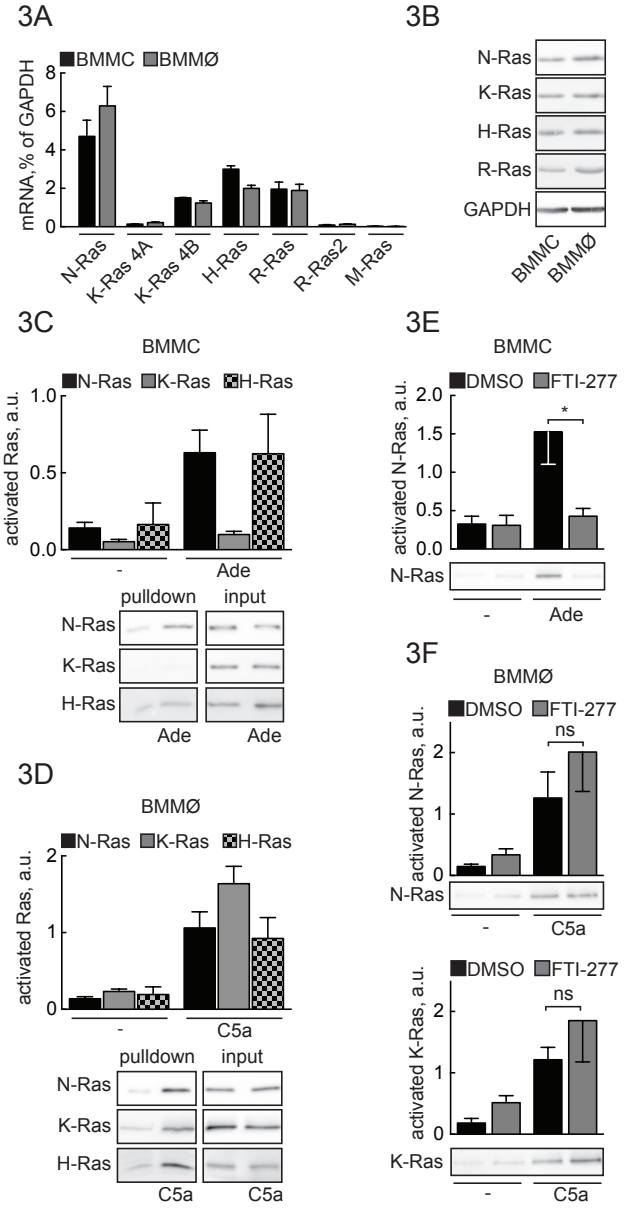

Fig. 3. Ras activation downstream of GPCR. (A) Relative mRNA abundance of N-Ras, K-Ras 4A, K-Ras 4B, H-Ras, R-Ras, R-Ras2 and M-Ras was assessed in BMMCs and BMMØ by qPCR and normalized to GAPDH expression level. (B) N-Ras, K-Ras, H-Ras and R-Ras proteins were detected in BMMC and BMMØ lysates by Western blotting using corresponding isoform-specific antibodies. (C and D) Ras activation assay in BMMC (C) and BMMØ (D). Cells were starved in the corresponding starvation medium for 4 hours before stimulation with 10nM C5a (BMMØ) or 4μM Ade (BMMC). GST-tagged Ras binding domain (RBD) of Raf1 was used to pull-down GTP-loaded activated Ras. N-Ras, K-Ras and H-Ras were subsequently detected in the pulled down fraction by Western blotting using isoform-specific antibodies and presented as a percentage of total amount of corresponding isoform in the lysate used for the pulldown experiment (input). (E and F) Effect of FTI-277 on Ras activation in BMMC (E) and BMMØ (F). Cells were treated with DMSO or 5μM FTI-277 for 72 hours. Ras activation assay was performed as in (C and D). Data is presented as mean ± standard error of mean (SEM) (n>3; p-values are *: $p<0.05$)

Ras knockout leads to the compensation of its function by another isoform.

We have demonstrated that two Ras isoforms, N- and H-Ras, are activated downstream of GPCR, and, therefore, might be involved in PI3Kγ signalling in mast cells. To further analyse the physiologic importance of these isoforms for mast cells activity, we obtained BMMCs from N-Ras$^{-/-}$ (34) and HRas$^{-/-}$ (35) mice, and assessed the effect of Ras knockout on the PI3Kγ activation in these cells. To our surprise neither Ade-induced signalling to PKB (Fig. 4A) or MAPK (fig. S7), nor PI3Kγ-dependent migration (Fig. 4B,C) or degranulation (Fig. 4D) were affected in any of the analysed genotypes. However, when we measured the protein abundance of different isoforms in knockout cells, we found out that elimination of one isoform leads to the increase in expression of another Ras protein (Fig. 4E). K-Ras was twice more abundant in N-Ras$^{-/-}$ or H-Ras$^{-/-}$ cells, and H-Ras showed approximately 1.5-fold increase of expression in N-Ras$^{-/-}$ cells. The only exception was N-Ras, whose expression level did not significantly change in H-Ras$^{-/-}$ BMMCs. The increase in K- and H-Ras level in N-Ras$^{-/-}$ cells and the absence of any effect of H-Ras knockdown on N-Ras expression might indicate that in mast cells N-Ras plays the major role, and its loss has to be compensated by another isoform. In line with this idea, in contrast to wild type BMMCs that previously showed no K-Ras activation, K-Ras became activated upon Ade stimulation of N-Ras knockout mast cells (Fig. 4F). Although these data suggest the more important role of N-Ras for PI3Kγ activation in mast cells, the final conclusion about the major role of one isoform cannot be made due to the observed compensation effect.

Figure 4

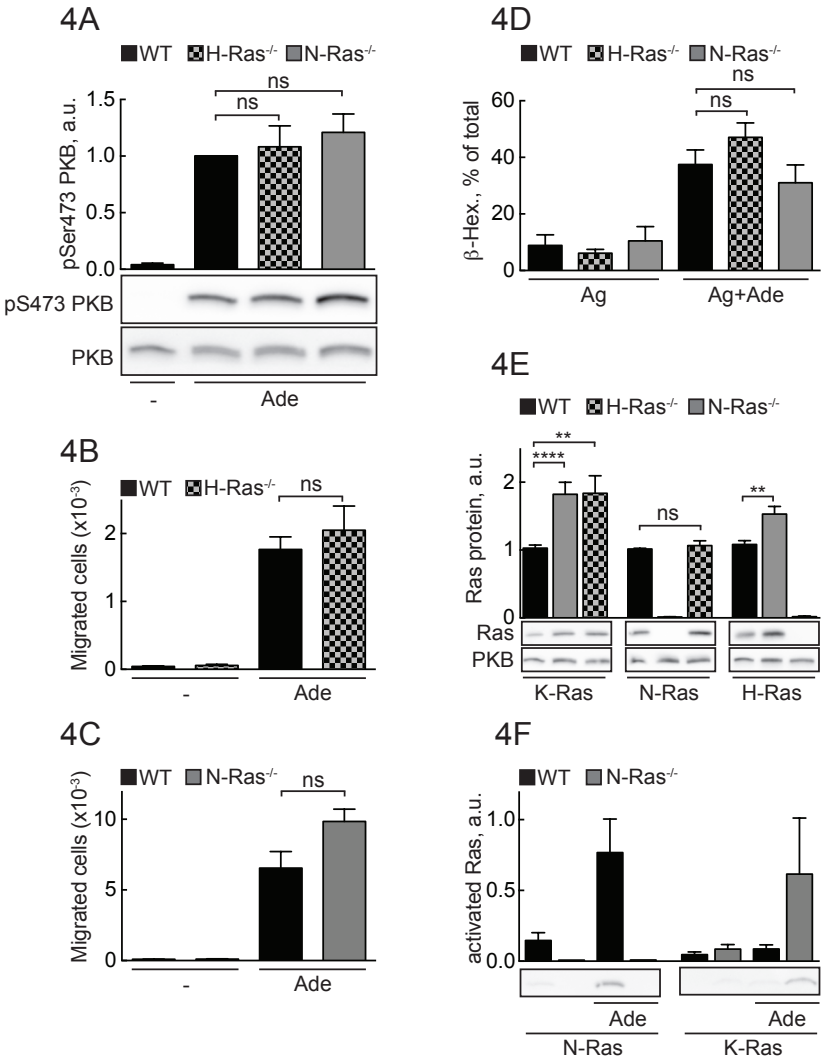

Fig. 4. The lack of an effect of N- or H-Ras knockout on PI3Kg signalling is a result of Ras isoforms compensation effect. (A) Wild type (WT), H-Ras$^{-/-}$ and N-Ras$^{-/-}$ BMMCs were starved in IL3-free medium containing 2% FCS for 4 hours and stimulated with 2μM Ade for 2 min at 37°C. Phosphorylation of PKB at Ser473 was determined by Western blotting and normalized to the total PKB levels. (B and C) Migration of wild type (WT), HRas$^{-/-}$ (B) and N-Ras$^{-/-}$ (C) cells was assessed in Transwell chambers for 6h at 37°C with 2μM Ade in the lower well. (D) Wild type (WT), H-Ras$^{-/-}$ and N-Ras$^{-/-}$ BMMCs were loaded overnight with anti-DNP IgE (100ng/ml) followed by stimulation with DNP-HSA (Ag, 2ng/ml) alone or in combination with 2μM Ade. Release of b-Hexosaminidase was quantified 20 min after stimulation. (E) K-Ras, N-Ras and H-Ras proteins were detected in the lysates from N-Ras$^{-/-}$ or H-Ras$^{-/-}$ BMMCs by Western blotting using isoform-specific antibodies and normalized to the corresponding protein amount in wild type (WT) cells. (F) Wild type (WT) and N-Ras$^{-/-}$ BMMCs were starved in IL3-free medium containing 2% FCS for 4 hours and stimulated with 4μM Ade for 1 min at 37°C. Active Ras was pulled-down from the lysates using GST-tagged Ras binding domain of Raf1 protein, detected by Western blotting with isoform-specific Ras antibodies and presented as a percentage of the total amount of the corresponding Ras isoform in the lysate used for the pulldown experiment. All data is presented as mean ± standard error of mean (SEM) (n>3; p-values are **: p<0.01, ****: p<0.0001).

Discussion

From the discovery of the second possible adaptor subunit for PI3Kγ (12, 13) the questions regarding the physiological importance of having two regulatory proteins were arising. In the recent years it has become evident that although some of the p84 and p101 functions are overlapping, adaptor subunits could also confer specific properties to PI3Kγ that result in diverse cellular responses (10, 11, 20, 22). Diverse outputs triggered by two complexes are most likely explained by differences in the spatiotemporal distribution of PtdIns(3,4,5)P_3 derived from either p101/p110γ or p84/p110γ (10). Ras was shown to be indispensable for membrane recruitment and activation of p84/p110γ, but not p101/p110γ complexes (22). Ras, therefore, could contribute to the differential coupling of PI3Kg heterodimers to downstream responses by ensuring their distribution to different membrane compartments. This suggests that targeting Ras activation might allow specific modulation of p84/p110γ-triggered cellular outputs.

p84 and p101 are not equally distributed in PI3Kγ expressing cells. Mast cells possess only p84 adaptor protein. In accordance with the role of Ras in the activation of p84/p110γ, all PI3Kγ-mediated responses in mast cells (phosphorylation of downstream target PKB, migration, cytokines production and degranulation) were blocked upon Ras inhibition with farnesyltransferase inhibitor FTI-277. At the same time, although FTI-277 blocked protein farnesylation in macrophages to the similar extend as in mast cells (as controlled by the accumulation of prelamin A), it had no effect on the PI3Kγ signalling in macrophages.

A possible explanation for the absence of FTI-277 effect on macrophages lies in the fact that in contrast to p84-only containing mast cells, macrophages are p101-dominated. We showed that p101 represents about 90% of p110γ adaptor protein in macrophages. In line with our suggestion, reconstitution of p110γ $^{-/-}$ mast cells with p101/p110γ complex made them insensitive to Ras inhibition with FTI-277 or to overexpression of GTPase activating domain of NF1. It is however likely that adaptor subunit is not the only reason for differential FTI-277 action on mast cells and macrophages. In contrast to mast cells, where FTI-277 caused significant decrease in the amount of activated N-Ras, N- and K-Ras activation in

macrophages was not affected by the inhibitor. These results show that there is an additional mechanism in macrophages protecting them from inhibition of Ras signalling. In caner cells treatment with FTIs can stimulate alternative geranylgeranylation of N-Ras and K-Ras (26). Higher rate of alternative geranylgeranylation in macrophages compared to mast cells could hypothetically be a reason for the observed insensitivity of Ras activation to FTI. Additionally, varying half-life of Ras proteins in different cells might also explain the differential susceptibility to farnesyltransferase inhibitors. Further studies are needed to elucidate the mechanism behind the resistance of Ras proteins to FTIs in macrophages, and whether it could be used for developing strategies for cell-specific Ras targeting.

In order to get a deeper understanding of PI3Kγ signalling in mast cells, we tried to determine the specific Ras isoform involved in the activation of p84/p110γ complex. Among seven Ras isoforms that were previously shown to interact with PI3K and activate it (N-, H-, K4A-, K4B-, R-, R1, and MRas) (33), only N-Ras and H-Ras were found to be activated downstream of GPCR. N- and H-Ras, but not K-Ras, were previously reported to be associated with cholesterol-rich lipid raft domains at the plasma membrane (reviewed in (36)). Their activation downstream of the GPCR, therefore, is in line with the lipid-raft associated activation of p84/p110γ complex (10). However, H-Ras is mainly localized to lipid rafts in inactive GDP-bound state, and is redistributed to non-raft microdomains of plasma membrane upon its activation (37, 38), making N-Ras more probable candidate for p84/p110γ activation. However, we were not able to verify which Ras protein plays the major role, since knockout of either of the two isoforms did not have any effect on the PI3Kg signalling. The lack of the effect can be explained by an isoform compensation mechanism: depletion of one isoform leads to increase in expression and activation of another Ras protein.

Mast cells have a central role in the pathogenesis of allergic disease. Many anti-allergic therapies target tissues mast cell activation or block the effects of mast cell mediators on adjacent tissues (39, 40). However, there is still no drug that can solely and specifically inhibit mast cell activation. PI3Kγ is a central enzyme in mast cell recruitment, activation and degranulation (2, 7), and the only adaptor subunit present in mast cells – p84 – is absolutely essential for PI3K activation

(10). The ability of Ras inhibition to block activation of p84/p110γ, but not p101/p110γ complexes could, therefore, represent a promising strategy for specific modulation of mast cell activation in allergic disease. Insensitivity of macrophages to FTIs suggests that this kind of treatment might leave the host defence system active. However, analysis of neutrophils from p110γ $^{DASAA/DASAA}$ (p110γ version incapable of binding Ras) or p84 $^{-/-}$ mice revealed various defects in PI3Kγ activation, and showed that p84 is the adaptor protein responsible for ROS production in neutrophils (20, 21). Although recombinant p110γ ^{DASAA} had the same basal catalytic activity, could bind 101 and was activated by Gβγ, expression of p110γ isoform in p110γ $^{DASAA/DASAA}$ knock-in mice was reduced potentially due to some changes in the stability of p110γ ^{DASAA} protein. It is therefore difficult to rule out the possibility that impaired PI3Kγ signalling in p110γ $^{DASAA/DASAA}$ cells did not result from the reduced protein expression. In our work, due to the long incubation time with FTI-277, it was impossible to analyse the effect of the inhibitor on primary neutrophils. Future *in vivo* analysis of the effect of Ras inhibition on the recruitment and activation of different immune cells will show if it is indeed only mast-cell specific or whether it could as well affect other immune responses, and will demonstrate the feasibility and potential of inhibiting Ras for the treatment of allergic disease.

MATERIAL AND METHODS

Cell Culture, Isolation and Differentiation:

Bone marrow-derived mast cells (BMMCs) and macrophages (BMMØ) were derived from bone marrow of 8-12 weeks old C57BL/6J wild type, p110γ $^{-/-}$, N-Ras $^{-/-}$ and H-Ras $^{-/-}$ mice (N-Ras $^{-/-}$ and H-Ras $^{-/-}$ mice were obtained from RIKEN BRC, Japan). To obtain BMMCs fresh bone marrow (BM) was resuspended in complete IMDM (10% HIFCS, 2mM L-Gln, 10mg/ml PEST, 50mM b-mercaptoethanol, 2 ng/ml recombinant murine IL-3 [Peprotech, #213-13, Rocky Hill, NJ]) supplemented with 5 ng/ml murine SCF (Peprotech, #250-03, Rocky Hill, NJ) and cultivated at 37°C, 5% CO_2 for four days. Subsequently, BMMCs were diluted weekly to 0.5×10^6 cells/ml with a mixture of 80% fresh complete IMDM and 20% recycled medium, with IL-3 added every 3rd day (2).

To derive BMMØ fresh BM was resuspended in complete RPMI (10% HIFCS, 2mM L-Gln, 10mg/ml PEST, 50mM b-mercaptoethanol) supplemented with 20% L929-conditioned medium. Cells were cultivated at 37°C, 5% CO_2 for five days and used immediately for experiments. The human embryonic kidney 293 (*HEK293*) cell line was grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% HI-FCS, 2 mM L-Gln, 10mg/ml PEST at 37°C, 5% CO2.

Stimulation of BMMCs and BMMØ

BMMCs and BMMØ treated with DMSO (Sigma, #34869) or FTI-277 (Sigma, #F9803) were collected by centrifugation (900rpm, 5min.), washed and resuspended in starvation medium (BMMC - IL-3 free growth medium containing 2% FCS; BMMØ – RPMI, 1% FCS, no L929-conditioned medium). After 4 hours cells were stimulated for 2min at 37°C with adenosine (Ade, 2μM, Sigma #01890) or stem cell factor (SCF; 10 ng/ml, Peprotech #300-07) in case of BMMC, or 10nM C5a (R&D Systems #2150-C5-025) or 30ng/ml M-CSF (Peprotech #315-02) in case of BMMØ. Stimulation was stopped on ice, the cells collected by centrifugation (1min., 4°C, 14000 rpm), washed in 1xPBS and lysed at $1x10^7$ cells/ml in 2x Laemmli sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 10% b-Mercaptoethanol, 20% Glycerol, bromphenolblue). Proteins were denatured at 95° for 7 min., subjected to SDS-PAGE and immunoblotting.

For degranulation experiments or analysis of cytokines expression BMMCs were incubated in complete IMDM with 100 ng/ml mouse anti-DNP IgE (clone SPE-7, Sigma #D8406) overnight, and then stimulated with 1 ng/ml DNP-HSA (Sigma #A-6661) with or without 2 μM adenosine or 10ng/ml SCF for 20 min (degranulation) or 1 hour (cytokines expression) at 37°C.

Immunoblotting and antibodies

Proteins were separated by SDS-PAGE and transferred to Immobilon PVDF membranes (Millipore). Primary antibodies used: mouse monoclonal anti-p110γ (clone H1, Alexis), rabbit monoclonal anti-pSer473-PKB/Akt (#4058), rabbit monoclonal anti-pThr308-PKB/Akt (#4056), mouse monoclonal anti-PKB/Akt (#2920), rabbit monoclonal anti-p101 (#5569), rabbit monoclonal anti-pCREB (#9198), rabbit polyclonal anti-R-Ras (#8446, all Cell Signaling Technology), mouse monoclonal anti-pMAPK (#M8159), rabbit polyclonal anti-MAPK (#M7927), mouse monoclonal anti-GAPDH (#G8795, all Sigma), rabbit monoclonal anti-H-Ras (ab32417, abcam), mouse monoclonal anti-N-Ras (#sc-31), mouse monoclonal anti-K-Ras (#sc-30), goat polyclonal anti- Lamin A (C-20) (Prelamin, #sc-6214, Santa Cruz Biotechnology), anti-p84 (rabbit antisera (10), mouse monoclonal anti HA (HA.11, #MMS-101R, Covance). Secondary antibodies such as horseradish peroxidase (HRPO)-coupled rabbit anti-mouse IgG and goat anti-rabbit IgG antibodies (Sigma) were visualized using enhanced chemiluminescence (#WBKLS0500, Millipore).

Cell migration assay

Migration of BMMCs and BMMØ was assayed in 24-well Transwell Supports (#3421, Corning) with 5.0 μm pore polycarbonate membranes for 6 hours.

BMMC: Before the experiment Transwell membranes were coated overnight with 1 mg/ml fibronectin/PBS at 4°C, then blocked with 4% BSA/PBS for 1 hour at 37°C and equilibrated in migration medium (IMDM supplemented with L-Gln, PEST, 1% BSA, 50 μM β-mercaptoethanol, and 20mM Hepes, pH 7.4) for 30 minutes at 37°C. BMMC were washed twice in migration medium. Cell suspensions (0.25 x 10^6 cells in 200 μl) were applied to the upper compartment of

the Transwell inserts already containing migration medium supplemented with or without 1 μM adenosine or 10ng/ml SCF in the lower well.

BMMØ: Transwells were equilibrated in migration medium (RPMI, supplemented with L-Gln, PEST, 1% FCS, 50 μM β-mercaptoethanol) for 30 minutes at 37°C. BMMØs were washed twice in migration medium. Cell suspensions (0.5 x 10^5 cells in 200 μl) were applied to the upper compartment of the Transwell inserts already containing migration medium supplemented with or without 10nM C5a, 10ng/ml Rantes or 30ng/ml M-CSF in the lower well.

After 6 hours cells were fixed with 4%PFA and stained with Hoechst. Bottom of the inserts as well as lower wells of the transwells were scanned using fluorescent microscope and cells were quantified with ImageJ plugin.

Transfection experiments

BMMCs were transfected using the Nucleofector™ protocol (Amaxa). BMMCs (7-10x10^6) were resuspended in 100ml nucleofection solution T (#VCA-1002, Amaxa), then 10-15 mg plasmid DNA (in 15 ml 10 mM TRIS/1 mM EDTA pH 8.0) were added. Immediately after electroporation cells were cultured in 5 ml complete IMDM at 37°C, 5% CO_2 for 5 hours. After a medium change with complete IMDM with DMSO or 5mM FTI-277 transfected cell populations were cultured for 24h. Stimulations occurred 24 h post-transfection.

HEK293 cells were seeded into 6 cm dishes and were transfected with JetPEI™ (Polyplus-transfection) using 2.5 μg of total plasmid DNA. Codon optimized 3xHA-Ras sequences (Table S2) used for expression in HEK293 cells were synthesized by GenScript and cloned into pcDNA3.1 plasmid.

Mast cell degranulation

Release of histamine containing granules was quantified by the determination of b-hexosaminidase in the cell supernatants, slightly modified from {Laffargue et al., 2002,}. BMMC were incubated with 100 ng/ml anti-DNP IgE overnight and cells resuspended in modified Tyrode's buffer at 0.5-1.0x 10^6 cells/ml, 37°C, 5% CO_2. Degranulation was induced with 1 ng/ml DNP-HSA with or without 2 μM adenosine or 10ng/ml SCF for 20 min at 37°C, 5% CO_2. The reaction was stopped

and β-hexosaminidase activity measured with p-nitrophenyl-N-acetyl-b-D-glucosaminide (#N9376, Sigma). Results are given as the percentage of total TritonX-100 releasable b-hexosaminidase.

Quantitative real-time PCR

RNA was isolated with RNeasy mini kit (#74104, Qiagen) according to manufecturer's instructions. cDNA was generated with 2 mg of total RNA using M-MVL reverse transcriptase (#28025-013, RT-buffer #18057-018 Invitrogen; RNAsin, #N2111, Promega) and stored at -80°C until use. A StepOnePlus (Applied Biosystems, Foster City, CA) and MESA Green qPCR MasterMix Plus for SYBR assay (#RT-SY2X-20+WOU, Eurogentec) was used for qPCR. Sequences of the primers used could be found in the Table S1.

Ras activation assay

BMMC: BMMCs ($2x10^7$/stimulation) were starved in IL-3 free growth medium containing 2% FCS for 4 hours. Cells were then washed twice with HBBSS buffer (15mM HEPES, 140mM NaCl, 5mM KCl, 2.8mM NaHCO3, 1.5mM $CaCl_2$, 1mM $MgCl_2$, 0.06mM $MgSO_4$, 5.6mM Glucose, 0.1% BSA pH 7.2). After final resuspension cells were kept on ice for 10min, and then incubated at 37°C 5 min prior to stimulations. Cells were stimulated with 4μM adenosine for 1 min at 37°C, centrifuged at 13000rpm for 10sec and resuspended in lysis buffer (50mM Tris pH7.5, 150mM NaCl, 5mM $MgCl_2$, 1mM EGTA, 1%NP-40, protease inhibitors) containing 20μg GST-tagged Raf-RBD. Lysates were kept on ice for 10 min, followed by 15 min centrifugation at 14000 rpm, 4°C. Cleared lysates were transferred to new 2ml tubes, and 40μl GSH-sepharose beads (#17-0756-01, GE Healthcare) preequilibrated in lysis buffer were added to each reaction. After 1 hour incubation on a wheel at 4°C, beads were washed 3 times with ice-cold lysis buffer and resuspended in Laemmli sample buffer.

BMMØ: One day before experiment $10x10^6$ BMMØs were seeded in a 10cm plate. Next day cells were starved in RPMI with 1% FCS and no L929-conditioned medium for 4 hours followed by a stimulation with 10nM C5a for 2min at 37°C. After stimulation cells were washed with ice-cold PBS and lysed in lysis buffer

supplemented with 20μg GST-tagged Raf-RBD. Lysates were transferred to 1.5ml tubes, incubated on ice for 10 min, and centrifuged for 15 min at 14000 rpm, 4°C. Cleared lysates were transferred to new 2ml tubes, and 40μl GSH-sepharose beads preequilibrated in lysis buffer were added to each reaction. After 1 hour incubation on a wheel at 4°C, beads were washed 3 times with ice-cold lysis buffer and resuspended in Laemmli sample buffer.

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Supplementary figures

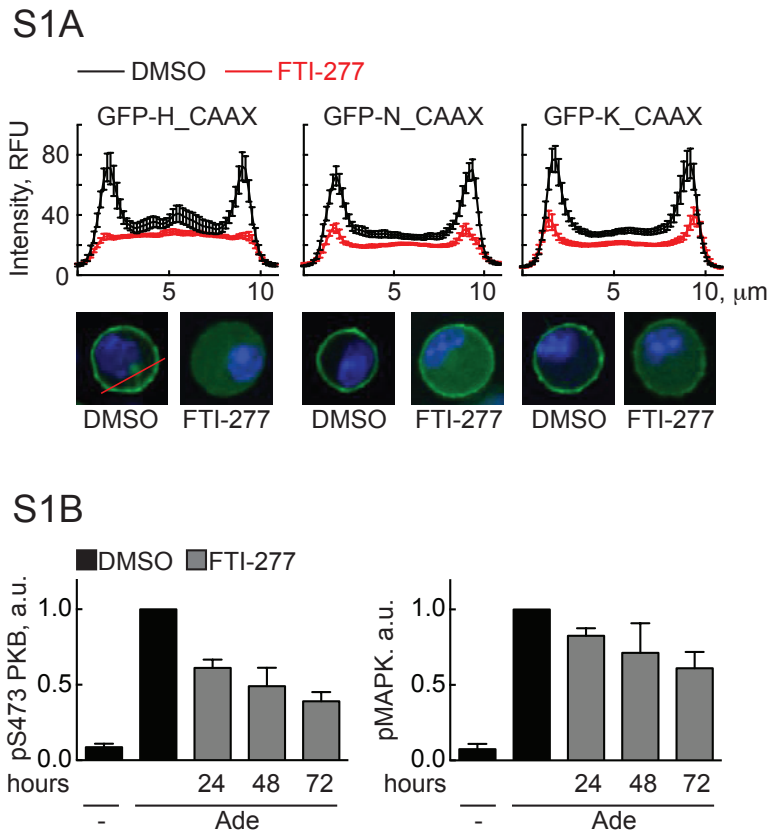

Fig. S1. FTI-277 affects Ras localization and PI3Kγ signalling in mast cells. (A) BMMCs were transfected with constructs expressing GFP-tagged C-terminal 25 aminoacids of H-Ras, N-Ras or K-Ras and treated with 5μM FTI-277. 24 hours later cells were fixed in 4% p-formaldehyde, and images were acquired by confocal microscopy. Distribution of Ras proteins along a predefined line spanning cells was analyzed (number of cells analysed per condition ≥18). (B) BMMCs were treated with DMSO or 5μM FTI-277 for 24, 48 or 72 hours. Cells were then starved for 4 hours in IL3-free medium containing 2%FCS and either DMSO or 5μM FTI-277, followed by stimulation with 2μM Ade for 2 min at 37°C. pSer473-PKB and pMAPK were determined by Western blotting and normalized to the total PKB or MAPK levels, correspondingly.

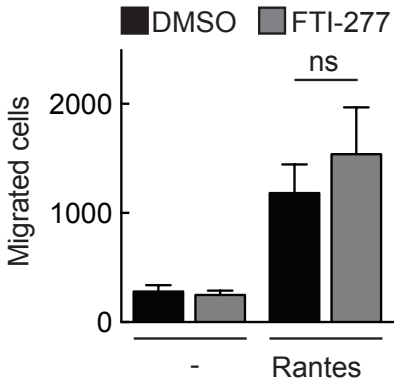

Fig. S2. Macrophages migration toward GPCR ligand Rantes is not affected by FTI-277. Migration of BMMØ was assessed in Transwell chambers for 6h at 37°C with 10ng/ml Rantes in the lower well, followed by quantification of migrated cells.

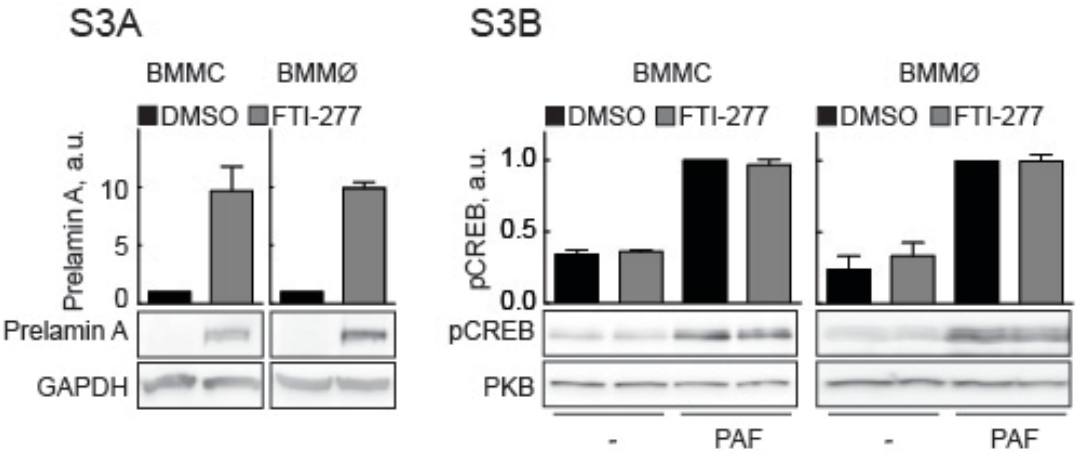

Fig. S3. FTI-277 inhibits protein farnesylation, but not Gγ functioning in mast cells and macrophages. (A) Prelamin A was detected in the lysates from DMSO or FTI-277 treated BMMCs and BMMØ and normalized to the level of GAPDH protein. Fold increase of prelamin A upon FTI-277 treatment was quantified and normalized to the DMSO-treated control. (B) BMMCs and BMMØ were treated with DMSO or 5μM FTI-277 for 72 hours, starved for 4 hours and activated with 1μM PAF. Phosphorylation of cyclic AMP-responsive element-binding protein (CREB) at Ser133 was determined by Western blot analysis of cell lysates and normalized to the total PKB level.

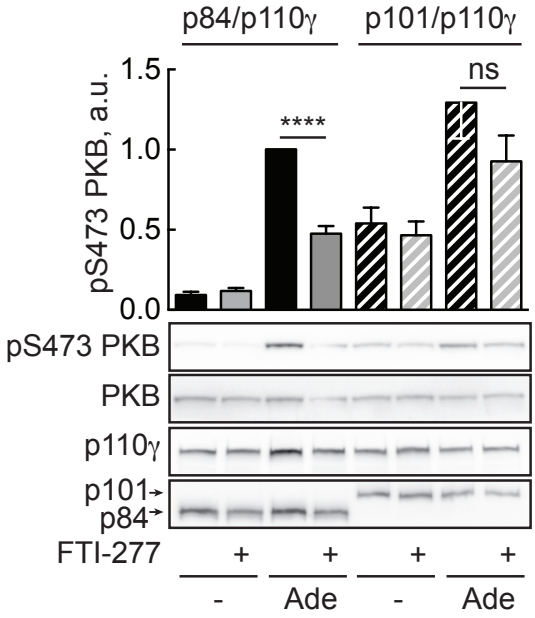

Fig. S4. Expression of p101 adaptor subunit makes PI3Kγ activation in mast cells insensitive to FTI-277. p110γ $^{-/-}$ BMMCs were transfected with plasmids encoding functional p110γ and either HA-tagged p84 or p101. 5 hours after transfection cells were put in a fresh medium containing DMSO or 5μM FTI-277. Next day cells were starved in IL3-free medium containing 2% FCS and stimulated with 2μM Ade for 2 min at 37°C. Phosphorylation of PKB at Ser473 was determined by Western blotting and normalized to the total PKB level. The abundance of p110γ was assessed with an anti-p110γ, whereas p84 and p101 were detected with anti-HA antibodies.

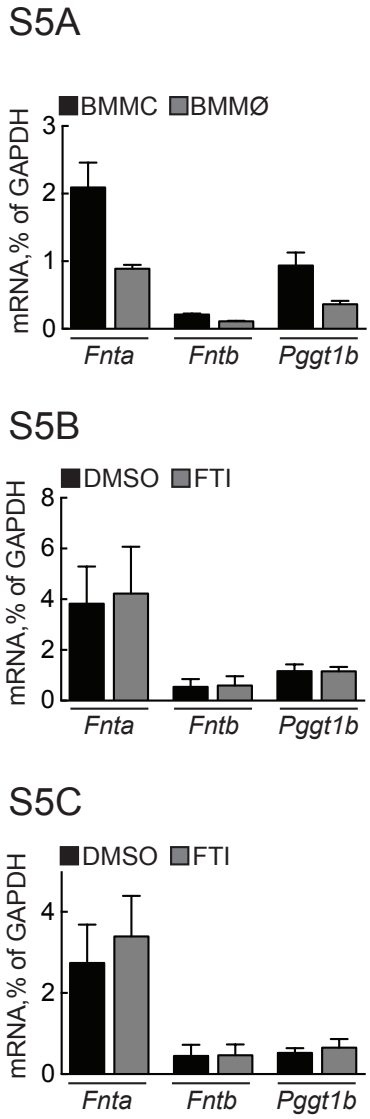

Fig. S5. Expression of farnesyltransferase (FTase) and geranylgeranyltransferase (GGTase) in mast cells and macrophages. (A) mRNA abundance of *Fnta* (a subunit of FTAse and GGTase), *Fntb* (b subunit of FTAse) and *Pggt1b* (b subunit of GGTase) in BMMCs and BMMØ was assessed by qPCR and normalized to the corresponding level of GAPDH expression. (B and C) Effect of FTI-277 on FTase and GGTase expression. BMMCs (B) and BMMØ (C) were treated with DMSO or 5μM FTI-277 for 72 hours. Expression of *Fnta*, *Fntb* and *Pggt1b* genes was assessed by qPCR and normalized to GAPDH.

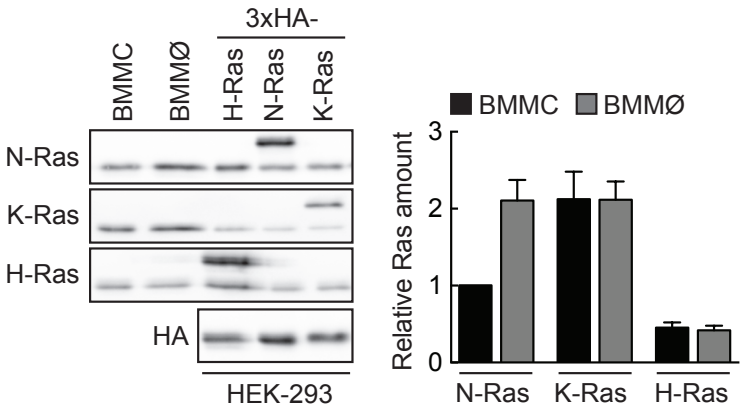

Fig. S6. Ras isoforms abundance in mast cells and macrophages. Plasmids encoding HA-tagged codon-optimized N-, K-, and H-Ras were transfected into HEK293 cells. Protein expression levels of three isoforms in HEK293 lysates were equalized using anti-HA antibodies. These lysates were then used as "standards" for quantification of relative amount of Ras isoforms in BMMCs and BMMØ, with N-Ras expression used as a reference point (=1).

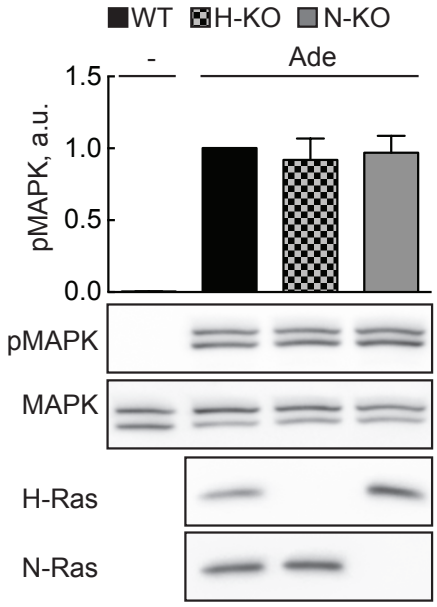

Fig. S7 Ade-induced MAPK activation is not affected in N-Ras$^{-/-}$ and H-Ras$^{-/-}$ BMMCs. Wild type (WT), H-Ras$^{-/-}$ and N-Ras$^{-/-}$ BMMCs were starved in IL3-free medium containing 2% FCS for 4 hours and stimulated with 2μM Ade for 2 min at 37°C. Phosphorylation of MAPK was determined in cell lysates by Western blotting and normalized to the total MAPK levels.

Table S1. Sequence of primers used for qPCR

Gene	Accession-No	Primer sequence 5' to 3'
Mm_GAPDH	NM_008084	F: CTGCACCACCAACTGCTTAG R: CCATCCACAGTCTTCTGGGTG
Mm_p84	AY753194	F: ATAGAGCAGGTGGCTAGCGA R:CAGAAGGTCACCGACACAGTG
Mm_p101	NM_177320	F: GACATCCTACAGGAAGTCCTTCTC R: TCAGCGCAATGCCTGTCCAT
Mm_p110g	NM_020272	F: CCCTGGTGATCGAGAAATGC R: GTCTTGGCGCAGATCATCAC
Mm_NRas	NM_010937	F: ATGAGGACAGGCGAAGGGTTC R: TCACACTTGTTGCCTACCAGCAC
Mm_KRas4A	XM_006506919	F: GATGTGCCTATGGTCCTGGTAGG R: GCATCCTCCACTCTCTGTCTTGTC
Mm_KRas4B	NM_021284	F: GATGTGCCTATGGTCCTGGTAGG R: GCATCGTCAACACCCTGTCTTGTC
Mm_HRas	NM_008284	F: GGCAGGGCGTGGAGGATG R: GCAGCCAGGACCACTCTCATCG
Mm_RRas	NM_009101	F: TGCCATTAACGACAGGCAG R: TGTTCCCAACCAACACAATG
Mm_RRas2	NM_025846	F: GGCAATAAAGCTGACCTGGA R: ATCCTGATCTTTGCCGATG
Mm_MRas	NM_008624	F: CCACCAGCTCATTCTGCGTGTCAAGG R: CCTTGGTCCCTGGTGACTTTCCTTAGG
Mm_TNFalpha	NM_013693	F: ATCCGCGACGTGGAACTG R: CGAAGTTCAGTAGACAGAAGA
Mm_IL6	NM_031168	F: ACAACCACGGCCTTCCCTACTT R: CACGATTTCCCAGAGAACATGTG
Mm_Fnta	NM_008033	F: AGCATCGACAGTGGGTCATTC R: GACGAAGTGTCTTTGGTTCCAC
Mm_Fntb	NM_145927	F: GAGAAGATCCAGGAGGTCTTCAG R: CTCATAGGCATCTGTCAGTTGTC
Mm_Pggt1b	NM_172627	F: CCATCAAAGAATCCAGGAGCAG R: ATCCACACGGCCTAAGTCATCTC

Mm, *mus musculus*; F, forward primer; R, reverse primer

Appendix I:

- Generation of PIK3r5 (p101) and PI3Kr6 (p84) knockout mouse strains

- Generation of p84 Knock in mouse strain

- Next generation engineering of conditional mouse alleles with loxP and FRT sites by dual RMCE

Generation of PIK3r5 (p101) and PI3Kr6 (p84) knockout mouse strains.

The sole member of class IB PI3K family, PI3Kγ, is a heterodimeric enzyme formed by a catalytic subunit, p110γ, and an adaptor subunit between p84 and p101. The information available regarding the role and the specificity of the two adaptor subunits *in vivo* are currently limited, probably due to a certain level of redundancy in their biological activity that make difficult the analysis of the adaptor subunit specific responses. In order to investigate the different roles of p101 and p84 in PI3Kγ signalling, we attempted to generate both a p101 and a p84 knock out mouse strain.

While the product of the PIK3r5 gene, p101, was isolated for the first time from pig neutrophils, in association with p110γ (Stephens et al., 1997), p84 (or $p87^{PIKAP}$) was identified almost 10 years later (Suire et al., 2005; Voigt et al., 2006). In 2006 a mouse strain lacking p101 had already been characterised (Suire et al., 2006) while the generation of a p84 knock-out mouse strain have only been published in 2015 (Deladeriere et al., 2015), but was not yet available while this project was in progress.

The p101 knockout mouse strain has been successfully generated starting from a JM8A3.N1 subline of embryonic stem (ES) cells, derived by C57BL/6N mice and targeted with the "knockout-first allele" method obtained by EUCOMM. The blastocyst injection and the generation of chimeric mice was carried out at the "Transgenic facility" of the Biozentrum, Basel, under the supervision of Dr. Daniela Klewe-Nebenius. The p84 knockout mouse strain project started with the ordering of two clones of E14TG2a.4 ES cells, derived by 129P2/OlaHsd mice. The two clones "E017F02" and "E324E05" were both genetically modified with the "Gene Trap" technology (Skarnes, W. C. et al., 2004) at the level of the same genomic locus, with different targeting vectors. The PIK3r6 gene of the clone "E017F02" was targeted with the "rFROSAbgeo+2s" vector and the clone "E324E05" with the "rsFlipROSACeo0" vector (http://www.informatics.jax.org). Classical PCR, 3'Race and western blotting analysis of the two ES cell clones and of the bone marrow cells isolated from the mice derived from the two clones

revealed that the targeting vector was only present in one of the two clones and the second one, even in presence of the targeting vector, did not show any reduction of expression of the protein of interest.

Rapresentation of the targeted PI3Kr5 locus (p101)

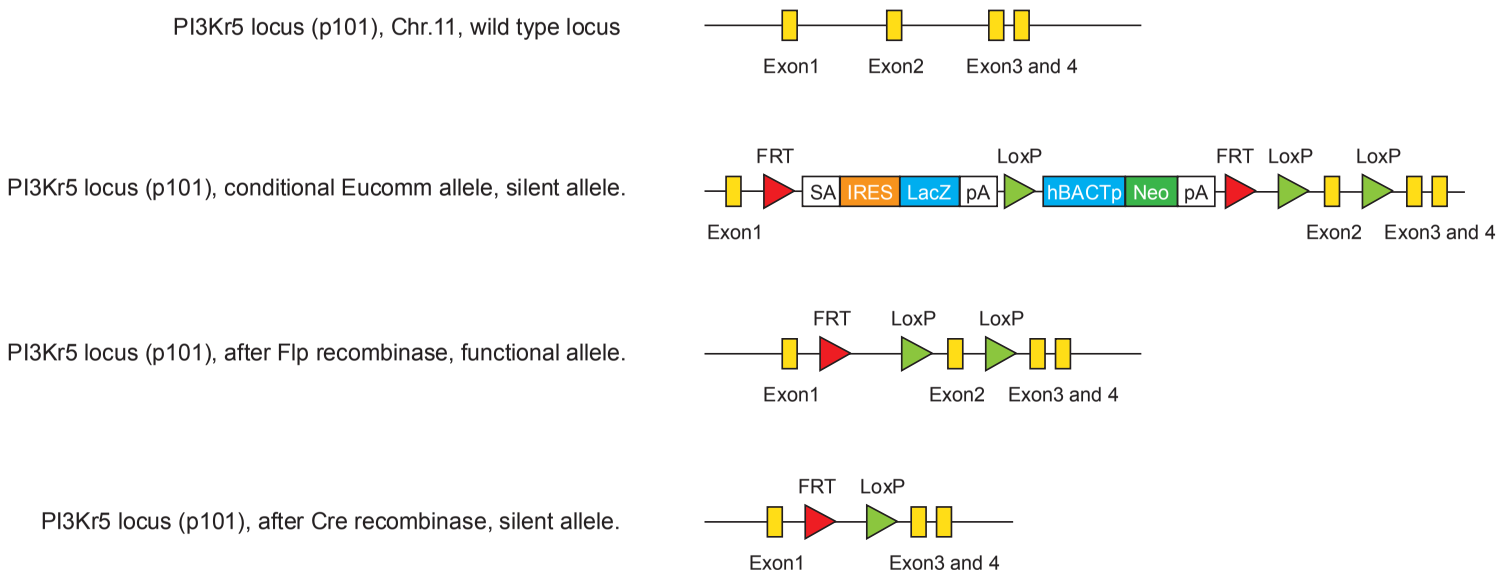

Figure 1, appendix I: Gene targeting of PIK3r5 (p101) genetic locus.Graphic representation of the PIK3r5 targeting gene cassette for the generation of the p101 knock out mouse strain with the "knock out first allele" technology developed by Eucomm. **FRT**: flippase recognition target site. **Loxp**: locus of X-over P1, is a short DNA sequence derived from the Bacteriophage P1, recognised by the Cre recombinase enzyme. **SA**: Splicing Acceptor. **IRES**: Internal Ribosome Entry Site. **LacZ**: beta-galactosidase. **pA**: polyadenilation site. **hBACTp**: human beta-actin promoter. **Neo:** neomicine resistence gene.

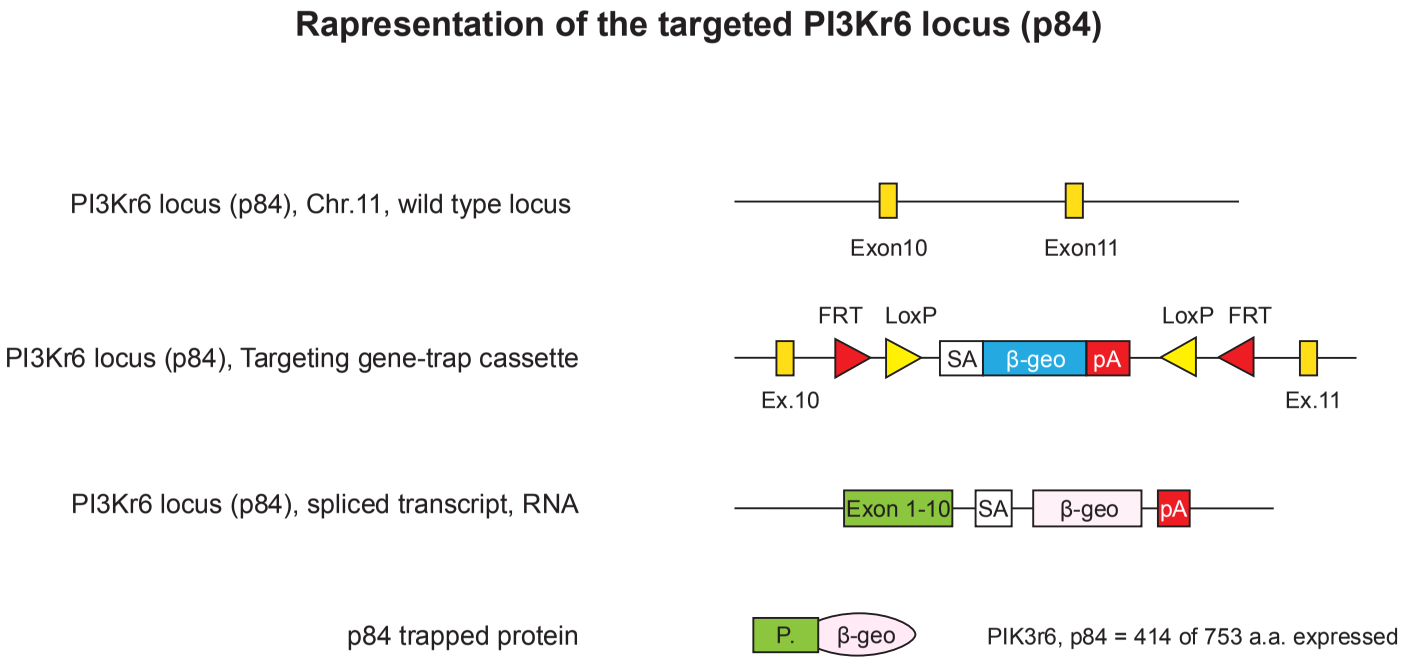

Figure 2, appendix I: Gene targeting of PIK3r6 (p84) genetic locus.Graphic representation of the PIK3r6 targeting gene cassette for the generation of the p84 knock out mouse strain with the gene-trap technology[44]. **FRT**: flippase recognition target site. **Loxp**: locus of X-over P1, is a short DNA sequence derived from the Bacteriophage P1, recognised by the Cre recombinase enzyme. **SA**: Splicing Acceptor. **Beta-geo**: Beta-galactosidase-neomicin resistence phusion gene. **pA**: polyadenylation site.

Generation of p84 Knock in mouse strain

Although the tissue specific expression of p84 and p101, the two adaptor subunits of PI3Kγ, has been investigated both in mouse (Bohnacker, T. et al., 2009) and human (Shymanets, A., 2013) the biological role of two proteins and their specificity *in vivo* is not understood yet. In order to investigate the physiological functionality of the two proteins in a mouse model, we thought about generating a new mouse strain bearing the particular feature of expressing the p84 adaptor protein in cells that physiologically express p101. In particular we tried to modify the PIK3r5 gene sequence, that generates p101, in order to silent the expression of p101 and to insert, at the same time, p84 coding sequence (cDNA of the PIK3r6 gene) under the control of the promoter of PIK3r5. To do so we made use of a technology developed by R. Zeller Lab to target ES cells whose genetic loci were already modified with the "knockout-first allele". The technic is called dRMCE, dual recombines cassette exchange and involves a site specific genomic recombination mediated by the presence of FRT and Loxp sites in ES cell (Osterwalder, M. et al., 2013). Starting from the same ES cell clone used for the generation of the p101 knock out mouse (JM8A3.N1), we performed an ES cells electroporation and a selection of the positive clones by pcr screening. The clones that correctly undergone dual recombines cassette exchange were sent to the "Transgenic facility" of the Biozentrum, Basel, for the blastocyst injection and generation of the chimeric mice. Due to a not previously identified mutation in one of the plasmids used for the ES cells electroporation, a mutation that interfered with the exogenous expression of the p84 protein, the mouse strains obtained did not over-expressed any p84 exogenous protein but showed only a deletion of the targeted gene locus. As a consequence of this mistake, the generation of the p84 knock-in mouse strain was arrested.

Next generation engineering of conditional mouse alleles with loxP and FRT sites by dual RMCE (protocol elaborated by: Marco Osterwalder , Javier Lopez-Rios & Rolf Zeller).

Reagents:

- ES cell and feeder cell cultures

D-PBS (1X), liquid (Gibco, 14190-144)
D-PBS with Mg^{2+}/Ca^{2+} (1X), liquid (Gibco 14040-117)
D-MEM (1X), liquid (High Glucose; 4.5g/l; Gibco 41966-029)
Fetal Bovine Serum (FBS; Gibco 10270-106)

TIP: Is essential to use a batch of FBS that supports the optimal growth of ES cells and keeps them in an undifferentiated state so that their germ-line transmission potential is not affected. We recommend that users either acquire batches of FBS for ES cell culture from the transgenic core facility that will produce their chimeric mice or ask them for advice on where to buy serum that meets these criteria.

Penicillin-Streptomycin (10,000 units-10 mg/ml; 100x; Gibco 15140-122)
L-Glutamine (200 mM; 100x; Gibco 25030-024)
β-Mercaptoethanol (50 mM stock; 500x; Gibco 31350-010)
Leukemia Inhibitory Factor (LIF; 10^7 units/ml; 10,000x; ESGRO® Chemicon ESG1107)
MEM Non Essential Amino Acids (10 mM; 100x; Gibco 11140-035)
MEM Sodium Pyruvate (100 mM; 100x; Gibco 11360-039)
Trypsin-EDTA 1X (Gibco 25300-054)
NOTE: this is a low-concentration Trypsin-EDTA solution.
DMSO (Sigma D-8418)
Mitomycin C (Sigma M-0503). Dissolve in D-PBS (Gibco, 14190-144) at 1 mg/ml (100x stock solution). Store at 4°C, light-sensitive and stable for up to 2 weeks.
Hygromycin (Sigma H3274). Dissolve in H_2O at 25 mg/ml. Sterile filter the solution using a 0.2 μm filter and store at 4°C in the dark. The working

concentration for selection of resistant R1 ES cell colonies is 175 µg/ml.
Puromycin (Sigma P9620; 10 mg/ml). Store frozen in small aliquots. The working concentration for selection of resistant ES cell colonies is 1 µg/ml for R1 cells and 0.5 µg/ml for ES cell colonies obtained by transfection of IKMC ES cell lines.
Gelatin (Sigma G-2500). Dissolve at 0.1% in H_2O.

- Prepared reagents
ES cell culture medium (store at 4°C)
DMEM (high glucose), 15% FBS, 1x Penicillin-Streptomycin (stock is 100x), 2 mM **L-Glutamine** (stock is 100x), 0.1 mM β-mercaptoethanol (stock is 500x), 0.1 mM **MEM Non Essential Amino Acids** (stock is 100x), 1 mM MEM Sodium Pyruvate (1mM; stock 100x), 10^3 units/ml LIF (stock is 10,000x).
ES cell freezing medium (prepare fresh and sterilize the solution using a 0.2 µm filter; keep on ice)
50% ES cell culture medium
40% FBS
10% DMSO
Feeder cell culture medium (store at 4°C)
DMEM (high glucose), 10% FBS, 1x Penicillin-Streptomycin (stock is 100x), 2 mM L-Glutamine (stock is 100x)

- Plasmid vectors for dRMCE
The dRMCE vectors are available as soon as possible from Addgene and are protected by an MTA. The plasmids have to be prepared as transfection grade DNA using e.g. Nucleobond PC 100 columns (Macherey-Nagel 740573) according to the manufacturer instructions. After washing with 70% ethanol, the DNA pellets should be dried under a laminar flow hood for 10-15 minutes. Subsequently, a small amount (100-200 µl) of sterile H_2O is added to the tube, the tube is covered with Parafilm and the plasmid DNA left to dissolve overnight at 4°C. The next day, the plasmid solution is transferred with minimal pipetting into a 1.5 ml tube and the concentration is determined using a UV spectrophotometer (a typical working concentration is 1-4 µg/µl). Contrary to targeting by

homologous recombination, plasmids are not linearized for dRMCE approaches.

- pDIRE

This plasmid expresses the iCre and FLPo recombinases in mouse ES cells. Experimental evidence indicates that the use of this plasmid is essential for efficient and correct dRMCE-mediated replacement.

- pDRAV series

These plasmids encode single loxP and FRT sites flanking a multiple cloning site and a hygromycin-resistance cassette that can be removed using the ϕ*C31 recombinase. The four available variants differ in the relative orientation of the loxP and FRT sites; *therefore is essential to select the appropriate version for custom modification.

- pDREV series

These plasmids are designed to modify the IKMC "knock-out first" mouse alleles. In addition to the 5' FRT and 3' loxP sites, the pDREV backbone encodes a H2B-Venus reporter protein that is expressed under the control of the endogenous locus due to the presence of upstream splice acceptor and T2A sequences. The three pDREV variants (0, 1, and 2) correspond to the different open reading frames and great care should be taken to select the appropriate vector. The pDREV plasmids can be further modified by substituting the H2B-Venus open reading frame with the coding sequences of choice in the form of an RsrII/PacI or RsrII/SapI restriction fragment. The selection cassette renders ES cells resistant to Puromycin and can be removed using the Dre recombinase. The pDREV plasmids are propagated in bacteria in the presence of 20 μg/ml nourseothricin (ClonNAT; WERNER BioAgents 5001000).

- Screening reagents

Specific PCR primers that allow discrimination of the wild-type and conditional from the deleted and correctly replaced alleles need to be designed. PCR-grade water, 10x PCR buffer, dNTPs, Taq polymerase and reagents and buffers for

agarose gel electrophoresis. Reagents, buffers and custom-designed probes for Southern blotting.

- Reagent setup

The primary screening to detect correct replacement following dRMCE is done using PCR. The basic strategy aims to detect a correct recombination event at the 3' end, which corresponds to the location at which the selection cassette is inserted into the pDRAV and pDREV plasmids. In a second step, all ES cell colonies with correct 3' replacement are re-screened to detect proper recombination at the 5' end and checked for insertion of the pDIRE plasmid (using primers amplifying *iCre* or *Flpo* coding sequences). ES cell colonies containing pDIRE sequences should be excluded due to potential insertional mutagenesis or instability. Three to five locus-specific primers located outside of the replaced region are typically designed to amplify the region of interest in combination with cassette-specific oligos. In general, primer pairs designed using specific software (Vector NTI, Primer3, etc) reliably detect the desired alterations. In addition, primer pairs that amplify the parental conditional and wild-type allele must be designed and tested on genomic DNA from the parental ES cell line. Primer pairs detecting the in cis-recombined, i.e. deleted allele, must be used for screening as a certain number of dRMCE positive ES cell colonies will be of mixed origin carrying either the correctly replaced or deleted locus. These sets of oligos must be tested prior to the actual screening of ES cell colonies (see step 4.3).

CRITICAL: Make sure all necessary oligo pairs are designed and synthesized before the ES cell culture and dRMCE experiments are initiated. In particular, several primer pairs should be designed and tested for each of the alleles to be probed.

TIP: When considering the configuration of the deleted locus, keep in mind that several possible configurations of the locus of interest may result from simultaneous recombination by iCre and Flpo. This is particularly important when using IKMC alleles with promoter-driven selection cassettes, as they encode three loxP and two FRT sites. We have evidence that the iCre recombinase acts faster than the Flpo

recombinase, which can result in a partially recombined target allele in which e.g. the lacZ cassette remains .

CRITICAL: Correct, dRMCE-mediated replacement events are identified by short-range PCR, but candidate ES cell clones have to be validated by Southern blotting using external probes at both the 5' and 3' ends. In addition, these blots can be used to exclude additional integration events using either a hygromycin or puromycin probe. Both pDRAV and pDREV plasmids include some restriction sites than can be used for Southern blot analysis. If no convenient restriction sites are available, we encourage the introduction of appropriate restriction sites, which permit verification of candidate ES cell clones by Southern blot analysis. Tests using genomic DNA from the parental ES cell line must be done prior to electroporation to validate the probes and the restriction patterns indicative of correct 5' and 3' replacement events.

Equipment:

Electroporator:
We use a Biorad Gene Pulser II model with 0.4 cm electrode gap cuvettes (Biorad 165-2088). The conditions of electroporation given in this protocol were optimized for this type of electroporator. Please refer to the manufacturer's instructions/protocol when using a different electroporation system.

Tissue culture and molecular biology equipment:
Laminar flow tissue culture hoods, CO_2 incubators (7.5% CO_2, 37°C for R1 ES cells; 5% CO_2, 37°C for feeder cells), stereo microscope, inverted microscope, tissue culture dishes, tissue culture 48, 24 and 6-well plates, 96-well U-bottom plates, PP tubes (15 and 50 ml), sterile pipettes (1, 2, 5, 10, 25 and 50 ml), micropipettes, sterile tips, PCR machine, equipment for agarose gel electrophoresis and Southern blotting transfer and hybridization.

Procedures:

Successful ES cell culture requires great care and dedication. For a full description on how to handle ES cells for optimal growth and maintenance of pluripotency, please refer to the laboratory manual "Manipulating the mouse embryo" (CSHL Press, 2003; Ref. 4). As with any other manipulation of ES cells for the generation of chimeric mice, previous training in general and dedicated ES cell culture techniques is required. The materials/procedures described here are the ones we routinely use for R1 ES cells and represent general guidelines. However, as different ES cell lines may have slightly different culture/handling procedures, is important that the supplying laboratory provides the necessary information together with the ES cells. For example, all the "knock-out first" alleles provided to the research community by the International Knockout Mouse Consortium; IKMC) have been generated in mouse ES cell lines derived from embryos with a C57BL/6N genetic background. We advise to exactly follow the cell line-specific protocols and culture conditions provided by EUCOMM for these ES-cell lines.

Independent of their origin, we recommend users to verify the germ-line transmission potential of the parental ES cell-line they plan to use for dRMCE or alternatively use ES cell-lines with proven germ-line transmission. This is particularly important in the case of conditional alleles generated by high-throughput approaches, as the resulting ES-cell clones may not have been characterized in great detail with respect to their germ-line transmission potential and/or karyotype. If the necessary information is not available, we recommend that minimally two independent parental ES cell lines carrying the conditional allele for the locus of choice are characterized by Southern blot analysis to verify correct targeting. In parallel, these parental ES-cell lines should be karyotyped to exclude clones with apparent chromosomal abnormalities.

In case the germ-line transmission potential is not known, we recommend the use of two independent parental ES-cell lines for parallel manipulation by dRMCE. Detailed protocols describing the PCR, Southern blotting and other molecular techniques can be found in the appropriate molecular biology laboratory manuals such as e.g. the Current Protocols in Molecular Biology series.

CRITICAL: treat ES cells gently and maintain optimal growth conditions at all times by only using freshly prepared media, minimizing handling times and taking utmost care during trypsinizing and transfer of cells, etc. ES cells have to be cultured at relatively high density and are typically split 1:3 to 1:6 every second day onto a layer of mitotically-arrested feeder cells. Only during drug selection after electroporation are ES cells grown without feeder cells on gelatin-coated dishes. ES cells grow in dense colonies with very defined borders and should never be grown to more than 70-80% confluency; individual colonies should never touch each other. The culture media have to be changed daily. When ES cells are split, is very important to dissociate them well by gentle pipetting such that the creation of air bubbles is avoided. The quality of the single-cell suspension has to be checked under the microscope. The generation of a single cell suspension by gentle pipetting not only prevents ES cell differentiation (and potential loss of pluripotency), but is important for preparing cells for electroporation. Sterile conditions must be assured at all times. For more details on ES cell culturing and passaging, we refer the user to the laboratory manual "Manipulating the mouse embryo".

1. Expansion of the parental ES cell line heterozygous for the conditional allele.

Always pre-warm the media, Trypsin-EDTA (1x) and D-PBS. ES cells must be split every two days. Make sure that a sufficient number of dishes with confluent and mitotically-arrested feeder cells are available on the day ES cells are split.

Mitotically-arrested mouse embryonic fibroblast feeder cells are prepared as follows: the feeder cell medium is replaced by medium containing 10 µg/ml of Mitomycin C (100x stock at 1 mg/ml) and the plates are incubated for 2 hours at 37°C in a tissue culture incubator. Then the Mitomycin C medium (toxic) is removed and the plates with the firmly adhered feeder cells are washed 3 times with D-PBS (with Mg^{2+}/Ca^{2+}). Add feeder cell medium and return the plates to the incubator and/or seed ES cells onto them. When using multi-well plates (after ES-cell colony picking, see below), it is better to Mitomycin C treat the feeder cells in 10-cm tissue culture plates, trypsinize and plate them into 48 well plates after washing. One confluent 10-cm dish of feeder cells is sufficient to seed cells into all wells of one multi-well plate with 12 to 96 wells. Plates with confluent layers of

Mitomycin C treated feeder cells can be used up to maximally 3 days for seeding with ES cells. Just prior to plating the ES cells, it is essential to change the feeder cell medium to ES cell medium.

Step 1.1 (day 1): thaw one aliquot of frozen parental ES cell (~3×10^6 cells/vial) into one 6-cm dish of mitotically-arrested confluent feeder cells.

Thaw the vial in a waterbath at 37°C and transfer the contents into a 15-ml tube containing 10 ml of ES cell medium. Centrifuge at 1000 rpm for 5 minutes and resuspend the pellet in 1ml ES cell medium and plate onto the 6-cm dish containing already 3ml of ES cell medium.

Step 1.2 (day 2): check the cells under the microscope and refresh the medium.

Step 1.3 (day 3): passage the cells from a 6-cm to a 10-cm dish.

Wash the plate with 2 ml Trypsin-EDTA (1x, do not use any other type of Trypsin – for details see reagent list). Replace with 1.5 ml of Trypsin-EDTA (1x) and return to the incubator for 15 minutes at 37°C. Tap the plate gently and pipette the ES cells up and down 5-7 times with a 2 ml pipette. Check the single cell status under a microscope. Add 4 ml of ES cell medium and pipette 2-3 more times using a 5 ml pipette. Return the dish to the incubator for 15 minutes. This will allow the feeder cells to re-attach and allow transfer of the ES cells without carrying over too many feeder cells. Transfer the ES cells by gently swirling the dish and collecting the 4.5 ml ES cell medium into a 15-ml tube. Centrifuge the cells (see step 1.1) and resuspend the pellet in 2ml of ES cell medium. Plate the harvested ES cells into one 10-cm dish with mitotically-arrested feeder cells and 8 ml of ES cell medium. In case the ES cell in the original 6-cm dish have grown almost to 80% confluency, then it may be necessary to split the cells into two 10-cm dishes (at 1:3 to 1:6 ratios).

Step 1.4 (day 4): monitor the cells for potential confluency and colony morphology. Refresh the medium.

Step 1.5 (day 5): split the cells onto four to six 10-cm dishes with feeder cells (at 1:4 to 1:6 ratios). For details see step 1.3, resuspend the cells in 7ml ES cell medium for plating.

Step 1.6 (day 6): monitor the cells and refresh the medium. The electroporation will be done the next day.

2.Electroporation.

Typical electroporation conditions for ES cells are 0.24 kV and 475 µF (high capacitance) using the Biorad Gene Pulser II system. About 1.5×10^7 cells in 0.8ml D-PBS are used per 0.4-cm cuvette. As rule of thumb, one about 70% confluent 10-cm dish provides enough ES cells for one cuvette. Grow always 1-2 additional dishes to ensure that sufficient amounts of ES cells are available on the day of electroporation.

Experimental design:

Experimental cuvette 1: ES cells (1.5×10^7) +pDIRE (50 µg), + replacement vector (50 µg). In general, a single cuvette is enough per replacement construct when using dRMCE.

Control cuvette: ES cells (1.5×10^7) + pDIRE (50 µg).

Optional: Control 2 (no DNA) and Control 3 (no electroporation). These controls are in general not needed as they mainly serve to monitor the performance of the electroporator or the quality of the media.

Step 2.1: trypsinize enough 10-cm dishes of approximately 70% confluent ES cells (for details see step 1.3 and 1.5).

CRITICAL: assure that ES cells form a single-cell suspension. If necessary, pipette them further in the Trypsin-EDTA (1x) solution using a 2-ml pipette (maximally 12 times in total).

TIP: prepare enough 10-cm dishes coated with 0.1% gelatin during the trypsinization step. Cover the surface of the tissue culture dishes with the gelatin solution, incubate them for 5 minutes at room temperature and then aspirate the solution. Leave the plates to dry in the hood for 10 minutes.

Step 2.2: Collect the ES cells after 10 min adherence to remove feeder cells (as described in step 1.3). Take great care not to transfer too many feeder cells. Pool the ES cell suspensions from various dishes in 50-ml tubes. Make sure the ES cell suspension is homogeneous and transfer a 100 µl aliquot into a 1.5-ml tube containing 0.9 ml of ES cell medium (1/10 dilution). Count the cells and calculate the total amount of ES cells in each 50 ml-tube.

TIP: take care to not let the cells re-adhere for too long as otherwise a significant fraction of the ES cells will be lost.

Step 2.3: Centrifuge the ES cells at 1000 rpm for 10 minutes. Resuspend the pellet in D-PBS (without Mg^{2+}/Ca^{2+}- this is crucial) at a final concentration of 1.875×10^7 cells per ml, which corresponds to 1.5×10^7 cells in 0.8ml D-PBS.

TIP: during the centrifugation step, open and label the cuvettes. Pipette the appropriate amount of DNA into each cuvette. Prepare a bucket with ice.

Step 2.4: add 0.8ml (1.5×10^7) ES cell suspension to each DNA-containing cuvette using a 1-ml cell culture grade plastic pipette (this is important). Mix the solution by pipetting up and down 2 to 3 times, which ensures homogenous dispersion of the DNA. Prepare all the cuvettes first, then electroporate one after the other.

Step 2.5: pulse each of the cuvettes and place them on ice immediately after electroporation. Typical time constants range between 6.0 to 6.8 milliseconds, although this may vary depending on the electroporator used. Following electroporation, let the cuvettes rest on ice for 20 minutes.

TIP: during this time, add 10 ml of ES cell medium (WITHOUT selective drug) to each of the gelatinized 10-cm dishes. In addition, prepare one 15-ml Falcon tube with 10 ml fresh ES cell media for each cuvette.

Step 2.6: transfer the content of a single cuvette into a 15-ml Falcon tube containing 10 ml of ES cell medium with a 1-ml plastic pipette. Rinse each cuvette twice with 1 ml of ES cell medium to recover additional cells. Do not centrifuge these tubes. Plate 2 ml aliquots of each experimental cuvette (1:5 ratio) into three 10-cm dishes. For the control cuvette (pDIRE only) plate two dishes (2 ml each). Place the dishes into the tissue culture incubator.

3. Recovery.

After at least 24 hours, exchange the medium for fresh ES cell medium (WITHOUT selective drug). This allows the ES cells to recover from the electroporation and allows dRMCE-mediated replacement and expression of the selective drug resistance gene.

TIP: as ES cells attach directly to the gelatin, the medium has to be changed very gently. It should be done the earliest after 24 hrs, i.e. during the afternoon/evening of the day following electroporation.

4. Hygromycin/Puromycin drug selection.

Step 4.1: two days following electroporation, the medium in the experimental dishes (ES cells electroporated with the pDIRE and the replacement vectors) is exchanged for ES medium supplemented with the appropriate selective drug. In addition, one of the control dishes (ES cells electroporated with pDIRE only) also receives medium with the selective drug, while the other receives regular ES cell medium.

CRITICAL: The control plate receiving medium WITHOUT selective drug serves as important positive control for ES cell growth and should be clearly labeled as such.

TIP: As puromycin selection requires lower drug concentrations, this is a rather low cost drug selection.

Step 4.2: Change the selection and control medium daily. The first clones will become apparent after 7-8 days of selection.

CRITICAL: during selection make sure to produce enough Mitomycin C treated feeder cells for growing and expanding the ES clones. For isolation of ES cell colonies, two 48 multi-well plates containing treated feeder cells per dRMCE replacement experiment will be needed over a period of four days. Over a 2 days interval, generate at least one 24-well, one 6-well and one 10cm dish per ES cell colony that later needs to be expanded.

Step 4.3: The ES cells growing in the control dish without selective drug will become sub-confluent usually in 2-3 days. Once this happens, wash the plate three times with D-PBS and proceed to extraction of genomic DNA (see part 8). This DNA sample is critical as it allows testing of the specific PCR amplifications that detect the wild-type, conditional and deleted alleles. A fraction of ES cells will have been electroporated with the pDIRE vector, which will induce cis-recombination of the conditional locus and generation of the deleted allele.

CRITICAL: the PCR primer pairs that were designed to specifically detect the wild-type, conditional and deleted alleles (see Figure 1) have to be validated prior to starting the isolation of ES cell colonies, as the ones with successful replacement and clonal origin need to be identified rapidly.

Step 4.4: the second control dish that received medium with the selective drug should be devoid of ES cells after about 5 days when using puromycin or 7 days when using hygromycin. This is an important control as it indicates that the drug selection is working.

5. Isolation (picking) of ES cell colonies.

ES cell colonies are picked using a stereo-microscope (cleaned carefully with ethanol) placed under the tissue culture hood. In average, ES cell colonies are big enough for picking starting at day 9-10 of drug selection.

Step 5.1: ES cell colonies can be easily spotted by the naked eye through the bottom of the tissue culture dish. Circle the colonies using a marker and carefully screen them under the microscope for ES cell colonies with round, defined borders, but where individual cells are not easily distinguishable (Figure 2).

CRITICAL: Do not pick ES cell colonies with a flat architecture and/or that are much larger than average colonies. Due to the high number of ES cell colonies present and the relatively low number of colonies that need to be picked, many colonies with perfect morphology (see Figure 2 for examples) should be readily apparent in the three plates selected per dRMCE construct.

Step 5.2: Add 40µl of cold Trypsin-EDTA (1x) per well into two rows of a 96 multi-well plate (24 wells in total; with U-shaped bottom). Keep the plate on ice. Replace the medium in the dish with 8ml of D-PBS (with Mg^{2+}/Ca^{2+}). An isolated ES cell colony is picked using a Gilson P20 pipette set to 10 µl by aspiration and transferring it into a well of the 96-well plate on ice. After picking 24 ES cell colonies, incubate cells in the Trypsin solution for 7 minutes at 37°C . Replace the D-PBS in the plate that was used to pick the ES cell colonies with fresh medium containing the selective drug and put the plate back in the tissue culture incubator.

Step 5.3: after Trypsin digestion, add 175 µl of ES cell medium without selection to each well using a multi-dispenser pipette. Disperse the cells by pipetting them up and down gently a few times with a Gilson P200 pipette set at 170 µl. Transfer the single cell suspension to one well of a 48 multi-well plate with Mitomycin C-treated feeder cells in 300 µl of ES cell medium WITHOUT selective drug. Return the 48 multi-well plate to the incubator.

CRITICAL: From now on, only use medium WITHOUT selective drug, as feeder cells are not resistant.

Step 5.4: A similar number of ES cell colonies should be picked the two following days by repeating steps 5.1-5.3.

TIP: In some cases, we have observed that fast growing clones, which are first apparent, are negative for dRMCE-mediated replacement. These clones probably arise as a consequence of early, random-integration events resulting in rapid expression of the drug resistance gene. We therefore recommend picking ES cell colonies over a period of 2-3 days (i.e. about 15-20 clones each day). In our experience, picking 40-60 cell colonies is in general sufficient to yield several correctly replaced ES cell clones. Nevertheless, the plates containing additional ES cell colonies should be kept and the ES cell medium changed every second day until correctly replaced ES cell clones are identified by PCR analysis (part 8).

6. Expansion of isolated ES cell colonies (48-well plate).

The day after isolation, inspect the cells under the microscope for viability and density. Exchange the medium by adding 500 µl of fresh ES cell medium.

CRITICAL: To avoid cross-contamination between wells, the tip of the pipette used to aspirate the old culture medium has to be flamed after each aspiration. The new medium should be added to the side of the wells using a multi-dispenser pipette. Great care should be taken to avoid touching the borders of the wells.

7. Passaging the ES cell colonies into a new 48 multi-well plate (2 days after picking).

Step 7.1: aspirate the ES cell medium and wash the wells with 75 µl of Trypsin-EDTA (1x). Exchange the 75 µl Trypsin-EDTA (1x, 75 µl) solution once and incubate the plate at 37°C for 8-10 minutes.

TIP: if some clones have not grown enough, they can be left in the original well for an additional day after changing the medium. To avoid prolonged trypsinization, process only 4-6 wells at a time.

Step 7.2: during the incubation, remove media from a new 48 multi-well plate with Mitomycin C-treated feeder cells and replenish the well with 300 µl of ES cell medium.

Step 7.3: carefully tap the plate containing the ES cell colonies and gently pipette up and down three times using a Gilson P200 pipette set at 60 µl. Immediately add 600 µl of ES cell medium using a multi-dispenser pipette to inactivate the Trypsin. Gently pipette up and down four times using a Gilson P1000 pipette set

to 500 µl. Transfer 200 µl of ES cell suspension into a single well of the new 48 multi-well plate and return it to the incubator. Transfer the remaining ES cell suspension to a 1.5ml Eppendorf tube and proceed to DNA extraction (part 8).

Step 7.4: the following day, monitor the cells under the microscope and change the medium (further expansion is described in part 9).

8. PCR screening to detect correct dRMCE-mediated replacement events (in parallel with step 7).

Step 8.1: Process the 1.5-ml tubes containing ES cells for DNA extraction (step 7.3) in batches of 24 samples. Pellet the cells in a microcentrifuge; wash them once with D-PBS and re-centrifuge.

CRITICAL: it is important to prepare genomic DNA of good quality for reliable PCR identification of correct replacement events. Therefore, the use of the protocol described here is highly recommended. This protocol results in extraction of DNA suitable for PCR analysis that is free of major contaminants. Contaminated or low quality DNA prepared by so-called quick DNA extraction protocols will compromise PCR analysis.

Step 8.2: lyse the cell pellet using 400 µl of lysis buffer (10 mM Tris-HCl pH8.0; 50mM EDTA; 100mM NaCl; 0.5% SDS) supplemented with 1 mg/ml of Proteinase K (Merck 24568; stock solution 10 mg/ml in H_2O). Incubate for 2 hours at 55°C.

Step 8.3: spin briefly to concentrate the liquid at the bottom of the 1.5-ml tube. Add 350 µl of digestion buffer (without Proteinase K) and 250 µl of 6M NaCl. Mix by inverting the tube several times (do NOT vortex).

Step 8.4: centrifuge the tubes for 10 minutes at 13,000 rpm in a microcentrifuge. Transfer the supernatant (contains the genomic DNA) to a new 1.5-ml tube containing 500 µl of isopropanol. Avoid transferring any of the precipitate (contains proteins, etc). Mix by gentle inversion for two minutes and re-centrifuge for 10 minutes at 13,000 rpm.

Step 8.5: carefully decant the supernatant – pay special attention to not loose the DNA pellet. Add 1 ml of 70% ethanol and centrifuge for 2 minutes at 13'000 rpm. Remove the supernatant by careful decanting and assure that the DNA pellet is not lost. Re-centrifuge the tubes and remove any residual ethanol using a thin tip.

Step 8.6: air-dry the DNA pellets for 15 minutes and resuspend in 50-100 µl (depending on pellet size) of 10 mM Tris-Cl, 100 µM EDTA (pH 8.0). Leave the solution for several hours to overnight at 4°C to resuspend. Mix by brief vortexing and use 1-3 µl of DNA solution per PCR reaction (in 50 µl total volume).

Step 8.7: Use the previously designed and validated PCR strategy to identify ES cell colonies with correct replacement events. The initial screen should focus on detecting correct recombination at one end of the locus (i.e. 3' recombination). To control DNA quality, a PCR amplification detecting the wild-type locus should be included (all ES cell colonies are heterozygous). ES cell colonies with correct 3' replacement are then re-screened for 5' recombination. As a fraction of ES cell colonies might be of mixed nature, it is crucial to verify the absence of the deleted and conditional alleles (for more details see Osterwalder et al.). In addition, all positive ES cell colonies have to be back-screened for the lack of integrated pDIRE sequences. It is possible to complete the entire PCR screening procedure of 48 ES cell colonies in one day.

CRITICAL: Due to the two-step nature of the dRMCE procedure, there is a small fraction of correctly targeted ES cell colonies that are of mixed origin with cells carrying the deleted or conditional allele. These mixed colonies likely originate as a consequence of early unequal segregation of the replacement vector and/or pDIRE plasmid. Using the PCR strategy described here, mixed colonies are easily recognized and must be discarded.

ES cell colonies must be screened during the two days they grow in 48 multi-well plates (part 7). In the extreme situation that no correctly replaced ES cell colonies would be detected, more could be picked and analyzed (part 5-7). In case the PCR strategy is flawed and/or fails, candidate colonies for correct replacement could be tentatively identified by the absence of the conditional and deleted alleles in combination with amplification of the wild-type allele as a control. These ES cell colonies could be expanded and frozen in aliquots and the correct replacement proven by rigorous Southern blotting analysis at a later stage.

9. Expansion of correctly replaced ES cell clones.

Step 9.1: mark the correctly replaced clones (part 8) in the 48 multi-well plates.
Step 9.2: among them, select minimally 6-8 ES cell clones that have good colony morphology (colonies with defined round borders and with densely packed cells) and are ready to be split 48 hours after plating. Trypsinize these clones (as described in part 7) and transfer all the cells into one well of a 24 multi-well plate containing Mitomycin C-treated feeders in ES cell medium.

TIP: after removing the selected ES cell clones from the 48 multi-well plate, place the plate on ice. Remove the medium from the additional positive clones (in groups of 4-8) and gently add 400 µl of ice-cold freezing media. Seal the plate with Parafilm, wrap it in a plastic bag and transfer it to -80°C into a Styrofoam box. Additional positive clones can be recovered from plates stored -80°C in such a manner up to a few months later.
Step 9.3: change the ES cell medium daily and split the cells every 2 days as follows: from a single 24-well to a single 6 well and then into a 10-cm tissue culture dish (always containing Mitomycin C treated feeder cells). When the cells are ready to be split into a 10-cm dish, take 1/5 of the single cell suspension and plate it into one gelatin-coated well of a 6 multi-well plate. These cells will be used for karyotyping to detect potential chromosomal abnormalities that would interfere with the generation of chimeric mice. DNA for Southern blot analysis is prepared by replenishing the wells of 24 and 6 multi-well plates with new medium after transferring the trypsinized cells. There is always enough ES cells left behind that can be re-grown and used to prepare larger amounts of clean DNA for Southern Blot analysis. Confirmation of correct replacement by extensive Southern blot analysis is absolutely essential.
TIP: the ES cell clones that a) have a normal karyotype; b) harbor no insertions of the pDIRE vector or random integration of the replacement vector and c) have been validated by Southern blot analysis for correct recombination at both ends can be directly microinjected into mouse blastocysts to generate chimeric mice.

10. Freezing ES cell clones for long-term storage.

Label 5 cryotubes (Nunc) per 10-cm dish of ES cells to be frozen. Include the locus, code name of the clone, date and the initials of the researcher.

Step 10.1: check the 10-cm dishes of ES cells for optimal confluency (70%) and colony morphology. Change the media 2-3 hours before the freezing process is initiated.

Step 10.2: trypsinize the ES cells as described (step 1.5). Centrifuge the cells and resuspend the pellet in 2ml of ice-cold freezing medium in a 15-ml tube. Keep the tube on ice. Add an additional 3 ml of freezing media to the tube and aliquot the 5 ml of cell suspension into the 5 cryotubes (1 ml each). Transfer the tubes to a Styrofoam holder and wrap it with bubble-wrap. Place this package at -80°C. The next day transfer the cryotubes individually to into a cell freezing box that fits into liquid nitrogen tank for long-term storage. ES cell clones frozen in this manner can be stored for indefinite time.

TIP: One or several of these vials can be sent to the transgenic facility and the ES cells defrosted and expanded for the production of chimeric mice. One vial should be thawed into a 6-cm tissue culture dish containing Mitomycin C-treated feeder cells.

Timing

1. Generation of the dRMCE replacement vector: 1-2 months. The time needed to construct a particular dRMCE replacement vector can vary significantly as this depends on the custom design. However, the use of the pDRAV and pDREV plasmids reduces the required time significantly.
2. Expansion of ES cells harboring the conditional allele: 6-7 days.
3. Electroporation: 1 day.
4. Recovery phase: 2 days.
5. Hygromycin/Puromycin selection: 8-10 days.
6. Picking of ES cell colonies: 1-3 days.
7. Growing of ES cell colonies in 48-well plates: 2 days.
8. Transfer into new 48-well plates: 2 days.
9. PCR screening: 2 days (in parallel to part 8).

10. Expansion of verified ES-cell clones: 6 days. During the expansion phase, the PCR positive ES cell clones are validated by Southern Blot analysis and karyotyping.
11. Freezing of sufficient vials of cells for validated ES cell clones: 1 day.
Total time: approximately 5 weeks from thawing a single vial of the parental ES cell line until the validated and correctly replaced ES cell clones are ready for microinjection into recipient mouse blastocysts.

Anticipated Results

Targeting frequencies by dRMCE are difficult to predict, but are in the range of 10-70% correct replacement. Among the ES cell colonies analyzed there will be a fraction of "mixed" colonies that contain both correctly replaced cells and ones still carrying the conditional or deleted allele. Some ES cell colonies could also have undergone incomplete replacement (e.g. positive for *loxP* /Cre but negative for *FRT* /Flp-mediated recombination or vice versa). Furthermore a small fraction of ES cell colonies might have additional integration events or display chromosomal abnormalities. All the ES cell colonies that are of mixed origin or aberrant in any manner should be discarded.
Due to the high frequency of correct replacement using dRMCE, is relatively straightforward even for less experienced researchers to expand a small number of ES cell colonies under optimal conditions and complete the PCR screening within a day. Our experience is that the best possible conditions of ES cell culture are the main asset for maintaining the pluripotency of these ES cell clones

Rapresentation of the tergeted PI3Kr6 locus in dRMCE

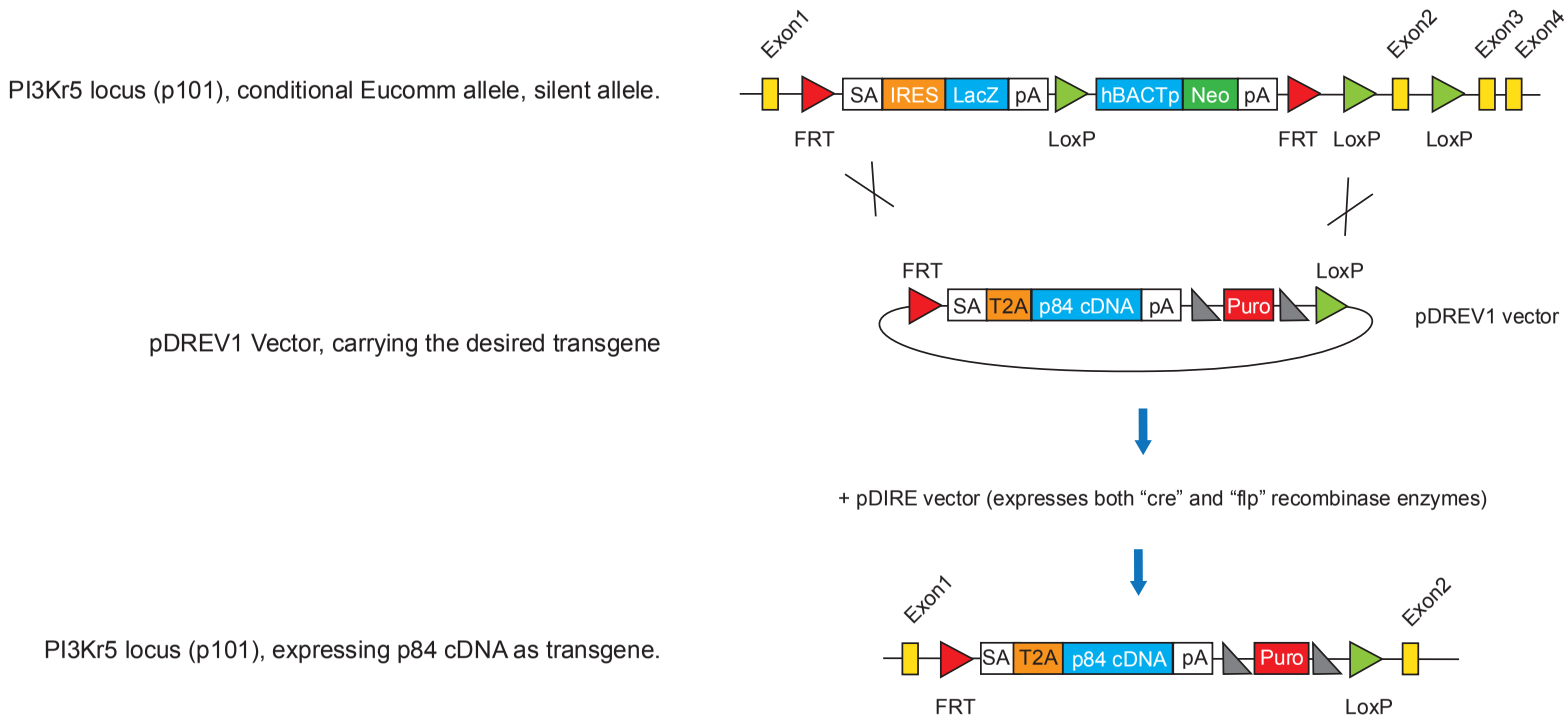

Figure 3, appendix I: Graphic representation of the dRMCE mediated targeting of the PIK3r5 locus (p101) for the generation of the p84 knock-in mouse strain. **FRT**: flippase recognition target site. **Loxp**: locus of X-over P1, is a short DNA sequence derived from the Bacteriophage P1, recognised by the Cre recombinase enzyme. **SA**: Splicing Acceptor. **IRES**: Internal Ribosome Entry Site. **LacZ**: beta-galactosidase. **pA**: poliadenilation site. **hBACTp**: human beta-actin promoter. **Neo:** neomicine resistence gene. **T2A:** self cleaving TA peptide.

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Abbreviations

AD	atopic dermatitis
APCs	antigen-presenting cells
AR	allergic rhinitis
ATP	adenosine triphosphate
BCR	B cell receptor
BH	breakpoint-cluster region homology
BM	bone marrow
BMMC	bone marrow-derived mast cells
Btk	Bruton's tyrosine kinase
BSA	bovine serum albumin
C2	domain protein kinase C homology domain 2
Ca^{2++}	calcium
CAMs	cell adhesion molecules
CCR	CC chemokine-binding receptor
CR3	complement receptor 3
CSR	class-switch recombination
CXCR	CXC chemokine-binding receptor
DAG	diacylglycerol
DC	dendritic cell
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DNP-HSA	dinitrophenyl human serum albumin
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
e.g.	exempli gratia
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGTA	ethylene-glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid

ELISA	enzyme linked immunosorbent assay
EtOH	ethanol
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FGFR	fibroblast growth factor receptor
FYVE	Fab1p, YOTB, Vac1p and EEA1
Gab2	Grb2-associated binding protein 2
Gads	Grb2-related adaptor protein 2
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine exchange factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
Grb2	growth factor receptor-bound protein 2
GPCR	G-protein coupled receptor
GTP	guanosine-5'-triphosphate
HC	highly cytokinergic
HIFCS	heat inactivated fetal calf serum
hr	hour(s)
HRPO	horseradish peroxidase
IC_{50}	50% inhibitory concentration
IFNγ	interferon-gamma
Ig	immunoglobulin
IgSF	immunoglobulin superfamily
IL	interleukin
IMDM	iscove's modified dulbecco's medium
IP_3	inositol 1,4,5-trisphosphate
IRS	insulin receptor substrate
iSH2	inter-SH2 domain
ITAMs	immunoreceptor tyrosine-based activation motifs
ITIMs	immunoreceptor tyrosine-based inhibiting motifs
kDa	kilo Dalton
KI	knock in
KO	knock out

LAT	linker for activation of T cells
Lck	lymphocyte-specific protein tyrosine kinase
L-Gln	L-glutamine
LPS	lipopolysaccharide
LT	leukotriene
mAb	monoclonal antibody
Mac-1	macrophage antigen-1
MAPK	mitogen-associated protein kinase
MCs	mast cells
MIP-1α	macrophage inflammatory protein-1 alpha
MIP-1β	macrophage inflammatory protein-1beta
mTOR	mammalian target of rapamycin
NRTK	non-receptor tyrosine kinase
NTAL	non-T cell activation linker
p85B	p85-binding site
PAF	platelet-activating receptor
PBS	phosphate buffered saline solution
PC	poorly cytokinergic
PCA	passive cutaneous anaphylaxis
PDK	phosphoinositide-dependent kinase
PEST	penicillin-streptomycin
PG	prostaglandin
PGN	peptiglycans
PH	pleckstrin homology
PKB	protein kinase B
PKC	protein kinase C
PI3K	phosphoinositide 3-kinase
PI4K	phosphoinositide 4-kinase
PLCγ	phospholipase C gamma
PMA	phorbol 12-myristate 13-acetate
PSA	passive systemic anaphylaxis
PtdIns	phosphatidylinositol
PtdIns(3)P	phosphatidylinositol 3-phosphate

PtdIns(3,4)P_2	phosphatidylinositol 3,4-bisphosphate
PtdIns(4,5)P_2	phosphatidylinositol 4,5-bisphosphate
PtdIns(3,4,5)P3	phosphatidylinositol 3,4,5-triphosphate
PTEN	phosphatase and tensin homologue deleted on chromosome 10
PVDF	polyvinylidene fluoride
PX	phox homology domain
RA	rheumatoid arthritis
RasB	Ras-binding domain
RNA	ribonucleic acid
rpm	rotations per minute
RTK	receptor tyrosine kinase
S6K	S6 kinase
SCF	stem cell factor
SDF-1α	stromal cell-derived factor 1alpha
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	Src homology 2
SH3	Src homology 3
Shc	Src-homologue and collagen-homologue
SHIP	SH2-containing inositol 5-phosphatase
Sl	steel locus
Sn	supernatant
SOCC	store-operated calcium channels
ssRNA	single-stranded ribonucleic acid
TBS	Tris buffered saline solution
TCR	T cell receptor
T_H	T helper
TH	tec homology
TLR	toll-like receptor
TNF-α	tumor necrosis factor-alpha
ZAP70	zeta-chain associated protein kinase 70 kD

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- Gogvadze E, Bohnacker T, **Botindari F**, Collmann E, Marone R, Volzmann J, Wymann MP. Blocking mast cell activation with Ras inhibitors – moving toward cell-specific PI3Kγ targeting (in preparation).
- Breasson L, Becattini B, Molinaro A, Zani F, Marone R, **Botindari F**, Wymann MP and Solinas G. PI3Kγ activity within multiple cell-types cooperatively promotes adipose tissue inflammation and insulin resistance during obesity (in preparation).

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